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IL-10–Induced miR-155 Targets SOCS1 To Enhance IgE-Mediated Mast Cell Function

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IL-10 is an important regulatory cytokine that modulates a wide range of immune cells. Whereas it is best known for its ability to suppress immune responses, IL-10 has been found to be pathogenic in several human and animal studies of immune-mediated diseases. There is a considerable gap in our understanding of the molecular mechanisms behind the stimulatory effects of IL-10 during allergic inflammation. IL-10 treatment has been shown to suppress mast cell TNF production. In this study, we report that whereas TNF secretion was reduced, IL-10 surprisingly enhanced IgE-mediated protease and cytokine production both in vitro and in vivo. This stimulatory effect was consistent in mouse and human skin mast cells. IL-10 enhanced activation of the key FcεRI signaling proteins Stat5, JNK, and ERK. We demonstrate that IL-10 effects are dependent on Stat3 activation, eliciting miR-155 expression, with a resulting loss of suppressor of cytokine signaling-1. The importance of miR-155 was demonstrated by the inability of IL-10 to enhance anaphylaxis in miR-155–deficient mice. Taken together, our results reveal an IL-10–induced, Stat3–miR-155 signaling pathway that can promote mast cell responses. *The Journal of Immunology*, 2016, 196: 4457–4467.

Interleukin-10 is a pleiotropic immunoregulatory cytokine that is secreted by macrophages, Th1 and Th2 cells, regulatory T and B cells, cytotoxic T cells, and mast cells (MCs) (1, 2). Traditionally, IL-10 is known to inhibit production of proinflammatory cytokines such as TNF, by altering Ag presentation in monocytes and macrophages (2, 3).

IL-10R is a member of the class II cytokine receptor family, employing JAK1 and Tyk2 kinases and signaling through several pathways, most notably the latent transcription factor, Stat3 (2, 3). The majority of preclinical data from patients and mouse models with defects in IL-10 or IL-10R suggest IL-10 is a major immunosuppressive cytokine, particularly in the context of intestinal mucosal homeostasis (4, 5). 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 proinflammatory properties. Several studies of IL-10 administration in experimental endotoxemia (6) as

well as studies of patients with Crohn's disease (7), systemic lupus erythematosus (8), and psoriasis (9) have shown immunostimulatory effects of IL-10 that correlated with disease severity.

MCs are master effector cells in allergic responses, present in skin and mucosal tissues. Along with producing IL-10, MCs are also responsive to IL-10. It is well established that the suppressive effects of IL-10 are largely Stat3 dependent (3). In MCs, we and others have shown that IL-10 suppresses FcεRI-mediated TNF production in a Stat3-dependent manner (10, 11), an effect noted after prolonged (3–4 d) treatment with IL-10 (10–12). In this study, we find that whereas IL-10 does reduce FcεRI-mediated TNF secretion, production of several inflammatory cytokines and chemokines is enhanced in a Stat3-dependent manner. Additional experiments suggest that Stat3 exerts its activity through the induction of miR-155, which suppresses suppressor of cytokine signaling (SOCS)1. These stimulatory effects were consistent among mouse and human MCs in vitro and in an in vivo mouse assay of systemic anaphylaxis. Taken together, these data show that IL-10 can promote IgE-mediated MC activation.

Materials and Methods

Reagents

Recombinant mouse IL-3, stem cell factor (SCF), and IL-10 were purchased from BioLegend (San Diego, CA). Purified mouse IgE (clone C38-2, κ isotype) was purchased from BD Biosciences (BD Pharmingen division, San Diego, CA). Dinitrophenyl (DNP)-coupled human serum albumin (HSA) and LPS (catalogue L-6529) were purchased from Sigma-Aldrich (St. Louis, MO). Histamine was purchased from Enzo Life Sciences (Farmingdale, NY). All Western blotting Abs were purchased from Cell Signaling Technology (Danvers, MA). D. Conrad (Virginia Commonwealth University) provided purified mouse anti-DNP IgE for in vivo experiments. Abs for c-Kit (PE anti-mouse CD117) and FcεRI (allophycocyanin anti-mouse FcεRIα) were purchased from BioLegend and used at a concentration of 1:100. LPS levels were tested using Toxin Sensor 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.

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Abbreviations used in this article: AHR, airway hyperresponsiveness; AUC, area under curve; BMMC, bone marrow–derived mast cell; DNP, dinitrophenyl; HSA, human serum albumin; KO, knockout; MC, mast cell; mMCPT-1, mouse MCPT-1; PSA, passive systemic anaphylaxis; qPCR, quantitative PCR; SCF, stem cell factor; siRNA, small interfering RNA; SOCS, suppressor of cytokine signaling.

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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 *t* test.

Animals

C57BL/6J and C57BL/6J-background miR-155^{-/-} (B6.Cg-Mir155^{tm1.1 Rsky/J}) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and used at a minimum of 8 wk old, with approval from the Virginia Commonwealth University Institutional Animal Care and Use Committee.

Mouse MC cultures

Mouse bone marrow-derived MCs (BMMCs) were cultured, as described (13). Briefly, BMMCs were derived by harvesting bone marrow from femurs of mice and culturing the bone marrow extract in complete RPMI 1640 medium (Invitrogen Life Technologies, Carlsbad, CA) containing 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 1 mM sodium pyruvate, and 10 mM HEPES (Biofluids, Rockville, MD). Complete RPMI 1640 was supplemented with IL-3 containing supernatant from WEHI-3 cells and SCF-containing supernatant from BHK-MKL cells for 21 d. The final concentration of IL-3 and SCF was adjusted to 1.5 and 15 ng/ml, respectively, as measured by ELISA. MC purity was determined to be 99%. Peritoneal cells were extracted in PBS and then expanded in culture for 12 d, as described for BMMCs. Peritoneal MC purity was 83% as determined via flow cytometry for c-Kit and FcεRI double-positive cells.

Human MC cultures

Protocols involving human tissues were approved by the human studies Internal Review Board at the University of South Carolina. Surgical skin samples were obtained from the Cooperative Human Tissue Network of the National Cancer Institute. Skin MCs were cultured, as described previously (14), and were used after 8–16 wk. The purity of MCs was 100%, as determined by toluidine blue staining.

IgE-mediated activation and cytokine, chemokine, protease measurement by ELISA

Human MCs, BMMCs, and peritoneal-derived MCs were sensitized overnight with DNP-specific mouse IgE (1.0 µg/ml for human MC; 0.5 µg/ml for BMMCs and peritoneal MCs). Cells were then washed and resuspended at 1×10^6 cells/ml in complete media with cytokines. Cells were stimulated for 16 h with DNP-HSA (30 ng/ml for human MC, and 50 ng/ml for mouse BMMCs) for assessment of cytokines in supernatant. 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. 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 Cysteineyl Leukotriene ELISA kit from Cayman Chemicals (Ann Arbor, MI), 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, 1×10^6 cells were treated and activated, as described above. Cells were pelleted and lysed in 1 ml PBS plus 1% Igepal CA-630 purchased from USB (Cleveland, OH). Supernatant and lysates were analyzed with mouse MCPT-1 (mMCPT-1) ELISA purchased from eBioscience. Percentage of 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.

Cytokine mRNA and miR-155 quantitative RT-PCR

BMMCs were cultured with or without 50 ng/ml IL-10 for the indicated time points. 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 ultraviolet-vis Spectrophotometer (Thermo Scientific, Waltham, MA), according to the manufacturer's recommended protocol. For cytokine mRNA detection, cDNA was synthesized using the qScript cDNA Synthesis from Quanta Biosciences (Gaithersburg, MD). For miR-155 detection, cDNA was synthesized using qScript microRNA Quantification System using mouse primers for miR-155-5p, miR-155-3p, and SNORD47 from Quanta Biosciences, according to the manufacturer's instructions. Bio-Rad 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 (forward, 5'-TCCAGTTGCTTCTTGGGAC-3'; reverse, 5'-TC-CAGTTGCTTCTTGGGAC-3'), TNF (forward, 5'-AGCACAGAAAG-CATCATCCGC-3'; reverse, 5'-TGCCACAAGCAGGAATGAGAAG-3'), β-actin (forward, 5'-GATGACGATATCGCTGCGC-3'; reverse, 5'-CTCGTCACCCACATAGGAGTC-3'), GAPDH (forward, 5'-GATGACA-TCAAGAAGGTGGTG-3'; reverse, 5'-GCTGTAGCCAAATTCGTTGTC-3'), SOCS1 (forward, 5'-CAGGTGGCAGCCGACAATGCGATC-3'; reverse, 5'-CGTAGTGCTCCAGCAGCTCGAAAA-3'), and SHIP-1 (forward, 5'-GGTGGTACGGTTTGGAGAGA-3'; reverse, 5'-ATGCTGAGCCTCTGTG-TGCT-3') were purchased from Eurofins MWG Operon (Huntsville, AL). Primers for mmu-miR-155-5p, 5'-UUAUUGCUAAUUGUGAUAGGGGU-3', mmu-miR-155-3p, 5'-CUCCUACCUGUAGCAUUAAC-3', and SNORD47, 5'-GUGAUGAUUCGCCAAUUGAUACAAAGUGAUACACCUUUAU-ACCGUUAUUAUUAUUCUGAGG-3' were purchased from Quanta Biosciences. Amplification conditions for microRNA detection were set to heat activation at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 5 s and annealing at 60°C for 30 s. Amplification conditions for all other reactions consisted of a heat-activation step at 95°C for 2 min, followed by 40 cycles of 95°C for 15 s, 55°C for 30 s, and 60°C for 1 min. All melting curve analysis was performed between 50°C and 95°C. Results were normalized to housekeeping genes using relative Livak Method.

