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IL-33 induces cellular and exosomal miR-146a expression as a feedback inhibitor of mast cell function

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Abstract

Interleukin 33 (IL-33) is an inflammatory cytokine that promotes allergic disease by activating ILC2, Th2 cells, and mast cells. IL-33 is increased in asthmatics, and its blockade suppresses asthma-like inflammation in mouse models. Homeostatic control of IL-33 signaling is poorly understood. Since the IL-33 receptor, ST2, acts via cascades used by the TLR family, similar feedback mechanisms may exist. MicroRNA-146a (miR-146a) is induced by LPS-mediated TLR4 signaling and serves as a feedback inhibitor. Therefore, we explored whether miR-146a has a role in IL-33 signaling. IL-33 induced cellular and exosomal miR-146a expression in mouse bone marrow-derived mast cells (BMMC). BMMC transfected with a miR-146a antagonist or derived from miR-146a KO mice showed enhanced cytokine expression in response to IL-33, suggesting that miR-146a is a negative regulator of IL-33-ST2 signaling. *In vivo*, miR-146a expression in plasma exosomes was elevated after intraperitoneal injection of IL-33 in wild type but not mast cell-deficient $Kit^{W\text{-}sh/W\text{-}sh}$ mice. Finally, $Kit^{W\text{-}sh/W\text{-}sh}$ mice acutely reconstituted with miR-146a KO BMMC prior to IL-33 challenge had elevated plasma IL-6 levels compared to littermates receiving WT BMMC. These results support the hypothesis that miR-146a is a feedback regulator of IL-33-mediated mast cell functions associated with allergic disease.

Introduction

Interleukin-33 (IL-33) is an alarmin cytokine in the IL-1 family. Both IL-33 and its receptor, ST2, have been consistently linked to asthma in genome-wide association studies [1]. In addition to allergy, IL-33-mediated signaling is associated with inflammatory responses to viral infection, chronic obstructive pulmonary disease, and parasite infection [2]. IL-33 is constitutively expressed as a nuclear protein in fibroblasts, endothelial, epithelial, stromal, and some immune cells, which release the protein upon cell damage, tissue injury, or exposure to enzymatically active antigens [3]. This elicits an inflammatory cascade by activating ILC2, Th2 cells, and mast cells as primary targets. Other cells activated include

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macrophages, neutrophils, and eosinophils [4]. In mast cells, IL-33 induces a variety of proinflammatory cytokines and chemokines that contribute to allergic disease in mouse models [5, 6]. Elevated serum IL-33 has been linked to asthma, atopic dermatitis, and allergic rhinitis [7–11], supporting efforts to target IL-33 clinically [12–14].

IL-33 signaling is limited partly by proteolytic degradation [15], but little is known about other mechanisms controlling this potent inflammatory factor. One way to regulate cell signaling is through the induction of microRNAs (miRs). These 18–23 nucleotide RNAs serve as post-transcriptional gene modifiers that are essential in cell development, function, and homeostasis [16, 17]. Numerous miRs regulate immune cell differentiation and function. For example, Mohammed et al. [18] recently showed that miR-155 induction promotes COX-2 expression during IgE-mediated mast cell activation. One miR that is known for its inhibitory effects is miR-146a, which regulates function or survival of B cells, CD4 T cells, macrophages, and mast cells [19, 20]. In macrophages, LPS-induced TLR4 signaling elicits not only proteolytic degradation of IRAK-1, but also NFκB-mediated miR-146a expression [21]. miR-146a in turn down-regulates TRAF6 and IRAK1 translation, resulting in diminished TLR4 function [22]. As evidence of its importance, miR-146a KO mice develop splenomegaly with lympho- and myeloproliferation progressing to autoimmune disease [23].

Recent studies have also linked miR-146a to atopic disease. In humans, a miR-146a polymorphism has been associated with asthma in several studies [24–26]. Further, miR-146a levels in pediatric PBMC were negatively correlated with the severity of allergic rhinitis and increased after subcutaneous or sublingual immunotherapy [27]. Murine models have found that miR-146a deficiency increases inflammation in atopic dermatitis, and miR-146a upregulation diminishes the severity of allergic dermatitis, rhinitis, conjunctivitis, and airway inflammation [28–30]. These studies support the concept that miR-146a limits allergic disease.

The IL-33 receptor, ST2, activates a TLR-like signaling cascade, including the MyD88-IRAK-TRAF pathway leading to NFκB function [2]. Homeostatic regulation of IL-33 is poorly understood and could be important for allergic disease, given the growing understanding of IL-33 function in these conditions. Because miR-146a has been shown to inhibit mast cell survival [31], we employed mast cells to test the hypothesis that IL-33-ST2 signaling induces miR-146a as a feedback regulator. Our data show that IL-33 elicits robust miR-146a expression that is critical for limiting mast cell-mediated inflammation. These results shed new light on how IL-33-induced inflammation is regulated to maintain immune homeostasis.

Materials and Methods

Animals

Mouse strains C57BL/6J, $Kit^{W\text{-}sh/W\text{-}sh}$ (hence referred to as Wsh), miR-146a KO, and MyD88 KO breeding pairs were purchased from The Jackson Laboratory (Bar Harbor, ME). Breeding colonies were maintained in a specific pathogen-free facility. Control mice (WT) for each experiment were age-, sex- and genotype-matched animals housed in the

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same facility. Bone marrow was extracted from mice at a minimum of 10 weeks old and IL-33-induced peritonitis studies were conducted on mice older than 12 weeks under a protocol approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee.

Mouse mast cell culture

Mouse bone marrow-derived mast cell (BMMC) cultures were developed by culturing bone marrow from mouse femurs in complete RPMI 1640 medium (cRPMI; Invitrogen Life Technologies, Carlsbad, CA) containing the following purchased from Corning, (Corning, NY): 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 1mM sodium pyruvate, and 1mM HEPES. Cultures were supplemented with supernatant from WEHI-3B cells adjusted to contain IL-3 at a final concentration of 1 ng/ml as measured by ELISA. The media also contained either supernatant from BHK-MKL cells adjusted to contain SCF at a final concentration of 10 ng/ml, or recombinant mouse SCF at 10 ng/ml final. BMMC were used after at least 21–28 days of culture and consisted of >95% mast cells based on staining for c-Kit and FcεRI. Peritoneal mast cells were enriched from peritoneal lavage samples by culture in the same media used for BMMC. After 5 days of culture, mast cells were identified as c-Kit-high, side scatter-high cells.

Cytokines and reagents

Recombinant mouse IL-3 and SCF were purchased from Shenandoah Biotechnology (Warwick, PA) for *in vitro* studies. Recombinant mouse IL-33 was purchased from Biolegend (San Diego, CA). APC-coupled anti-mouse ST2, PE-coupled anti-mouse Ly6G, APC-coupled anti-mouse CD45, PE-coupled anti-mouse CD117/c-Kit, FITC-coupled anti-mouse Ly6C, BV421-coupled anti-cKit, APC-coupled anti-CD107a, PE-coupled anti-CD63-PE, and corresponding isotype controls were purchased from Biolegend (San Diego, CA). Purified rat anti-mouse CD16/CD32 were purchased from BD Biosciences (San Jose, CA). IRAK-1/4 inhibitor was purchased from Cayman Chemical (catalog #17540).

Cytokine measurements

BMMC were washed and resuspended in cRPMI at a concentration of 1×10^6 cells/ml with 10 ng/ml of IL-3 and SCF. Cultures were stimulated with IL-33 (50 ng/ml unless otherwise stated) for 16 hours and supernatants were measured for IL-6, TNF, or MCP-1 levels using ELISA kits from Biolegend (San Diego, CA). IL-13 was quantified using an ELISA kit from Peprotech (Rocky Hill, NJ). ELISAs were developed using BD OptEIA reagents from BD Biosciences (Franklin Lakes, NJ).