Passive systemic anaphylaxis

Age-matched groups of mice (8–16 wk old) received three i.p. injections of 4 µg rIL-10 in 200 µl PBS 24 h prior to receiving DNP-HSA. Anti-DNP IgE (50 µg) was i.p. injected 11.5 h before i.p. injection of DNP-HSA (100 µg). For histamine-induced passive systemic anaphylaxis (PSA), mice were given IL-10 injections, as stated above, and 8 mg histamine was injected i.p. 24 h after the first IL-10 injection. The core body temperature of each mouse was measured using a rectal microprobe (Physitemp Instruments). Mice were euthanized with CO₂ asphyxiation, and blood was collected via cardiac puncture 120 or 180 min after DNP-HSA injection. ELISA was used to analyze plasma cytokine levels.

Western blot analysis

Western blotting was performed using 40 µg total cellular protein, as described previously (13), using the following Abs from BioLegend: phospho-p44/42 MAPK (Erk1/2) (Thr²⁰²/Tyr²⁰⁴) 9101, p44/42 MAPK (Erk1/2) 9102, phospho-stress-activated protein kinase/JNK (Thr¹⁸³/Tyr¹⁸⁵) (G9) mouse mAb 9255, stress-activated protein kinase/JNK Ab 9252, phospho-Stat5 (Tyr⁶⁹⁴) (C71E5) rabbit mAb 9314, Stat5 Ab 9363, Fyn Ab 4023, Lyn Ab 2732, Syk Ab 2712, GAPDH (14C10) rabbit mAb 2118, phospho-Stat3 (Tyr⁷⁰⁵) Ab 9131, and Stat3 Ab 9132. All primary Abs 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 h for pStat5 and Stat5) 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 h 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 (LiCor).

Cell transfection for small interfering RNA knockdown and miR-mimic

BMMCs were transfected with 100 nM STAT3-, or SOCS1-specific, or scrambled FlexiTube small interfering RNAs (siRNAs; a pool of four targeting sequences) from Qiagen (Valencia, CA). miR-155-5p and miR-155-3p mimics were purchased from Exiqon (Woburn, MA) and used at 50 nM concentration. All transfection experiments were done using Amaxa Nucleofector from Lonza (Allendale, NJ) with program T-5 in DMEM with 20% FBS and 50 mM HEPES (pH 7.5) (10). Cells were used 48 h after being transfected.

Flow cytometric analysis

For surface c-Kit and FcεRI expression, cells were treated with IL-10 (50 ng/ml) for 24 h and washed in PBS. Cell pellets were incubated in 10 µl rat anti-mouse FcγRII/III clone 2.4G2 (10 µg/ml) plus staining or isotype control Abs for 20 min at 4°C, washed with PBS, resuspended in FACS buffer (PBS, 3% FBS, 0.1% sodium azide), and analyzed by flow cytometry using a BD FACSCalibur.

Statistical analysis

Data presented are the mean $\pm$ SEM of at least three independent experiments (unless otherwise stated). Paired or unpaired Student *t* test, one-way ANOVA with Tukey post hoc tests, or area under curve (AUC) was used when appropriate, using GraphPad Prism software. Statistical significance was set at $p < 0.05$. In all figures, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

Results

IL-10 enhances Fc ϵ RI-mediated cytokine production in BMMCs

We previously reported IL-10 suppressed Fc ϵ RI-mediated TNF production in BMMCs in vitro and ameliorated PSA (11). In a follow-up study examining a broader array of MC mediators, we were surprised to find that IL-10 enhanced IgE-mediated IL-6 and MCP-1 production, with the greatest effects using 50 ng/ml IL-10 24 h prior to Ag exposure (Fig. 1A, 1B). IL-13 production was also enhanced, whereas TNF secretion was suppressed, as we previously

reported (11) (Fig. 1C). RT-quantitative PCR (qPCR) data showed that IL-6 but not TNF mRNA was upregulated by IL-10 treatment (Fig. 1D). IL-10 treatment for 24 h prior to activation also increased MC degranulation, as indicated by increased mMCPT-1 release (Fig. 1E). Interestingly, the percentage of mMCPT-1 release was unchanged by IL-10 addition, which was explained by an increase in total mMCPT-1 content induced by IL-10 (Fig. 1E).

It has been reported that MCs expanded in IL-3 alone have a mucosal MC phenotype, whereas cells grown in IL-3 plus SCF resemble a connective tissue phenotype (15, 16). IL-10 has also been shown to enhance the growth factor activity of SCF in rat MCs (17). To test whether our results were due to enhanced SCF signaling, we generated BMMCs in WEHI/IL-3 for 21 d with no SCF. These cells were then treated with IL-10 for 24 h. The stimulatory effects of IL-10 were preserved and comparable to SCF-treated cells (Supplemental Fig. 1A). Furthermore, we noted that 24-h treatment with IL-10 enhanced IL-6 production

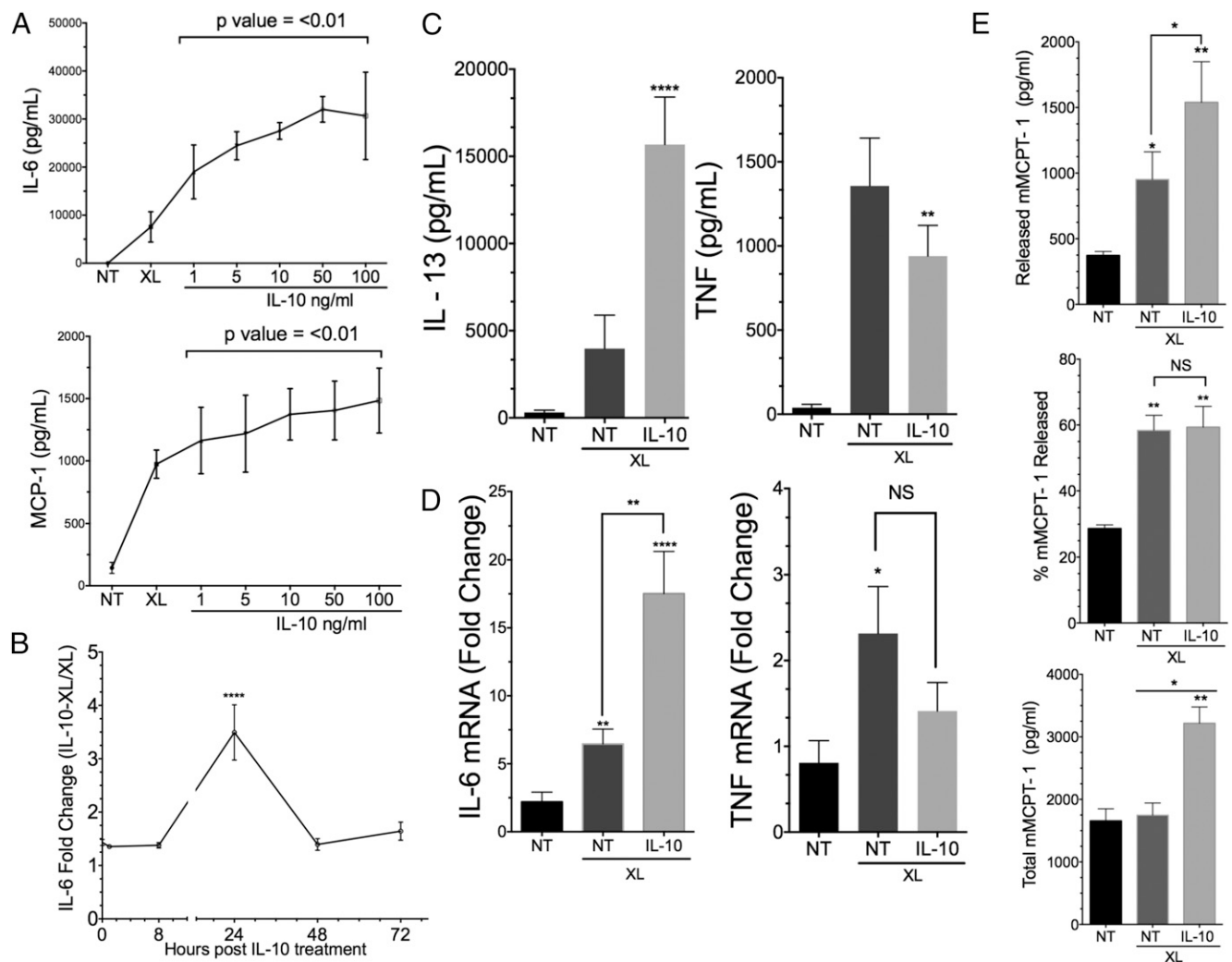


FIGURE 1. Twenty-four-hour IL-10 treatment enhances Fc ϵ RI-mediated cytokine production and degranulation in mouse BMMCs. **(A)** BMMCs were incubated with IgE and the indicated IL-10 concentrations for 24 h prior to Ag-induced IgE cross-linking (XL) for 16 h. Cytokines were measured using ELISA. **(B)** BMMCs were treated as in (A) using 50 ng/ml IL-10 for the indicated times. **(C)** BMMCs were treated as in (A) with 50 ng/ml IL-10 prior to IgE XL for 16 h to measure IL-13 and TNF release. **(D)** BMMCs were treated with IL-10 for 4 h, and mRNA levels were measured by RT-qPCR relative to β -actin. **(E)** BMMCs were treated with 50 ng/ml IL-10 or 24 h prior to IgE XL for 15 min to measure mMCPT-1 levels by ELISA. Cytokines were measured by ELISA. Data are means $\pm$ SEM of four (A and C) and three (B, D, and E) independent experiments done in triplicate. Post hoc comparisons using Tukey honest significant difference test indicated that the mean score for 24-h treatment was significantly different from all other treatment times. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$.

at 5 or 16 h after Ag-induced IgE cross-linking, whereas TNF suppression was only apparent 16 h after Ag addition (Supplemental Fig. 1B). We also tested BMMCs 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 h of IL-10 treatment have proinflammatory effects on IgE responses in mouse MCs, effects that do not require SCF.