RT-qPCR

RNA was harvested from BMMC using TRIzol reagent (Life Technologies, Grand Island, NY). Following RNA extraction, cDNA was produced using the qScript cDNA Synthesis Kit or qScript microRNA Quantification System (Quanta Biosciences, Gaithersburg, MD) following manufacturers' protocols. Amplification and qPCR analysis were conducted using Bio-Rad CFX96 Touch™ Real-Time PCR Detection System (Hercules, CA) and SYBR® Green detection. The reactions performed contained cDNA, PerfeCTa SYBR Green

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SuperMix (Quanta Biosciences, Gaithersburg, MD) and primers for the gene of interest and housekeeping genes. The gene of interest mRNA levels in each sample were normalized to the level of mRNA of the appropriate housekeeping gene using the Relative Livak Method (ΔΔCt).

PCR primers were purchased from Quanta biosciences (Gaithersburg, MD). These included: For *miR-146a-5p* qPCR, primers for *mmu-miR-146a-5p* (5'-UGAGAACUGAAUUCCAUGGGUU-3') and housekeeping gene *SNORD47* (5'-GUGAUGAUUCUGCCAAAUGAUACAAAGUGAUAUCACCUUUAAACCGUUCAUUUUAUUUCUGAGG-3') Amplification occurred under the following conditions: 95 °C for 2 min followed by 40 cycles of 95 °C for 5 s, 60°C for 30 s. Primers for *mmu-il33* qPCR were forward primer: 5'CTACTGCATGAGACTCCGTTCTG; reverse primer: 5'AGAATCCCGTGGATAGGCAGAG. Primers for *mmu-beta actin* were forward primer: 5'-GATGACGATATCGCTGCGC; reverse primer: 5'-CTCGTCACCCACATAGGAGTC. Amplification conditions for *il33* and *beta actin* were: 95 °C for 2 min followed by 40 cycles of 95 °C for 15 s, 55°C for 30 s, 60°C for 30 s.

Flow cytometric analysis

For surface staining, cells were cultured at 1×10^6 cells/ml in cRPMI with the indicated treatments prior to staining. Afterward, cells were washed twice, pelleted, and resuspended in PBS containing 3% fetal calf serum and 0.1% sodium azide (FACS buffer) and the indicated antibodies and anti-CD16/32 clone 2.4G2. Samples were incubated at 4°C for 45 minutes, washed and resuspended in FACS buffer. Samples were then analyzed to assess the expression of surface molecules. The gating strategy included doublet exclusion based on FSC-A × FSC-H analysis, followed by FSC-A/SSC-A gating to exclude dead cells. Samples were acquired on a BD FACSCelesta and examined using BD FACSDIVA™ (BD Biosciences, Franklin Lakes, NJ) or FlowJo (Ashland, OR) software.

siRNA, antagomir and miR mimics:

BMMC were transfected with 100 nMoles of siRNAs specific for Akt1/2 or with scrambled control sequences (FlexiTube LNA-RNAs, using a pool of 4 targeting sequences) from Qiagen (Valencia, CA). miR-146a-5p mimic and antagomir were purchased from Exiqon (now part of Qiagen) and used at 50 nMoles/transfection. 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). Cells were used 48 hours after being transfected, with transfection verified by Western blotting for proteins or qRT-PCR for microRNAs.

Western Blotting

Western blot analysis was performed as described previously [32]. Blots were visualized and quantified using a LiCor Odyssey CLx infrared imaging system (Lincoln, NE). Rabbit antibodies against IRAK-1 (clone D51G7), GAPDH (clone 14C10), phospho-p38-T180/Y182 (clone 12F8), and phospho-p65-S536 (clone 93H1) were from Cell Signaling Technology. Mouse monoclonal antibodies against NFκB p65 (clone L8F6 from Cell

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Signaling Technology) and p38 (clone A-12 from Santa Cruz Biotechnologies) were also used.

Tissue mast cell distribution

Age- and sex-matched groups of co-housed C57BL/6 and miR-146a KO mice of at least 8 weeks old were used. Tissue samples were harvested and placed into neutral-buffered formalin solution (Sigma-Aldrich, St. Louis, MO). Tissues were embedded in paraffin, sectioned, and mounted, then stained with pinacyanol erythrosinate by StageBio (Mount Jackson, VA). Mast cells found in four 40x fields were enumerated on slides that were blinded to the examiner. For peritoneal mast cells, 3 mL of PBS-EDTA (PBS, 1% EDTA) was injected into the peritoneal cavity, which was massaged before exudate was collected with a Pasteur pipette. Cells were stained for FcεRI and c-Kit expression and analyzed by flow cytometry.

Measurement of neutrophil recruitment and cytokines *in vivo*

Wsh mice were intraperitoneally reconstituted with 2×10^6 BMMC derived from WT or miR-146a KO mice 1 hour prior to IL-33 injections. Mice were injected via intraperitoneal (i.p.) route with 1 μg of IL-33 (in 100 μL PBS) or 100 μL of PBS. Four hours later, peritoneal lavage and cardiac puncture were performed. Peritoneal cells were stained with antibodies against CD45, Ly6G, and Ly6C prior to analysis by flow cytometry. Neutrophils were quantified by gating on FSC-A × FSC-H singlets, $CD45^+$, $Ly6G^{high}$, and $Ly6C^{low/-}$. Precision Count Beads™ (San Diego, CA) from Biolegend were used to count the absolute cell number. Peripheral blood plasma cytokine levels were determined by ELISA.

Exosome isolation and miR-146a detection

For *in vivo* exosome isolation, mice were injected i.p. with 1μg of IL-33 or phosphate buffered saline (PBS). Blood was collected 24 hours post-injection via cardiac puncture into tubes containing EDTA. Plasma was spun at 3000xg for 15 minutes to remove debris. Plasma (500μl) was treated with 5U/mL of thrombin liquid suspension solution 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 in a microcentrifuge. The supernatant was transferred to clean a 1.7 mL tube. Exosomes were isolated from plasma using ExoQuick (Systems Biosciences) according to the manufacturer's recommended protocol. Briefly, 120 μl of ExoQuick was added to the supernatant and mixed by inverting 3 times. The mixture was then stored for 1 hour at 4 °C and microcentrifuged at 13,000 rpm for 2 minutes (4 °C). The supernatant was aspirated, and residual liquid was spun at 1500xg for 5 minutes. The pellet was lysed with the 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 instructions, with a wash and elution step. miR-146a was detected using a TaqMan-based amplification kit from ThermoFisher Scientific, catalog number 4427975 using the manufacturer's recommended conditions.

For *in vitro* exosome isolation, BMMC (10 million cells at 1×10^6/mL) were treated +/− IL-33 (50ng/ml) as indicated above for 24 hours in cRPMI supplemented with 10%

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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. miR-146a was detected by RT-qPCR as described above.

Statistical analyses

P values were calculated with GraphPad Prism software by paired or unpaired two-tailed Student's t-test when comparing 2 samples. For a comparison of 3 or more samples, ANOVA was performed followed by Tukey's post hoc test. P values of <0.05 were considered statistically significant. Data are expressed as mean ± standard error of mean (SEM) with statistical significance: *p< .05, **p< .01, ***p< .001, ****p<.0001.

Results

IL-33-induced miR-146a-5p expression in mouse mast cells provides negative feedback for cytokine production.

To determine if IL-33 induces miR-146a production, BMMC were cultured with IL-33 and analyzed for miR-146a-5p expression at several time points. IL-33 treatment induced miR-146a as early as 2 hours following stimulation, with levels increasing >1000-fold by 24 hours (Figure 1A, B). Since miR-146a is a negative regulator of TLR4 signaling in macrophages [20, 32–35], we next determined if miR-146a suppressed IL-33-induced cytokine production. miR-146a KO BMMC stimulated with IL-33 exhibited significantly greater production of IL-6, IL-13, TNF, and MCP-1 than WT BMMC at multiple points in a 48-hour time course (Figure 1C).