IL-10 enhances FcεRI-mediated cytokine production in peritoneal-derived mouse MCs and human skin MCs

We next expanded peritoneal-derived MCs that matured in vivo and treated them with IL-10 for 24 h prior to IgE-induced activation. Peritoneal-derived MCs also showed enhanced inflammatory cytokines with IL-10 treatment (Fig. 2A), suggesting these effects were not due to in vitro MC differentiation. Finally, human skin MCs from five different donors treated with IL-10 24 h prior to Ag cross-linking showed significantly enhanced MCP-1 secretion, whereas TNF production was unchanged (Fig. 2B). These data suggest that the proinflammatory effects of IL-10 are not limited to BMMCs and can be observed in primary MCs from mouse or human sources.

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

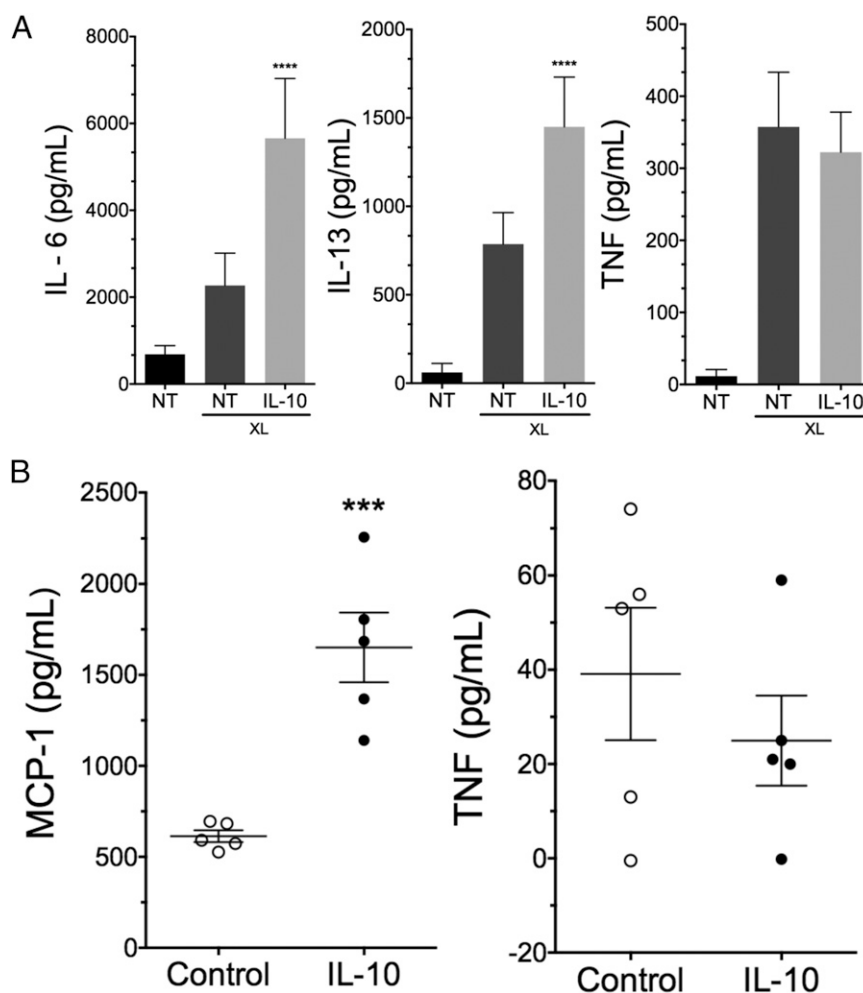
To test the functional relevance of IL-10 treatment under our conditions, we administered three IL-10 injections (4 μg each) prior to inducing IgE-mediated PSA, as described in Fig. 3A. Core body

temperature drop in IL-10-treated mice was almost double compared with controls (Fig. 3B). 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 min of Ag exposure (Fig. 3C) and elevated plasma IL-6 and MIP-1α after 120 min (Fig. 3D). The more severe drop in body temperature could reflect either greater MC activation in the presence of IL-10, or a stronger vascular response to MC mediators, particularly histamine. To assess the effects of IL-10 on the vasculature, we administered three IL-10 injections 24 h prior to injecting 8 mg histamine. No difference was seen between IL-10-treated and control mice (Fig. 3E), 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 proinflammatory effects of IL-10 are functionally significant in vivo, and that this is most likely due to exacerbated MC activation.

IL-10 enhances FcεRI signaling

We previously reported that 3-d culture with IL-10 suppressed FcεRI and c-Kit expression (11, 12), suggesting that perhaps 24-h IL-10 exposure might have an opposite effect. However, IL-10 treatment for 24 h did not alter c-Kit or FcεRI surface expression, as assessed by flow cytometry (Supplemental Fig. 2). 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

FIGURE 2. Twenty-four-hour IL-10 treatment enhances FcεRI-mediated cytokine production in peritoneal-derived mouse or human skin MCs. **(A)** Mouse peritoneal-derived MCs and **(B)** human skin MCs were incubated with IgE and 50 ng/ml IL-10 for 24 h prior to Ag-induced IgE cross-linking (XL) for 16 h. Data are means $\pm$ SEM of (A) three independent experiments done in triplicates or (B) cells from five donors tested six times. Cytokines were measured using ELISA. *** p < 0.001, **** p < 0.0001.



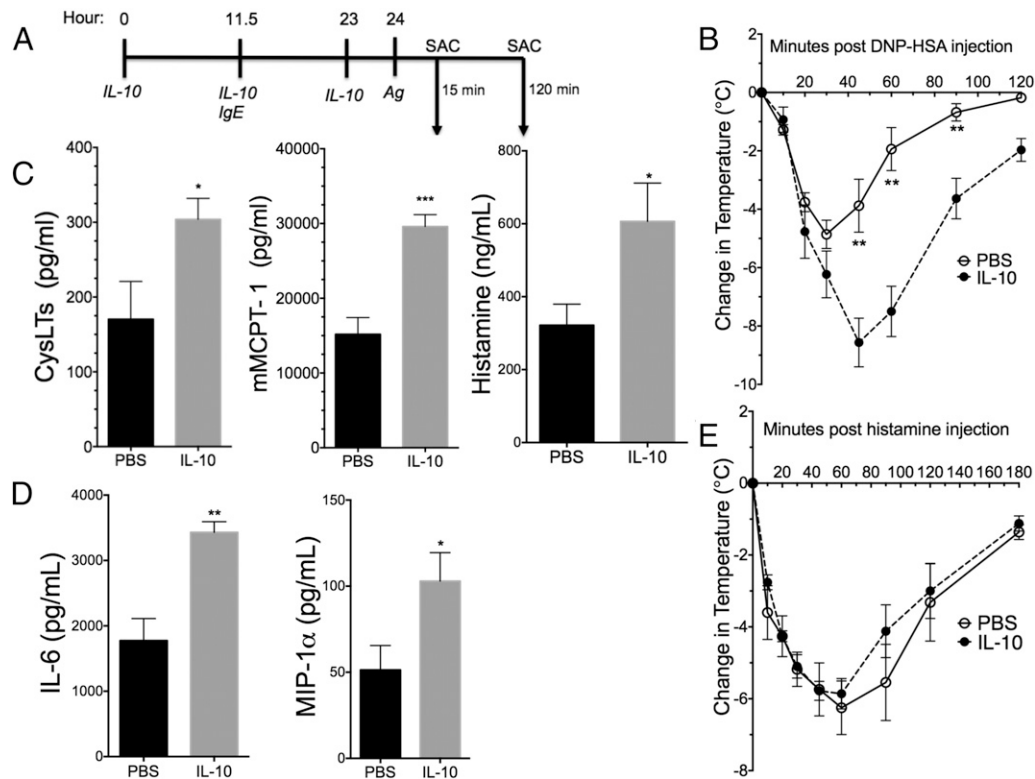


FIGURE 3. IL-10 treatment exacerbates PSA. **(A)** Schematic of PSA assay. SAC, sacrifice. C57BL/6 mice received IL-10 injections and were subjected to PSA, as described in **(A)** and *Materials and Methods*. **(B)** Depicts change in core body temperature. **(C)** Plasma CysLTs, mMCPT-1, and histamine levels from mice sacrificed 15 min post-Ag administration. **(D)** Plasma cytokine and chemokine levels 120 min 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 24 h in place of Ag, and mice were sacrificed 180 min posthistamine injection. **(B)** and **(C)** $n = 5$, representative of three independent experiments. **(D)** and **(E)** $n = 5$. Data are means $\pm$ SEM. In **(B)** and **(E)**, asterisk shows 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). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

receptor. Western blot data showed that IL-10 significantly increased IgE-mediated phosphorylation of JNK, ERK, and Stat5 (Fig. 4A). Although IL-10 has been reported to suppress total Fyn levels in MCs (10), we observed no significant changes in total Fyn, Lyn, and Syk levels with IL-10 treatment for 24 h (Fig. 4B).

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 (18). Twenty-four-hour IL-10 treatment enhanced Stat3 Tyr⁷⁰⁵ phosphorylation, as expected (Fig. 5A). We suppressed Stat3 expression via siRNA transfection (Fig. 5B), and found that the ability of IL-10 to enhance FcεRI-mediated IL-6 or MCP-1 was significantly reduced (Fig. 5C). Thus, Stat3 is critical for IL-10 to increase IgE-mediated cytokine production.

IL-10 induces mir-155

miR-155 is known to be a major contributor to inflammatory diseases and has recently been shown to control MC activation by FcεRI (19, 20). To determine the expression profile of miR-155 in IL-10-treated BMMCs, 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 h of treatment (Fig. 6A). It is noteworthy that miR-155-3p enhancement was several-fold higher than miR-155-5p (Fig. 6A). There is previous evidence that in bone marrow-derived macro-

phages, IL-10 suppresses LPS-induced miR-155 expression (21). To test the importance of lineage and stimulus, BMMCs were treated with 1 μ g/ml LPS $\pm$ 50 ng/ml IL-10. Under these conditions, IL-10 enhanced miR-155-5p and did not change miR-155-3p induction by LPS (Supplemental Fig. 3A), suggesting that cell lineage alters IL-10 effects on miR-155. To determine the functional significance of miR-155, we treated control and miR-155 knockout (KO) BMMCs with IL-10 for 24 h 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 (Fig. 6B).

Because miR-155-5p and -3p have complementary sequences, they most likely have distinct target sets. To determine the importance of each strand, miR-155 KO BMMCs were transfected with control (mock), miR-155-5p, and/or miR-155-3p mimics. Transfection efficiency was measured via qPCR (Supplemental Fig. 4). 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 Fig. 6C, 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.

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 (22). To determine

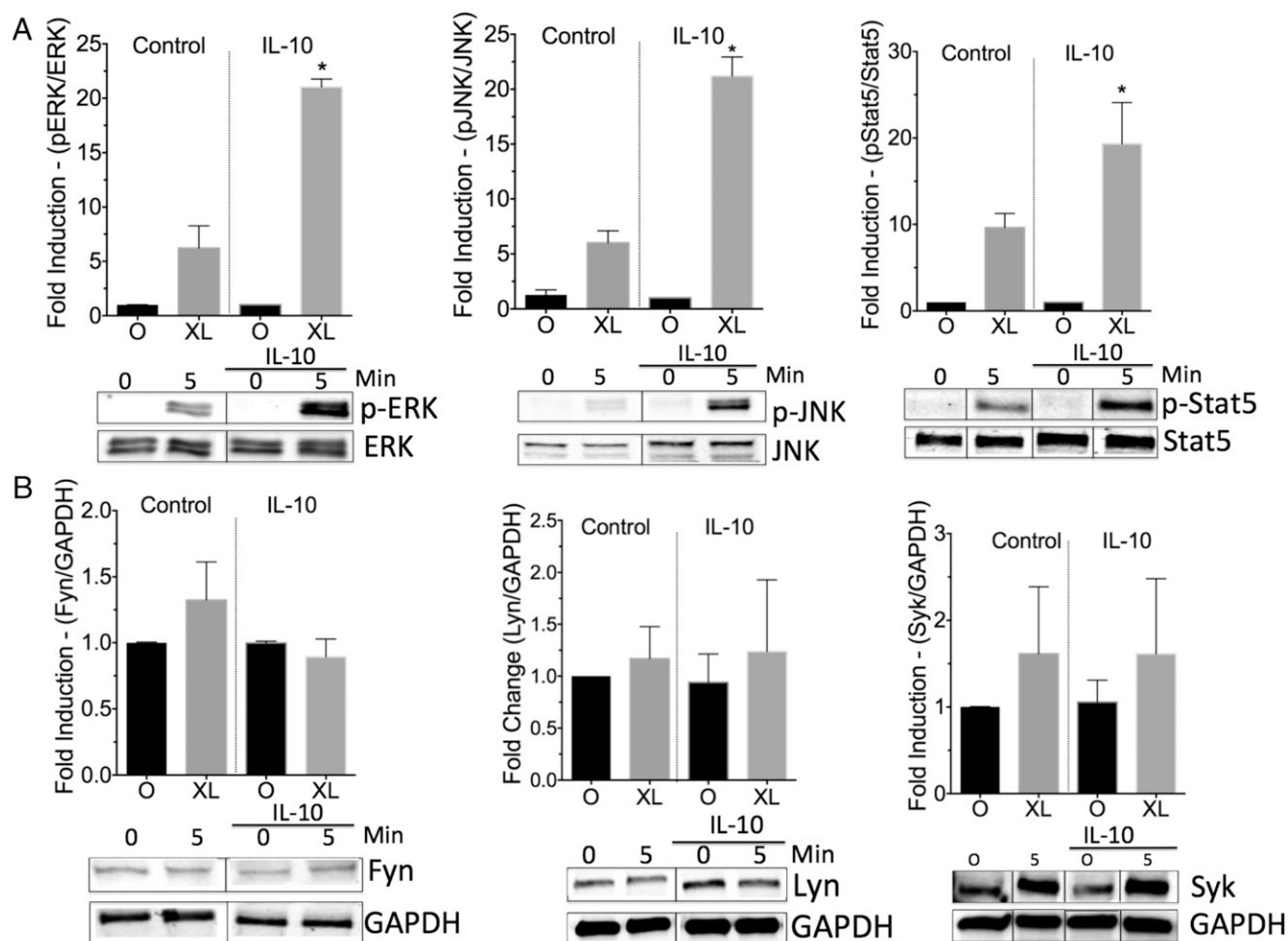


FIGURE 4. (A and B) Twenty-four-hour IL-10 treatment enhances FcεRI signaling. BMMCs were presensitized with IgE and treated with ± 50 ng/ml IL-10 for 24 h, then left unactivated (O) or activated by IgE cross-linking (XL) for 5 min. Lysates were collected and subjected to Western blotting using parameters described in *Materials and Methods*. Data are means $\pm$ 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. * $p < 0.05$.

whether Stat3 is required for IL-10-induced miR-155 expression in MCs, Stat3 was suppressed by siRNA, and miR-155 expression was measured 4 h after IL-10 treatment. Stat3 suppression diminished the ability of IL-10 to induce miR-155-5p and -3p (Fig. 7A). 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 (23–25). IL-10 significantly suppressed SOCS1 mRNA, an effect that was absent in miR-155 KO cells (Fig. 7B). To our surprise, IL-10 enhanced SHIP-1 mRNA levels, and this was conserved in miR-155 KO cells (Fig. 7B). Furthermore, IL-10-mediated SOCS1 suppression was reversed when Stat3 was knocked down (Fig. 7C). To confirm that the enhancement of cytokines with IL-10 treatment was due to SOCS1 suppression, we transfected C57BL/6-derived BMMCs 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 (Fig. 7D). These data support the hypothesis that IL-10 uses Stat3 to induce miR-155, which suppresses SOCS1, resulting in enhanced cytokine production.

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 h before inducing IgE-mediated PSA, as described in Fig. 3A. Similar to the results in Fig. 3, 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 (Fig. 8A). Drop in body temperature was analyzed using both ANOVA and AUC analysis. Consistent with temperature change, plasma mMCPT-1 and CysLTs were significantly elevated in IL-10-treated C57BL/6, but not miR-155 KO mice (Fig. 8B). Similarly, plasma IL-13, MCP-1, and IL-6 were significantly elevated by IL-10 treatment only among C57BL/6 mice (Fig. 8C). Baseline IL-13 and MCP-1 levels between C57BL/6 and miR-155 KO mice were comparable (Supplemental Fig. 3B). Baseline IL-6 levels were undetectable (assay limit of detection was ~ 60 pg/ml). 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 inhibitory effects.

Discussion

IL-10 was originally termed cytokine synthesis inhibitory factor in the context of Th1/Th2 cross-regulation (1). However, support for IL-10-mediated anti-inflammatory effects is not absolute. To our knowledge, this study shows for the first time