IL-33 does not induce degranulation of WT mast cells [33–35]. Because of the enhanced cytokine response in miR-146a KO mast cells, we measured IL-33-induced degranulation using peritoneal mast cells but found no changes in CD63 or CD107a expression, although we did note that miR-146a KO mast cells expressed CD63 and CD107a at higher levels than WT cells (Supplemental Figure 1). Collectively, these data support the hypothesis that miR-146a is required for normal feedback control of IL-33-induced cytokine production.

ST2 expression is unchanged in miR-146a KO BMMC.

miR-146a KO mice exhibit splenomegaly and myeloproliferative disorders [23]. It has also been shown that miR-146a KO deletion can affect surface receptor levels in adipocytes and macrophages [36, 37]. Increased expression of the IL-33 receptor ST2 could logically explain why miR-146a KO BMMC are hyperresponsive to IL-33. Further, because SCF augments IL-33 signaling [38], increased expression of the SCF receptor, c-Kit (CD117), could also be functionally relevant. WT and miR-146a KO BMMC were analyzed by flow cytometry for ST2 and CD117. We noted no difference in either the percent of cells staining

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positive or staining intensity between WT and miR-146a KO BMMC (Supplemental Figure 2). Hence, increased receptor expression does not explain the IL-33 hyperresponsiveness noted in these cells.

We also investigated the possibility that IL-33 treatment induces IL-33 expression, which has been reported for pulmonary cells [39, 40] and if miR-146a deficiency augments this effect. However, we found that IL-33 stimulation for 24 hours, the point at which miR-146a expression is highly elevated, did not induce *il33* mRNA expression in wild type BMMC and modestly decreased it in miR-146a KO cells (Supplemental Figure 3). Thus, an autocrine IL-33 signaling loop does not appear to explain enhanced cytokine production by miR-146a KO BMMC.

miR-146a-5p antagomir and mimic alter IL-33-induced cytokine production.

Because miR-146a deletion might alter BMMC differentiation, we corroborated our miR-146A KO data by transfection. First, we transfected C57BL/6 BMMC with an oligonucleotide inhibitor (antagomir) of miR-146a-5p prior to IL-33 stimulation. As shown in Figure 2A, antagomir treatment modestly but significantly reduced miR-146a-5p levels compared to the scrambled control treatment after 48 hours. This was associated with enhanced IL-33-mediated cytokine production (Figure 2B). The disproportionate increase in cytokine secretion relative to the decrease in miR-146a may indicate that the antagomir inhibited miR-146a function without inducing its degradation, as has been described [41].

To complement these data, a miR-146a-5p mimic was transfected into C57BL/6 BMMC (Figure 2C) to determine if overexpressing this miR reduces IL-33-mediated cytokine production. Compared to cells receiving the scrambled control miR, miR-146a transfected cells produced significantly less cytokine when stimulated with IL-33 (Figure 2B). These data collectively support our hypothesis that miR-146a negatively regulates IL-33-induced mast cell function.

miR-146a induction requires the IL-33 signaling proteins MyD88 and NFκB but not IRAK-1/4.

Previous studies in macrophages have shown TLR stimulation induces miR-146a through MyD88-dependent NFκB activation, corroborating known NFκB binding sites in the miR-146a promoter [22, 42, 43]. Since IL-33 also utilizes the MyD88-NFκB pathway, we investigated whether these signaling proteins were involved in miR-146a induction. BMMC cultured from MyD88 KO mice were completely unable to induce miR-146a following IL-33 stimulation, in contrast to WT BMMC (Figure 3A). The kinases IRAK-1 and IRAK-4 have been shown to be activated by IL-33/ST2 and act downstream of MyD88 [44, 45]. Surprisingly, an IRAK-1/4 inhibitor modestly increased IL-33-mediated miR-146a despite significantly reducing IL-6 secretion (Figure 3B). In contrast, the NFκB inhibitor BAY-117085 greatly reduced miR-146a expression as well as IL-6 secretion (Figure 3C). Thus, MyD88 and NFκB, but not IRAK-1/4 are required for IL-33-induced miR-146a expression in mast cells.

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miR-146a deficiency results in dysregulated IL-33 signaling

After determining the signals required for IL-33 to induce miR-146a, we investigated the reciprocal: how miR-146a affects IL-33 signaling. Because miR-146a has been shown to inhibit IRAK-1 expression [22], we assessed changes in IRAK-1 protein levels during IL-33 signaling. As shown in Figure 4A, unstimulated miR-146a KO BMMC expressed more than twice as much IRAK-1 than WT cultures, suggesting that miR-146a restrains IRAK-1 levels in resting mast cells. Upon IL-33 stimulation, IRAK-1 levels dropped precipitously, fitting the previous finding that IRAK-1 is degraded via ubiquitination during IL-1 and TLR4 signaling [21, 46]. Because we found robust miR-146a induction 12–24 hours after IL-33 stimulation (Figure 1), we anticipated that miR-146a KO BMMC might show higher IRAK-1 expression that WT cells at these time points. However, IRAK-1 protein levels did not recover, remaining very low in both WT and miR-146a KO cells at the 20-hour time point (Figure 4A). Thus, it appears that miR-146a restricts basal IRAK-1 expression, but a rapid, miR-146a-independent degradation limits IRAK-1 levels during IL-33 signaling.

We next examined MAPK activation, which lies downstream of IRAK-1 activation in the ST2 signaling cascade [47]. Although ERK, JNK, and p38 can be activated by ST2, we noted only consistent p38 phosphorylation (Figure 4B and data not shown). Because p38 has been associated with promoting IL-33/ST2 function [48–51], we expected to find greater p38 activation among miR-146a KO BMMC. Surprisingly, the opposite was found. miR-146a KO BMMC exhibited significantly less p38 phosphorylation than WT cells, especially at the early time points (Figure 4B). Separate from this counterintuitive outcome, NFκB activation as judged by p65/RelA phosphorylation was consistently greater in miR-146a KO BMMC, starting at triple the levels in WT cells in the first 10 minutes of IL-33 stimulation (Figure 4C). In summary, miR-146a deficiency led to higher basal IRAK-1 expression that correlated with enhanced NFκB activation but unexpectedly reduced p38 signaling.

miR-146a deficiency enhances IL-33-mediated peritonitis *in vivo*

To further our studies, we explored whether miR-146a contributes to IL-33-mediated mast cell function *in vivo*. In the IL-33-induced peritonitis model, intraperitoneal IL-33 injection recruits neutrophils to the peritoneal cavity through a process requiring mast cell-derived TNF [52] (Figure 5A). C57BL/6J or miR-146a KO mice were injected with IL-33, and peritoneal lavage neutrophils were quantified. Unexpectedly, miR-146a KO mice exhibited reduced neutrophil migration compared to WT mice (Figure 5B). Upon further investigation, we found that naïve miR-146a KO mice have approximately 60% fewer peritoneal mast cells than WT mice (Figure 5C and Supplemental Figure 4A). This likely explains the defective neutrophil recruitment. Interestingly, mast cell deficiency was also noted in ear tissue, but not in back skin or small intestine (Figure 5C). Because of these differences in mast cell numbers, we continued *in vivo* studies by repopulating Wsh mice.

Wsh mice have a c-Kit mutation that results in defective SCF signaling causing mast cell deficiency. The mice can be repopulated with BMMC, which is often accomplished by IV injection and an 8–12 week reconstitution period [53]. However, we found that miR-146a KO BMMC inefficiently repopulated Wsh mice under these conditions (data not shown).