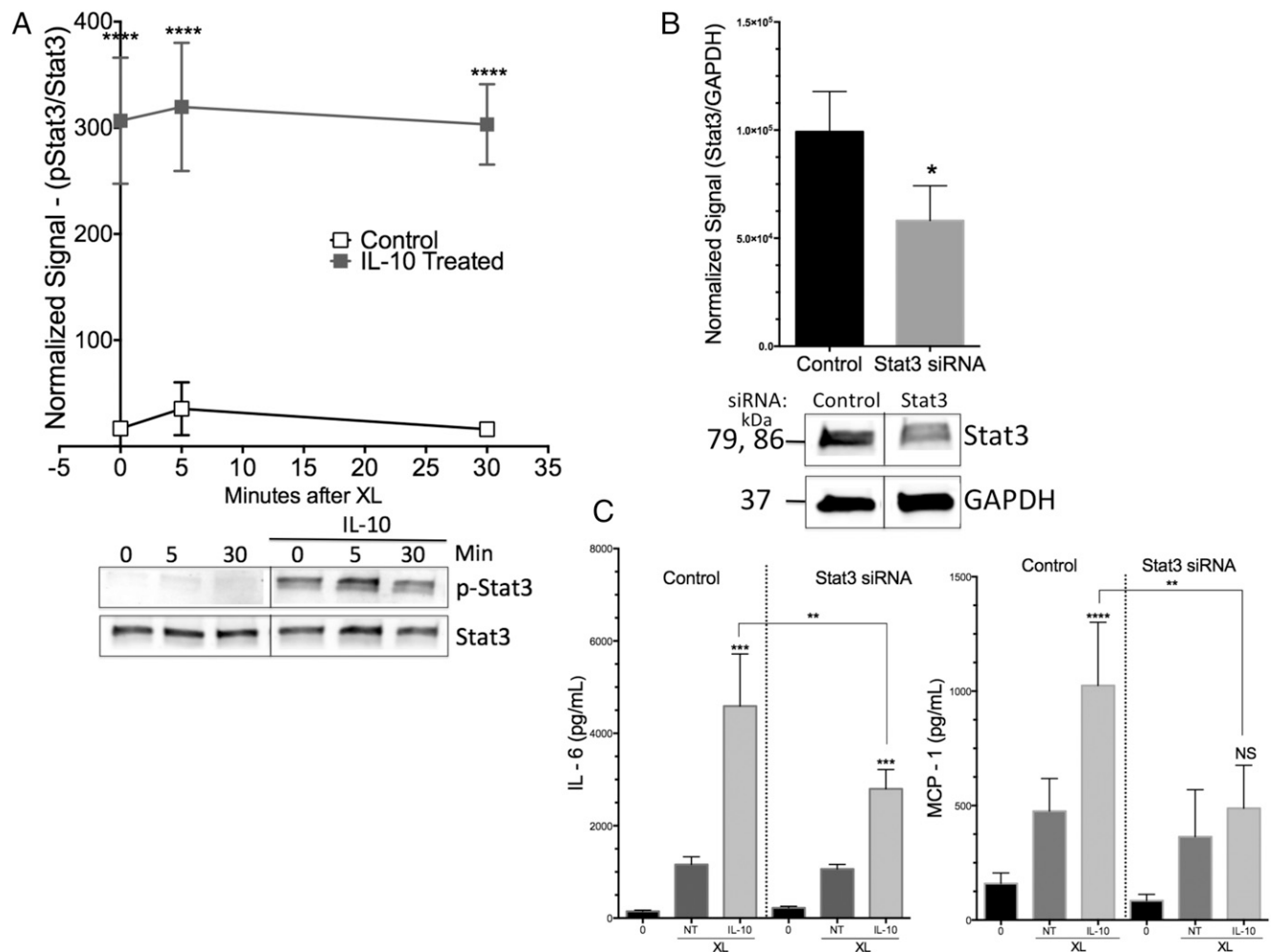


FIGURE 5. IL-10-mediated effects are Stat3 dependent. **(A)** BMMCs were presensitized with IgE and treated with ± 50 ng/ml IL-10 for 24 h prior to Ag-induced IgE cross-linking (XL) for indicated times (bands were cropped to show indicated time points). Lysates were collected and subjected to Western blotting. Graph shows normalized data from $n = 3$ samples. **(B)** BMMCs were transfected with control or Stat3-targeting siRNA, and lysates were collected 48 h later for Western blot analysis. Representative blot is shown for Stat3 suppression (bands were cropped to show one population). **(C)** Transfected cells were treated as described in (A), with IgE XL for 16 h. Supernatants were collected for ELISA analysis. Data are means $\pm$ SD of three (A) and two (B) independent experiments done in triplicate. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

that IL-10 can enhance MC activation and exacerbate anaphylaxis, through a Stat3- and miR-155-dependent process. Previous studies of IL-10 effects on MCs have focused mostly on TNF production. We previously showed that 4-d treatment with IL-10 suppresses TNF production in BMMCs (11). There are also data from rat peritoneal MCs that show TNF mRNA and protein suppression with 18 and 24 h of IL-10 treatment, respectively (17, 26). Interestingly, the same study also showed increased histamine production with 24-h IL-10 (50 ng/ml) treatment (17). 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 MC biology, and how IL-10 may participate in allergic and inflammatory diseases involving MC activation.

Similar to our earlier work addressing IL-10-mediated anti-inflammatory effects (11), we found that Stat3 expression is required for the ability of IL-10 to enhance MC 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 addressed Stat3 effects during IL-6 and IL-10 treatment, which cause proinflammatory and anti-inflammatory effects, respectively (18, 27). Braun et al. (27) 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 dendritic cells led to the same genome-wide proinflammatory transcriptional responses. However, at later time points, the transcriptional responses were the opposite, with IL-10-activated Stat3 upregulating anti-inflammatory genes. This is an attractive theory to explain the current data, as we have previously found that the suppressive effects of IL-10 on MCs require 3–4 d to manifest (11). One possibility is that the duration of Stat3 activation alters its pairing with other transcriptional regulators, allowing for differential gene expression.

MicroRNAs are small noncoding RNAs that are involved in many developmental and pathological processes. miR-155 expression is present in both myeloid and lymphoid cells at varying levels

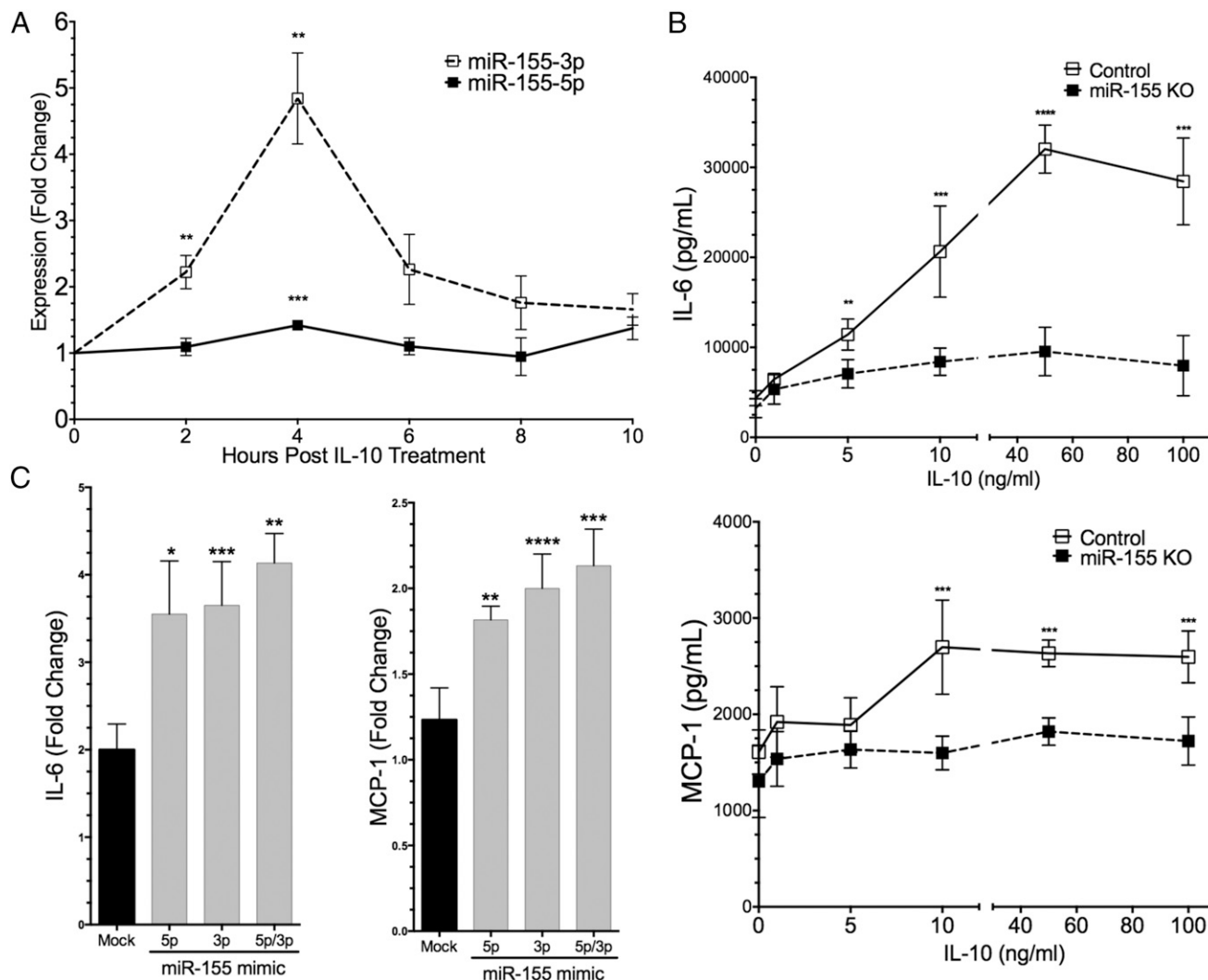


FIGURE 6. IL-10 induces miR-155 expression. **(A)** BMMCs were treated with 50 ng/ml IL-10 for indicated times. qPCR was used to measure miR-155-5p and miR-155-3p expression relative to Snord47. Fold change relative to untreated cells is shown. **(B)** C57BL/6 and miR-155KO BMMCs were presensitized with anti-DNP IgE and treated with $\pm$ IL-10 at the indicated concentrations for 24 h prior to IgE cross-linking (XL) for 16 h. Cytokines were measured via ELISA. **(C)** miR-155 KO BMMCs were mock transfected or received 50 nM miR-155-5p, miR-155-3p, or miR-155-5p/3p mimic 48 h prior to treatment with $\pm$ 50 ng/ml IL-10. Cells were activated, as described in (B), and cytokines were measured via ELISA. Fold change is relative to cells not receiving IL-10. Data are means $\pm$ SEM of five (A), three (B), or two (C) independent experiments done in triplicate. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

of expression. miR-155 has been shown to play a critical role in a variety of cancers, viral infections, and other inflammatory diseases (2). Like most miRs, miR-155 can bind the 3' untranslated region 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 (28). 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 (20). 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 (29, 30). There is also evidence that miR-155 can be anti-inflammatory. One study of MCs revealed that miR-155 targets PI3K γ (19), suppressing MC activation. Paradoxically, miR-155 can be both pro- and anti-inflammatory in the same

cell system. For example, it is well known that in LPS-activated macrophages, TLR-induced miR-155 inhibits TLR signaling by targeting IL-1R-associated kinase 1 and TNFR-associated factor in a negative feedback loop to suppress macrophage activation (31). However, miR-155 overexpression was shown to enhance LPS-induced TNF production both in vitro and in vivo (32).