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Also, this model has been shown to poorly restore peritoneal mast cells [53]. Therefore, we used an acute repopulation model. Wsh mice were intraperitoneally reconstituted with miR-146a KO or WT BMMC 1 hour prior to IL-33 injection (Figure 6A). Mast cell numbers in the peritoneal lavages were similar between the miR-146a KO and C57BL/6J repopulated mice (Figure 6B). Mice repopulated with miR-146a KO mast cells, without further stimulation, showed neutrophil recruitment equal to IL-33 treatment of Wsh mice complemented with WT BMMC (Figure 6C). While IL-33 injection increased neutrophil recruitment as expected in the WT BMMC group, IL-33 had marginal effects on the already elevated miR-146a BMMC reconstitution group. Interestingly, IL-33 did increase plasma IL-6, MCP-1, and IL-13 in miR146a KO-repopulated mice, and to a greater extent than repopulation with C57BL/6J BMMC (Figure 6D). TNF was not detectable in the plasma. These data suggest that miR-146a deficient mast cells had increased basal activation and enhanced IL-33 responsiveness.

IL-33 induces exosomes containing miR-146a *in vitro* and *in vivo.*

Exosomes are secreted vesicles that contribute to intercellular communication. Immune cells, including mast cells, have been shown to secrete and take up exosomes containing a variety of mediators and RNAs [54–56]. Therefore, we explored whether exosomes derived from BMMC contained miR146a *in vitro*. BMMC were stimulated with IL-33 for 24 hours, and exosomes were isolated from the culture supernatant. miR-146a was detectable intracellularly and in exosomes released from mast cells following IL-33 stimulation (Figure 7A). Both cellular and exosomal miR-146a levels were several-fold higher than the unstimulated control, supporting their induction by IL-33. We next determined whether IL-33 induced exosomes containing miR-146a *in vivo* and if this is dependent on mast cells. 24 hours after intraperitoneal injection of IL-33, miR-146a levels were modestly but significantly higher in serum exosomes when compared to mice injected with vehicle control (Figure 7B). By comparison, mast cell-deficient Wsh/Wsh mice showed no significant increase in plasma exosomal miR-146a expression. While we cannot conclude that mast cells are the direct source of plasma exosomal miR-146a, it is apparent that mast cells must be present for IL-33 to elicit this response *in vivo*.

Discussion

IL-33 is an alarmin that alerts the immune system to cellular damage. It is expressed in the nuclei of barrier tissues such as epithelial and endothelial cells and released when these cells are ruptured or damaged [1, 2]. Although it seems that IL-33 is essential for wound healing and tissue homeostasis [57], it has also been implicated in allergic disease. For example, in the house dust mite model of allergic airway inflammation, allergen elicits structural cells to release IL-33 that activates mast cells and ILC2 cells, promoting Th2 differentiation, pulmonary eosinophil recruitment, and lung remodeling that collectively are pathological [58]. Several studies have linked IL-33 to allergic diseases including allergic rhinitis, atopic dermatitis, and asthma [7–11], which is why pharmaceutical companies have entered IL-33 blockers into clinical trials [12–14]. Because of its importance in allergic disease, it is important to understand how IL-33 function is regulated.

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miRs provide one means of controlling cell signaling. They can be induced and selectively downregulate protein expression by binding mRNA and preventing translation. In TLR4 signaling, miR-146a induction inhibits IRAK1 and TRAF6 expression, effectively dampening TLR4 function [20, 32–35]. This is but one example of numerous miRs controlling TLR signaling [59]. Since ST2 and TLR4 have similar signaling pathways, we investigated whether miR-146a had a similar role in IL-33 signaling.

We demonstrate that IL-33 induces miR-146a-5p expression in mouse mast cells and in exosomes released from them. miR-146a expression appears to be functionally important, since miR-146a KO cells exhibited stronger IL-33 response than their WT counterparts. Similarly, a miR-146a antagomir enhanced IL-33 responses in WT BMMC, while a miR-146a mimic decreased IL-33 effects on miR-146a KO cells. We conclude that miR-146a serves as a feedback inhibitor of IL-33-induced mast cell function.

miR-146a induction required canonical proteins shared by TLR and ST2 signaling cascades. Mast cells lacking MyD88 or treated with an NFκB inhibitor had poor miR-146a expression in response to IL-33. Previous studies have found that NFκB transcribes miR-146a, whose promoter contains multiple NFκB binding sites [20, 42, 43]. It was surprising that IRAK-1/4 inhibition did not reduce IL-33-mediated miR-146a induction. The inhibitor suppressed IL-6 secretion, supporting its efficacy. The IRAK family consists of the active kinases IRAK-1 and −4, which have been associated with IL-33/ST2 signaling [44, 45], and the pseudokinases IRAK-2 and IRAK-M [60]. IRAK-2 has not been linked in to ST2 the literature. IRAK-M, whose expression is restricted to the monocyte/macrophage lineage, has been shown to inhibit ST2 signaling [54]. We conclude that while MyD88 and NFκB are required for miR-146a expression, IRAK-1/4 are either dispensable or their minimal activity in the presence of an inhibitor is sufficient for miR-146a induction. Since the canonical ST2 signaling cascade proceeds to NFκB via IRAK-1/4, these data are enigmatic and warrant further study. It is noteworthy that while miR-146a limits LPS effects partly by suppressing IRAK expression [43, 61], a role for IRAKs in LPS-mediated miR-146a expression has not been demonstrated.

We also studied the reciprocal relationship, asking how miR-146a deficiency alters IL-33/ST2 signaling. These data included both unexpected and anticipated outcomes. Because IL-33 elicits a dramatic increase in miR-146a expression over 12–24 hours, we expected to observe differences in signal transduction at these relatively late time points – but this was not the case. Instead, the most striking differences were noted at rest or within the first few minutes of signaling. Expression of IRAK-1, a miR-146a target [21], was elevated 2-fold in unstimulated KO cells compared to their WT counterpart. In keeping with its proteasome-mediated degradation during IL-1 and TLR4 signaling [21, 46], IRAK-1 protein levels rapidly declined upon IL-33 stimulation and were not restored after 20 hours in either WT or miR-146a KO cells. This suggests that miR-146a limits basal IRAK-1 expression but has little impact on this kinase during IL-33 signaling. Increased basal IRAK-1 seemed functionally important, since it correlated with a 3-fold greater phosphorylation of NFκB p65. This latter result fits the known role of NFκB in cytokine production and our Figure 3, showing that an NFκB inhibitor essentially ablated IL-6 secretion. An additional oddity in the signaling data was an approximate 50% decrease

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in IL-33-mediated p38 MAPK activation in miR-146a KO mast cells. This was unexpected, because p38 has been consistently associated with promoting IL-33 function; hence reduced p38 activation does not agree with greater cytokine secretion. We are left to hypothesize that p38 and NFκB activation can be disconnected downstream of ST2 and that, at least in mast cells, NFκB is the better predictor of inflammatory cytokine release.

A peritonitis model was used to determine if miR-146a controls IL-33-induced inflammation *in vivo*. This model requires mast cell-derived TNF secretion [58], but IL-33 acts on many cells and the model does not ascribe effects to the mast cell lineage exclusively. Unexpectedly and in opposition to our *in vitro* studies, we found miR-146a KO mice had decreased neutrophil recruitment in response to IL-33 injection. We subsequently found that miR-146a KO mice have a mast cell deficiency in selective tissues, with fewer mast cells in the peritoneal cavity and ear tissue than WT mice. Because miR-146a has been shown to inhibit mast cell survival and proliferation [37], we did not anticipate mast cell deficiency in miR-146a KO mice. However, miR-146a KO mice have myriad disorders, including splenomegaly, myeloproliferative disorders, and autoimmunity [23]. Thus, mast cell deficiency could be secondary to these defects, such as altered growth factor expression in select tissues.

Given the mast cell deficiency in miR-146a KO mice, we attempted to repopulate Wsh mice with miR-146a KO BMMC but found poor engraftment, suggesting a survival or migration defect in these cells. In the acute repopulation model, miR-146a KO adoptive transfer led to significant neutrophil recruitment even without IL-33 treatment. The act of injecting the cells could activate them, although this did not occur with WT BMMC transfer. One plausible theory is that the transfer process elicited Alarmin release, perhaps IL-33, which had a more potent effect on miR-146a KO cells and induced neutrophil infiltration. Subsequent IL-33 injection also induced greater plasma cytokine levels in mice repopulated with miR-146a KO BMMC than those repopulated with WT BMMC. By limiting miR-146a deficiency to the mast cell lineage, these data support the hypothesis that this microRNA normally inhibits IL-33-mediated mast cell function *in vivo*.

In addition to inducing cytoplasmic miR-146a, we found that IL-33 also elicited exosomal expression in BMMC, data that were consistent in exosomes purified from the peripheral blood of IL-33-injected mice. While we have not conclusively proven exosomal miR-146a is derived from mast cells, our data from mast cell-deficient Wsh mice supports this possibility and indicates that mast cells are necessary for exosomal miR-146a expression. Exosomes are being explored as therapeutic tools [62–64], raising the possibility they can be used to carry miRs regulating inflammation. Determining the role of exosomal miR-146a in allergic inflammation is an exciting area for future study.

In conclusion, this study supports the hypothesis that miR-146a is a negative feedback regulator of IL-33, limiting mast cell function *in vitro* and *in vivo*. These data provide new insight into the role of IL-33 in inflammatory disease, where it has generated interest as a therapeutic target. Because IL-33 induced exosomal miR-146a, these data also suggest that this microRNA could act on remote sites to regulate the systemic IL-33 response.

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Key Points:

- IL-33 induces miR-146a expression in mast cells, which reduces cytokine production.
- miR-146a KO mast cells have increased IRAK-1 expression and NFκB activation.
- IL-33 also induces release of exosomes containing miR-146a in vitro and in vivo.

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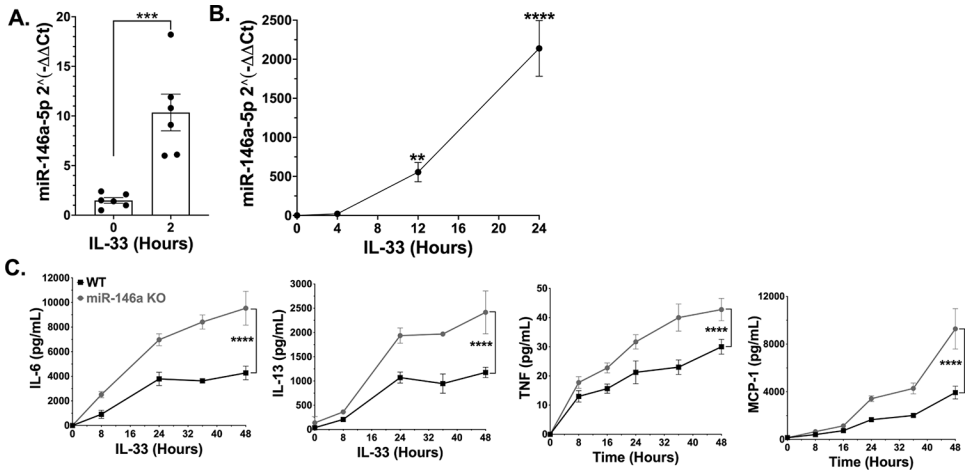

Figure 1. miR-146a-5p is induced by IL-33 and inhibits cytokine secretion.

(A) BMMC were stimulated with IL-33 (50 ng/ml) for the indicated times. Isolated RNA was used to measure miR-146a-5p by qPCR, normalized to miR-SNORD47. Data shown are means ± SEM of 6 samples from 2 separate experiments. **(B)** BMMC were treated as in (A) for the indicated times and miR-146a-5p was quantified by qPCR. Data shown are means ± SEM of 6 samples from 2 separate experiments. **(C)** BMMC derived from C57BL/6J or miR-146aKO mice were activated for 16 hours ± IL-33 (50ng/ml). Cytokine levels were quantified by ELISA. Data shown are means ± SEM of 4–10 samples from 2 separate experiments. ****p<.0001 using ANOVA to compare WT and miR-146a KO lines.

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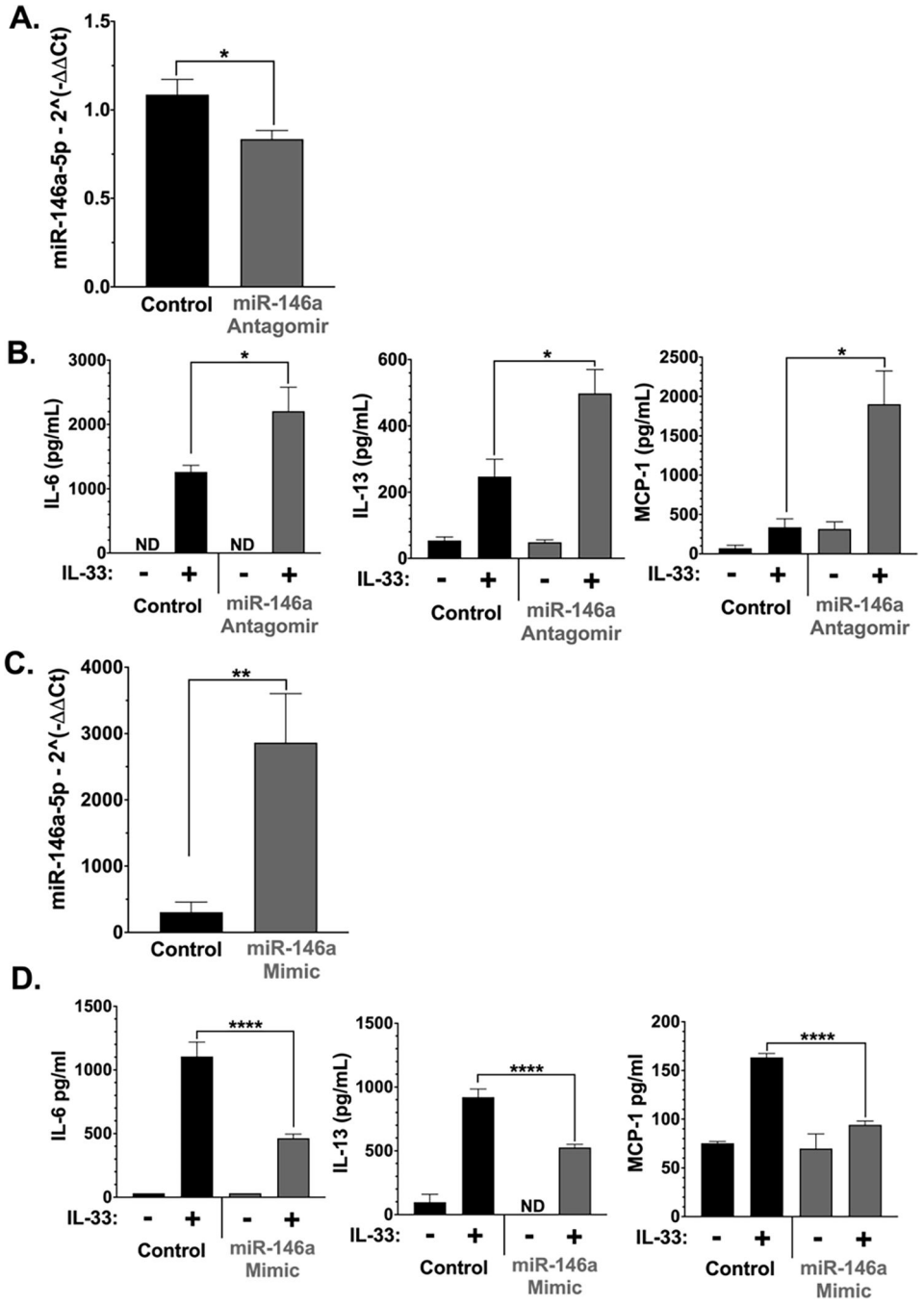

Figure 2. Altering miR-146a expression controls IL-33-induced cytokine production.