Our data show that Stat3-dependent miR-155 induction is required for IL-10 to enhance MC activation and exacerbate PSA. Recent data in miR-155 KO BMMCs confirm that miR-155 ablation does not alter MC numbers or Fc ϵ RI α and c-Kit expression (19). Other than the previously published PI3K γ (19), no other target of miR-155 has been identified in MCs. We found that IL-10 can suppress SOCS1 mRNA, a confirmed miR-155 target (23–25), in a Stat3-dependent manner, and that SOCS1 suppression is lost in miR-155 KO MCs. Because SOCS1 is an important negative regulator of MC signaling (33), reducing SOCS1 levels is a logical contributor to the inflammatory effects of IL-10. Our data confirmed this hypothesis,

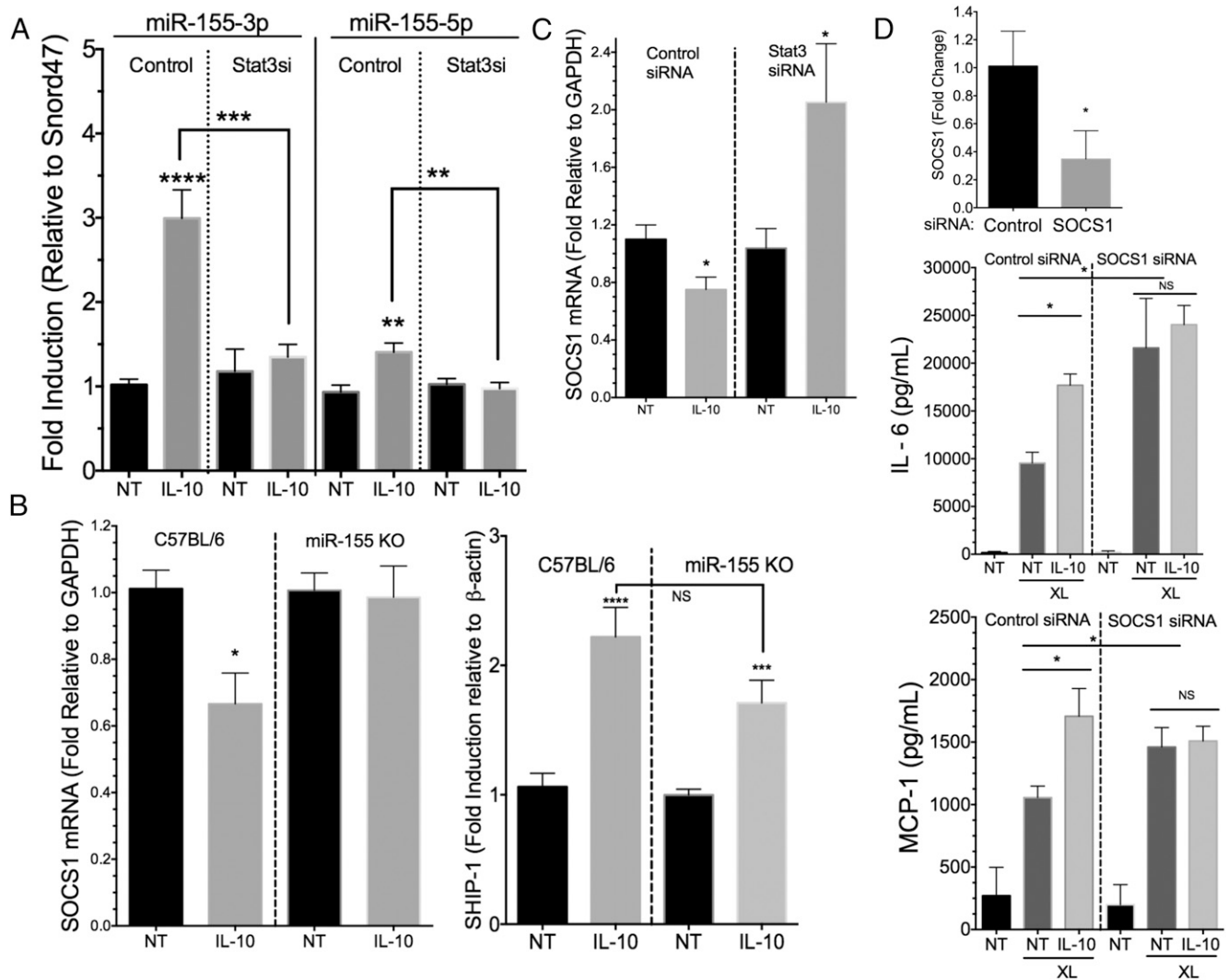


FIGURE 7. IL-10 induction of miR-155 is Stat3 dependent. **(A)** C57BL/6 BMMCs were transfected with control or Stat3 siRNA and treated with ± 50 ng/ml IL-10 for 4 h before mRNA was collected. qPCR was used to measure miR-155-5p and miR-155-3p levels relative to Snord47. **(B)** miR-155 KO and control BMMCs were treated with 50 ng/ml IL-10 for 4 h. SOCS1 and SHIP-1 mRNA were measured via RT-qPCR. **(C)** C57BL/6J BMMCs were transfected with control or Stat3 siRNA, as described in (A), and SOCS1 mRNA levels were measured using RT-qPCR. **(D)** C57BL/6J BMMCs were transfected with control or SOCS1 siRNA, and SOCS1 mRNA suppression was measured via RT-qPCR. Cells were treated with ± 50 ng/ml IL-10 for 24 h prior to IgE cross-linking (XL) for 16 h. Cytokines were measured by ELISA. Data are means $\pm$ SEM of three (A and B) or two (C and D) independent experiments done in triplicate. NT, not treated (no IL-10). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

because IL-10 was unable to enhance IgE-mediated cytokine production after SOCS-1 depletion.

To our surprise, IL-10 treatment enhanced SHIP-1 mRNA, an effect that persisted in miR-155 KO BMMCs. 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 (34). 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-h IL-10 treatment time.

IL-10 is upregulated in many human inflammatory diseases and in animal models such as endotoxemia (6). High serum IL-10 has been suggested as an indicator of poor prognosis in cancers (35) and sepsis (36). However, these data are mostly correlative. Therefore, it is still not understood whether 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 regard to MCs, that designates IL-10 as a possible inducer of inflammation.

One noteworthy example is the role of IL-10 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 rIL-10 (37–39). In animal models one mechanism of AHR development is through MC activation and Th2 cytokine release (40). Because allergic sensitization is required for IL-10 to induce AHR and IL-10 does not directly trigger smooth muscle contraction (39), we speculate that IL-10 might act partly by enhancing IgE-induced signaling in MCs to upregulate Th2 cytokine production. To our knowledge, there are no studies addressing the effect of

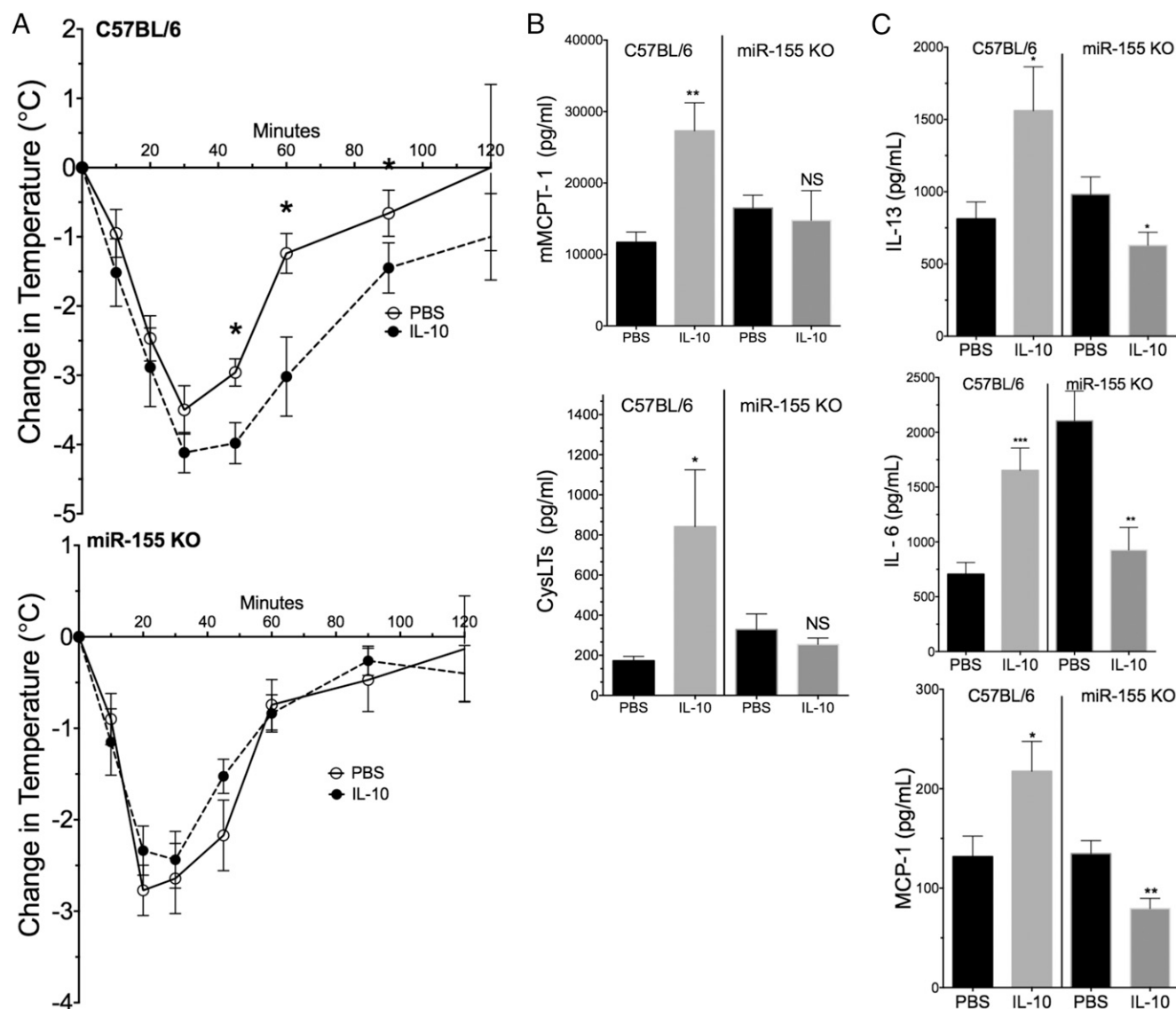


FIGURE 8. IL-10 treatment does not affect PSA in miR-155 KO mice. **(A)** C57BL/6J and miR-155KO mice were subjected to IL-10 or PBS i.p. injections and PSA, as described in Fig. 3A and 3B. **(B)** Plasma mMCPT-1/CysLTs (15 min post-Ag injection) and **(C)** cytokines (120 min post-Ag injection) were measured by ELISA. Data are means $\pm$ SEM of eight mice for temperature change and cytokine measurements and five mice for MCPT-1 and CysLT. In **(A)**, asterisk 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). * p < 0.05, ** p < 0.01, *** p < 0.001.

IL-10 on MCs 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 (41). 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 (37–39). There is, however, no substantial data on the role of rIL-10 therapy in asthmatic patients. It would be of interest to study the role of IL-10-induced miR-155 expression in a MC-dependent asthma model.

Our data show IL-10 stimulatory effects on human MCs, suggesting that IL-10 could exacerbate disease states in which MCs are active. There is support for this. For instance, human skin MCs have been found to be major producers of the IL-10 homolog IL-22 in psoriasis patients, leading to increased psoriatic plaques (42). One clinical study found that IL-10 therapy in psoriasis patients exacerbated inflammation and increased Th2 cytokines (9). Although miR-155 expression was found to be lower among benign

skin disorders (including psoriasis) than in cutaneous T cell lymphoma (43), 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 MCs. Although 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 MC activation coincide.

Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

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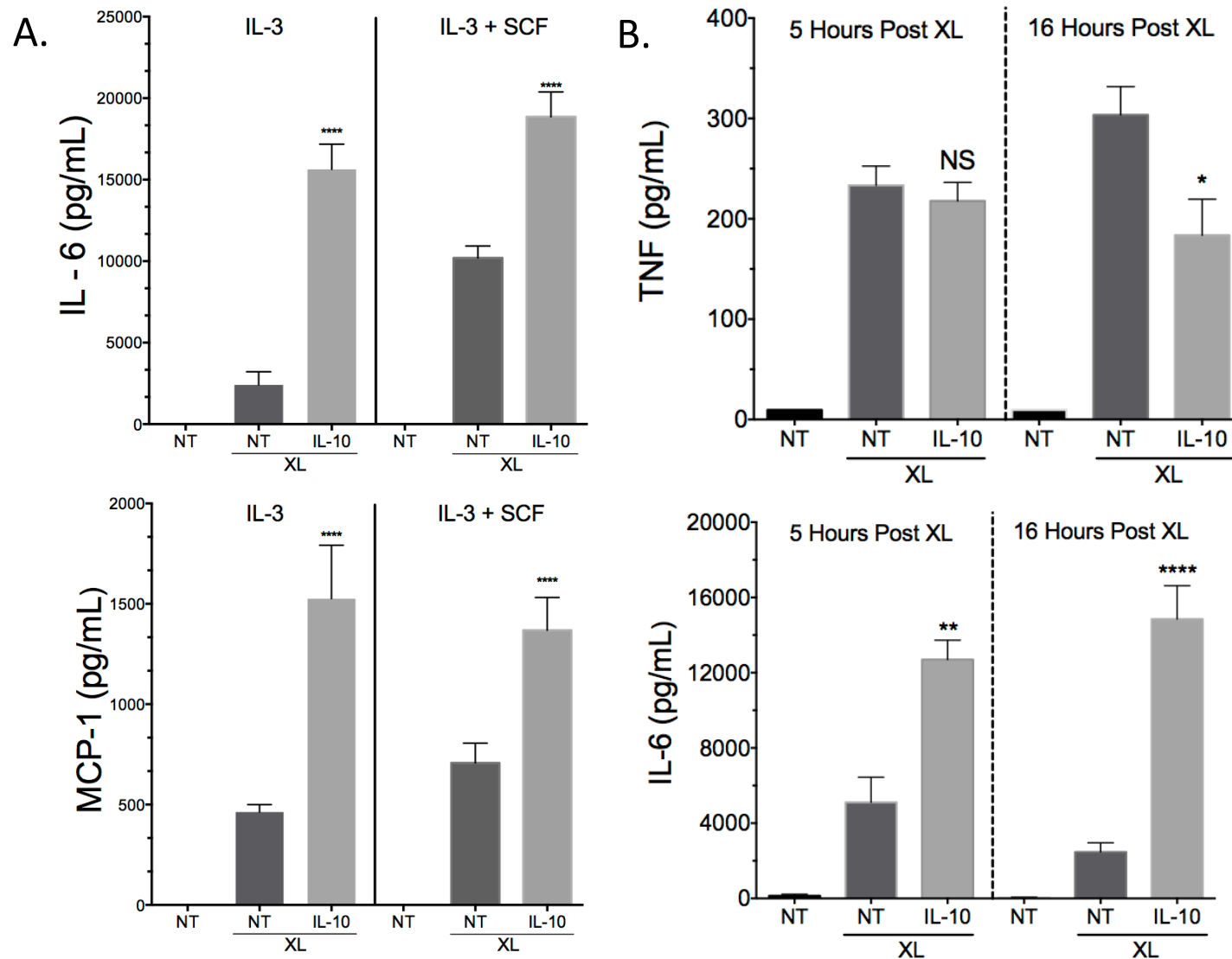


Figure S1: (A) BMMC were cultured as described previously with slight alternations. Briefly, bone marrow cells were collected from femurs of C57BL/6J mice and expanded in WEHI-IL-3 (1.5ng/ml) $\pm$ BHK-SCF (15ng/mL) for 21 days. Cells were then treated with 50ng/ml of IL-10 for 24 hours in the presence of 10ng/ml IL-3 prior to IgE XL for 16 hours. Data are means $\pm$ SEM of two independent experiments done in triplicate. p-value based on Student t test, between Ag-XL and Ag-XL with 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 $\pm$ SEM of 3 populations tested in triplicates.