(A) C57BL/6 BMMC were transfected with a miR-146a-5p antagomir or the scramble control analyzed for miR-146a-5p levels by RT-qPCR. **(B)** Cells from (A) were stimulated for 16 hours with IL-33 (50 ng/ml) and cytokines were measured by ELISA. Data are from three BMMC populations analyzed in triplicate from 1 of 3 independent experiments. **(C)** C57BL/6 BMMC were transfected with a miR-146a-5p mimic or the scrambled RNA sequence control. mRNA was collected after 48h hours and analyzed for miR-146a-5p levels by RT-qPCR. Data shown are from three BMMC populations analyzed in triplicate from 1 of

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2 independent experiments using, each using 3 independent BMMC populations. **(D)** Cells from (C) were stimulated for 16 hours with IL-33 (50 ng/ml) and cytokines were measured by ELISA. Data shown are from three BMMC populations analyzed in triplicate from 1 of 2 independent experiments, each using 3 independent BMMC populations. ND = not detectable. *p< .05, **p< .01, ***p< .001, ****p<.0001 when comparing the indicated bars by ANOVA.

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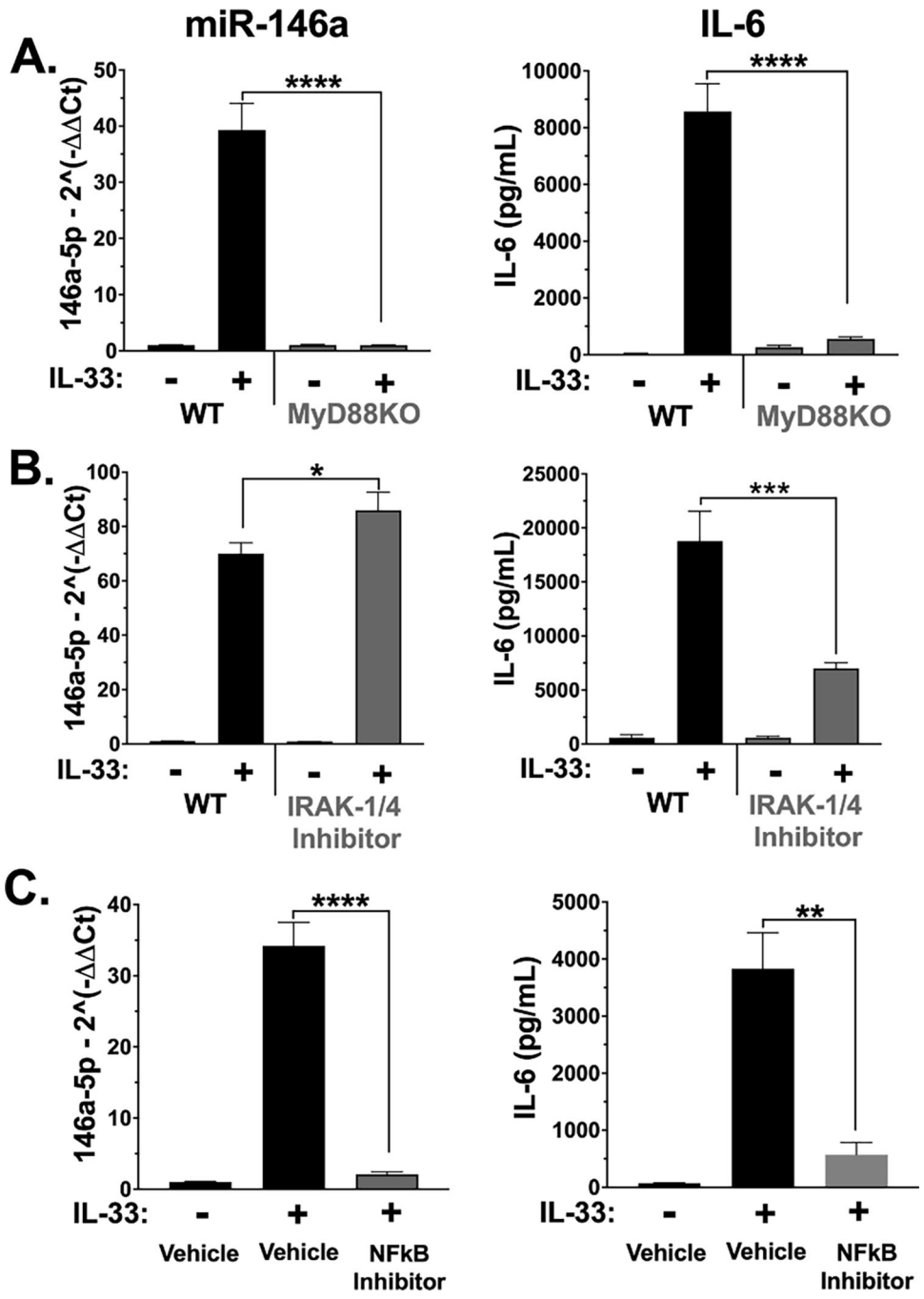

Figure 3. IL-33-mediated miR-146a-5p induction is dependent on MyD88 and NFκB signaling pathways.

(A) WT or MyD88 KO BMMC were stimulated with IL-33 (50 ng/ml) for 8 hours and miR-146a expression was measured by RT-qPCR. Data are means ± SEM of 6 populations from 2 independent experiments. **(B)** WT BMMC were treated for 2 hours with DMSO IRAK-1/4 inhibitor (10 μM) then stimulated for 24 hours with IL-33 (50 ng/ml) and miR-146a expression was measured by RT-qPCR. Data are means ± SEM from 2 independent BMMC populations analyzed in triplicate on two separate days for a total of

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12 samples. **(C)** WT BMMC were treated with NFκB inhibitor bay11–7082 (2 μM) for 1 hour prior to IL-33 stimulation. Data are means ± SEM of 6 populations from 2 independent experiments. The right side of the figure shows IL-6 detected in culture supernatants by ELISA under the same conditions used to detect miR-146a. *p< .05, **p< .01, ***p< .001, ****p<.0001 when comparing the indicated bars by ANOVA.