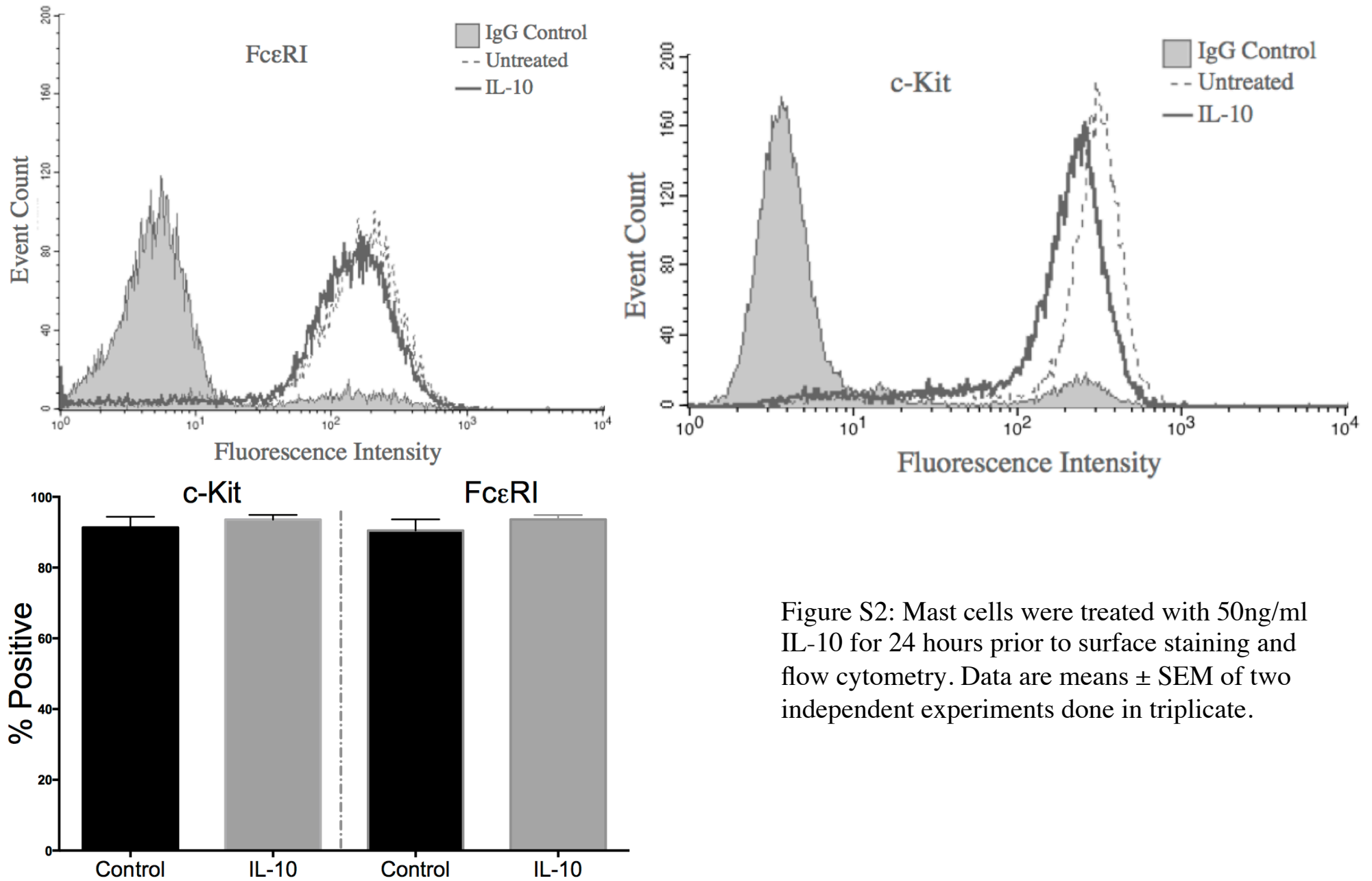


Figure S2: Mast cells were treated with 50ng/ml IL-10 for 24 hours prior to surface staining and flow cytometry. Data are means $\pm$ SEM of two independent experiments done in triplicate.

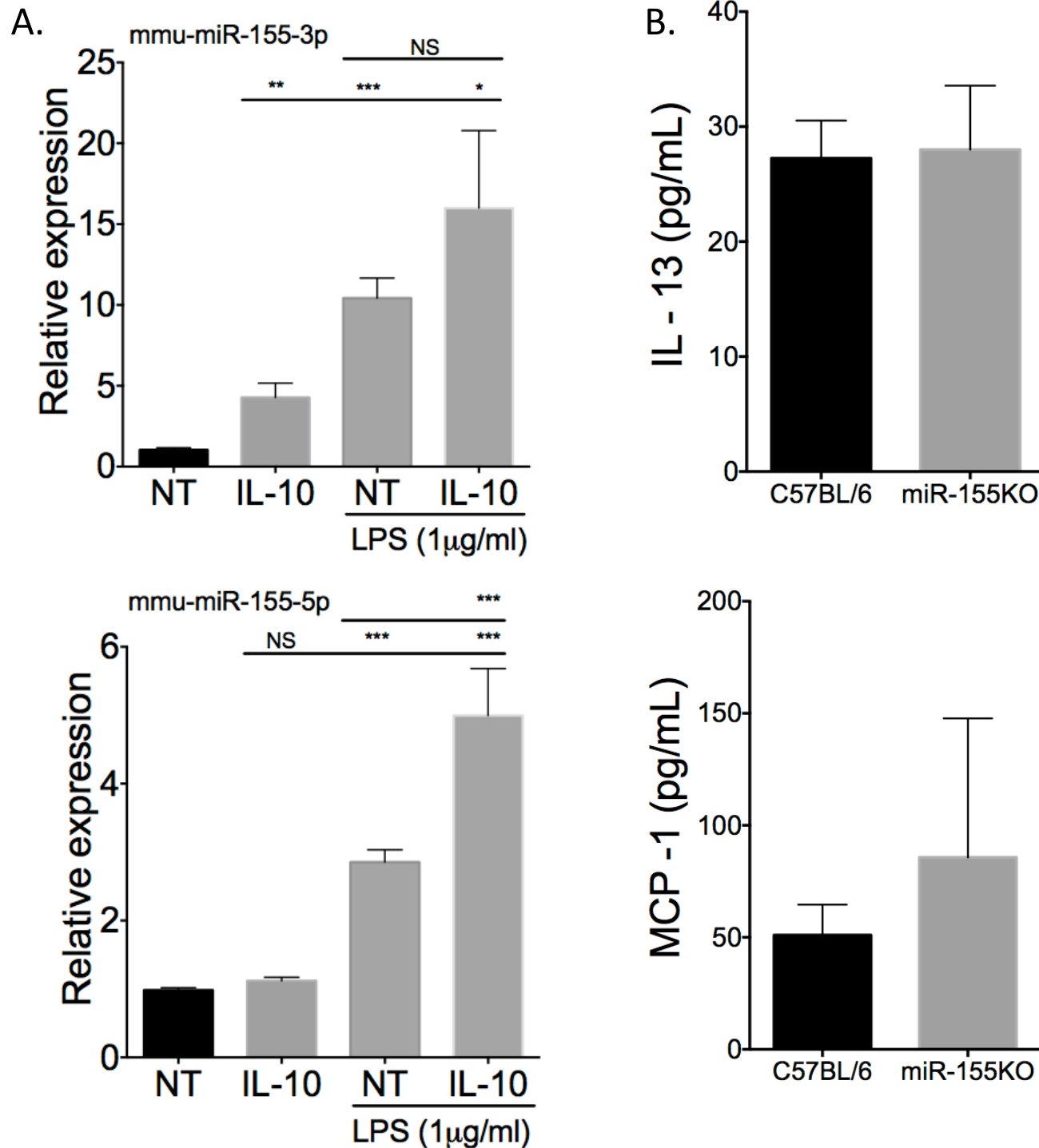


Figure S3: (A) BMMC were treated with 1µg/ml LPS and/or 50ng/ml IL-10 for 4 hours. qPCR was used to measure miR-155-5p and miR-155-3p expression relative to Snord47. Fold change relative to untreated cells is shown. Data is mean $\pm$ SEM of three populations analyzed in triplicates. (B) Plasma was collected 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.

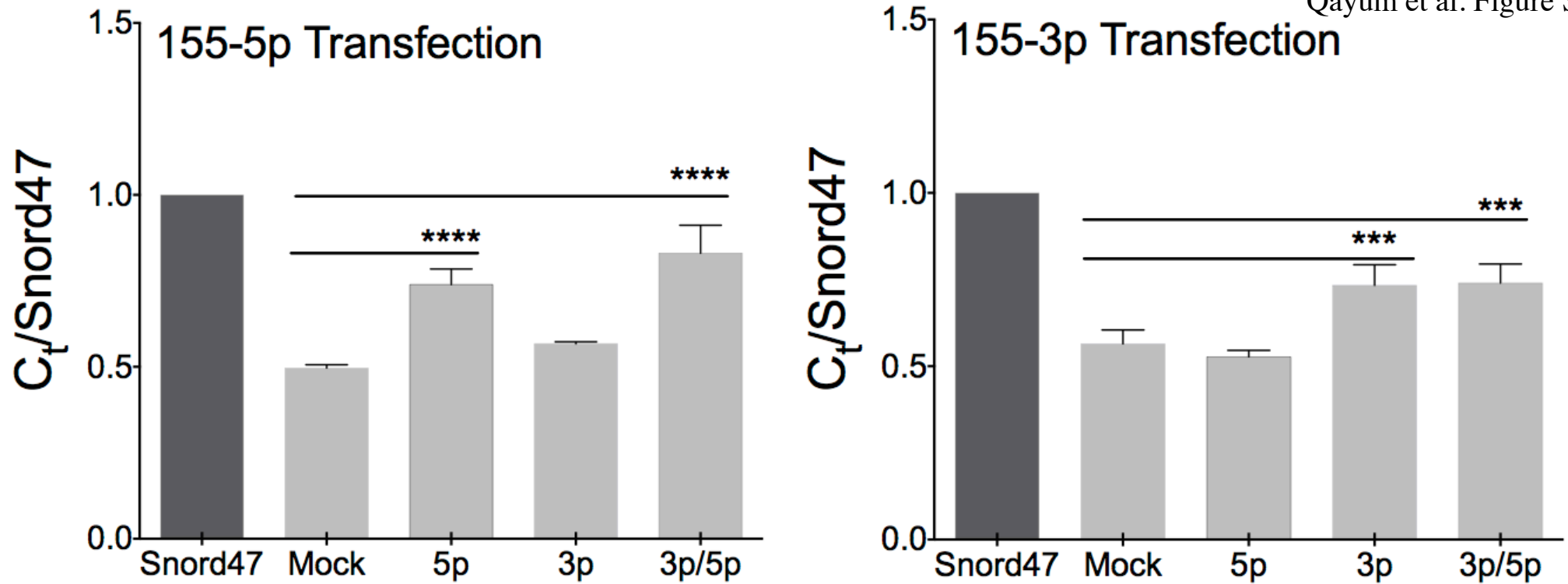


Figure S4: BMMC from miR-155 KO mice were transfected with Mock (control), miR-155-5p, miR-155-3p and miR-155-5p/3p mimics. miR transfection was validated 48 hours post transfection via qRT-PCR. Data are means $\pm$ SEM of two independent experiments done in triplicate with representative of one experiment shown.