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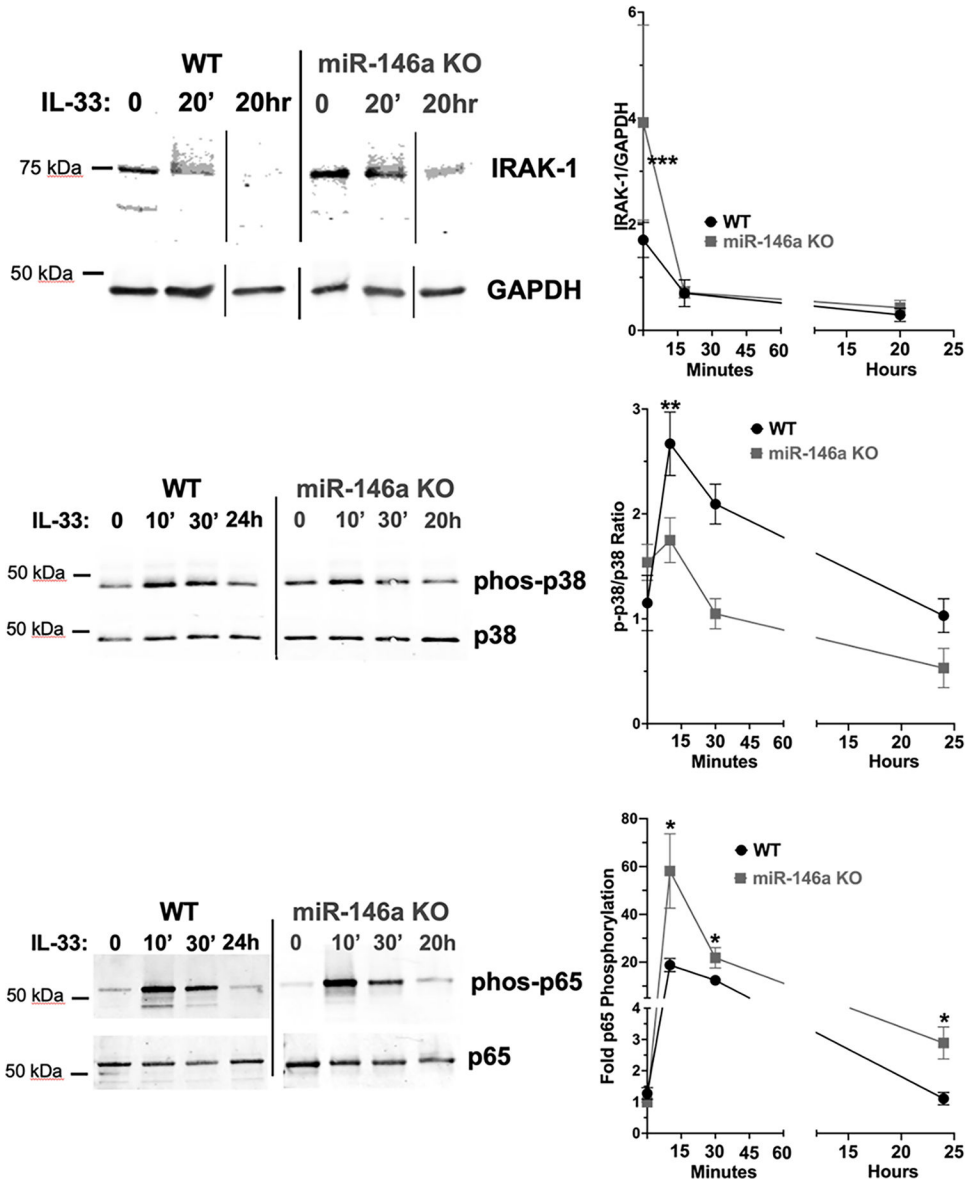

Figure 4. miR-146a deficiency alters early IL-33-ST2 signaling events.

WT and miR-146a KO BMMC were stimulated with 50ng/ml IL-33 for the indicated times. Expression of the indicated proteins was measured by Western blotting. Example blots are shown next to normalized data from three independent experiments. *p< .05, **p< .01, ***p< .001 when comparing the indicated WT and miR-146a KO sample pairs by ANOVA.

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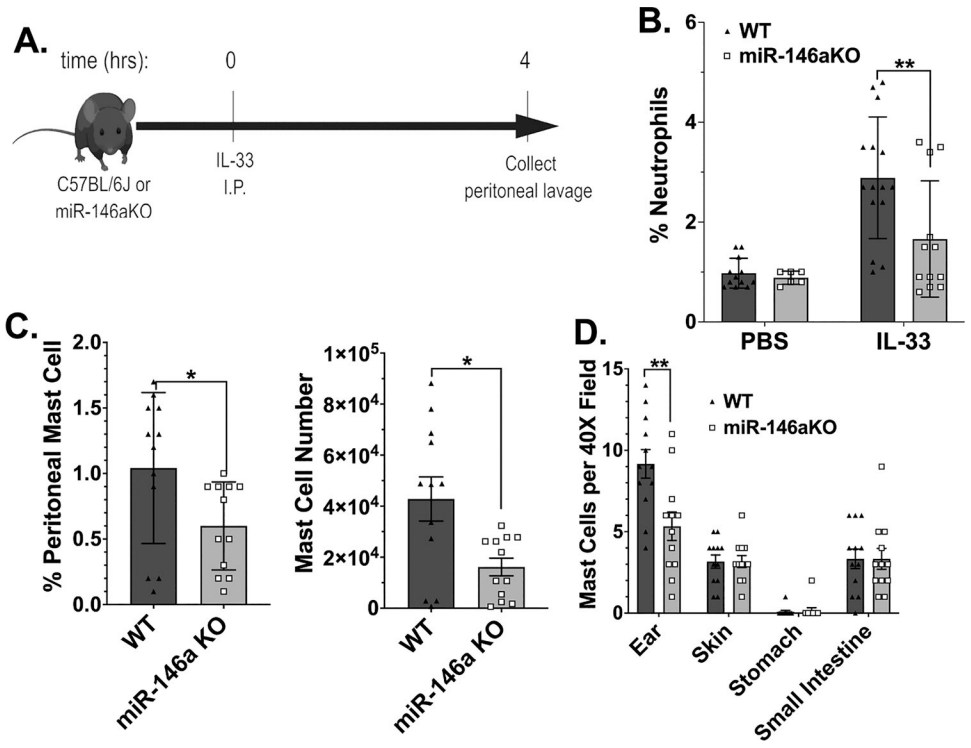

Figure 5. miR-146a KO mice have decreased IL-33 induced neutrophilia.

(A) Design of the model. Age and sex-matched WT and miR-146a KO mice were injected intraperitoneally with IL-33 (1 μg) or PBS. Four hours later, peritoneal lavages were collected, and cells were stained for neutrophils prior to flow cytometry analysis. **(B and C)** Peritoneal lavages were harvested from WT or miR-146a KO mice, and cells were stained for neutrophil and mast cell surface markers prior to analyzing by flow cytometer. **(D)** Tissues from WT or miR-146a KO mice were collected and stained with pinacyanol erythrosine. Mast cell numbers were counted from four 40x fields. Data are means ± SEM of 5 (A, B) or 3 (C) mice. Schematic was created with Biorender. *p< .05, **p< .01 when comparing the indicated bars by ANOVA.

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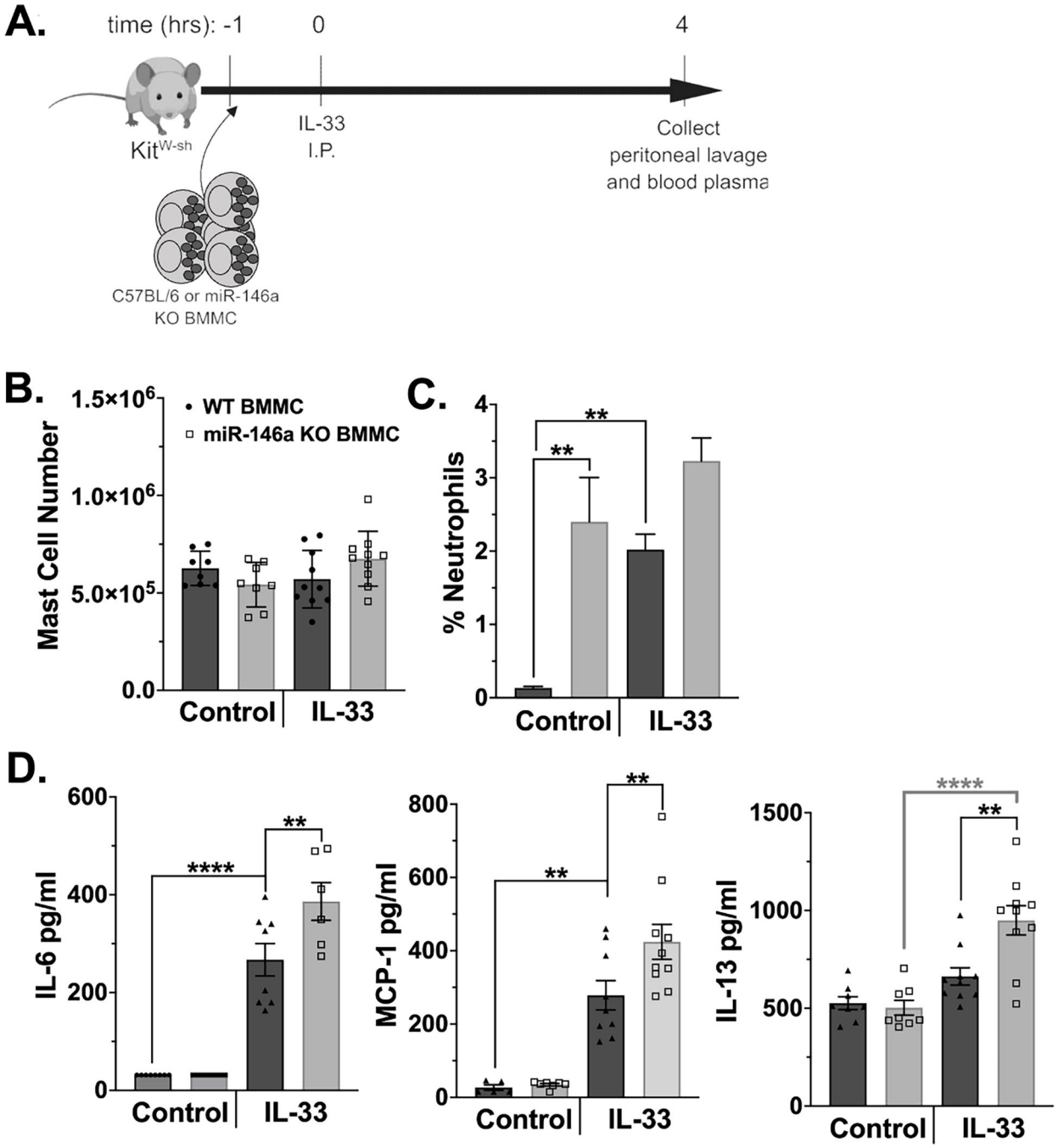

Figure 6. Kit$^{W\text{-}sh/W\text{-}sh}$ mice reconstituted with miR-146a KO BMMC have increased IL-33-induced peritonitis.

(A) The acute mast cell reconstitution model (Image created with Biorender). Age and sex-matched Kit$^{W\text{-}sh/W\text{-}sh}$ mice were intraperitoneally reconstituted with WT or miR-146a KO BMMC (2×10^6 cells/mouse) for 1 prior to IL-33 (1 μg/ml) or PBS injection. Four hours later, peritoneal lavages **(B and C)** and plasma **(D)** were collected. Peritoneal cells were stained for mast cell and neutrophil surface markers prior to analysis by flow cytometry.

Plasma cytokines were quantified by ELISA. Data are means ± SEM of 4 mice per group. **p< .01, ****p<.0001 when comparing the indicated bars by ANOVA.

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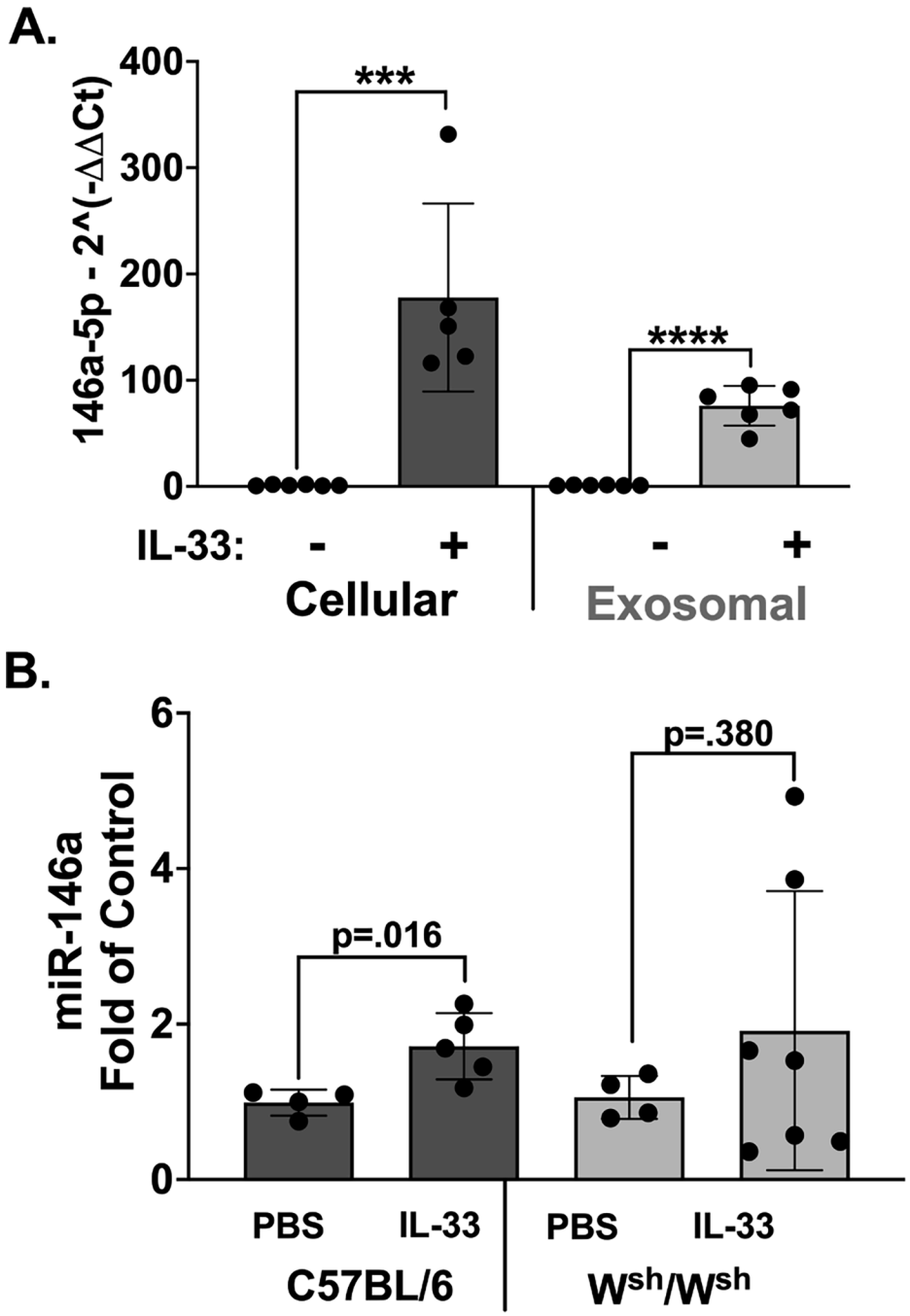

Figure 7. IL-33 induces miR-146a-5p expression in exosomes *in vitro* and *in vivo*.

(A) BMMC were treated with IL-33 (50 ng/mL) for 24 hours. Exosomes were isolated and miR-146a-5p was measured from cellular and exosomal mRNA by RT-qPCR. Data are means ± SEM of 5–6 populations analyzed in two experiments. (B) C57BL/6 and Wsh mice were intraperitoneally injected with IL-33 (1 μg) 24 hours before collecting plasma exosomes. miR-146a-5p was quantified by RT-qPCR. Data are means ± SEM of duplicate

samples from individual animals. ***p< .001, ****p<.0001 when comparing the indicated bars by ANOVA.

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