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ADAM10: a Novel Regulator of Mast Cell Function and Activation

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

by

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Bachelor of Science in Biology

Virginia Commonwealth University, 2008

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List of Abbreviations

ADAM10- A Disintegrin and Metalloproteinase 10

ADAM17 (TACE)- A Disintegrin and Metalloproteinase 17

AHR- Airway hyper-responsiveness,

BHK- Supernatant from SCF producing cells

BMMC- Bone marrow-derived mast cells

B220- A B cell marker

CD3- Cluster of Differentiation 3, a T cell marker

CD4- Cluster of Differentiation 4, a T helper cell marker

CD8- Cluster of Differentiation 8, a CTL marker

CD11b- A myeloid cell marker

CD23- Cluster of Differentiation 23, aka FcεRII

CD44- Cluster of Differentiation 44

c-Kit- SCF receptor

Cre- A recombinase that recognizes DNA segments flanked by loxP sites

CTL- Cytotoxic T cell

DNP-HSA- Dinitrophenylated human serum albumin

DL1- Delta-like 1, a Notch ligand

EGF- Epidermal growth factor

FcεRI- High-affinity IgE receptor

FcεRII- Low-affinity IgE receptor, aka CD23

F4/80- A macrophage marker

Gr1- A granulocyte cell marker

IgE- Immunoglobulin E

IgG- Immunoglobulin G

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

IL-4- Interleukin 4, promotes IgE production by B cells

IL-5- Interleukin 5, attracts eosinophils

IL-6- Interleukin 6, a proinflammatory cytokine

IL-6R- Interleukin 6 receptor

IL-10- Interleukin 10, an inhibitory cytokine

IL-12- Interleukin 12, important in T helper cell 1 differentiation and NK cell activation

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

IL-17- Interleukin 17, a proinflammatory cytokine

KO- Knockout

MAPK- Mitogen-activated protein kinases

MICA/MICB- Molecular Histocompatibility Complex- Class I-Related Chain A or B

MIP-1 α - Macrophage inflammatory protein 1 α

Mx1- An Interferon-inducible promoter

NF κ B- Nuclear factor κ B

NK cell- Natural Killer cell

NKG2D- Natural Killer G2D (a receptor on NK cells)

N2ICD- Notch 2 Intracellular Domain

RIP- Intramembrane proteolysis

SCF- Stem cell factor, vital for mast cell survival

STAT3- Signal transducer and activator of transcription 3

Tg- Transgene/Transgenic

TGF- β 1- Tumor Growth Factor β 1, an inhibitory cytokine

TNF- α - Tumor Necrosis Factor α , an proinflammatory cytokine

Treg- T regulatory helper cells

WEHI- Supernatant from IL-3 producing cells

Abstract

ADAM10: A NOVEL REGULATOR OF MAST CELL FUNCTION AND ACTIVATION

Travis Wright Faber, Bachelor of Science in Biology

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biology at Virginia Commonwealth University, 2012.

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

In this study we show, to our knowledge, the first description of the role ADAM10 plays on mast cells. ADAM10 is abundantly expressed on mast cells both in vitro and in vivo. Its expression is inhibited by IL-10, a suppressive cytokine. siRNA depletion of ADAM10 on bone marrow-derived mast cells (BMMC) caused decreased IL-6 production following IgE cross-linking and also impaired BMMC stem cell factor (SCF)-induced migration through collagen IV. Mast cells and T helper cells (Th cells) in the peritoneum were reduced in ADAM10 KO mice. In addition, ADAM10 KO BMMC produced significantly less of all cytokines measured following IgE cross-linking, including IL-6, TNF- α , IL-13, and MCP-1, compared to wild type

BMMC. Collectively these data show that mast cell ADAM10 can be regulated by a T regulatory cell cytokine, IL-10, and describes key ways in which ADAM10 loss affects prototypical mast cell functions and distribution.

Introduction

Mast cells are sentinels of the innate immune system, lying in wait to wreak havoc upon invading pathogens. Unfortunately, mast cells are also able to respond to innocuous airborne allergens. They do this primarily through antigen (Ag)-induced cross-linking of IgE bound to the high-affinity IgE receptor, FcεRI (1-6). The resulting signal cascade induces mast cells to release a myriad of early and late phase mediators. These include tryptases, chymases, histamine, prostaglandins, leukotrienes, and Platelet Activating Factor, which are released in the first few minutes after activation, and cytokines such as IL-4, IL-5, IL-6, IL-10, IL-13, tumor necrosis factor- α (TNF- α), and macrophage inflammatory protein-1 α (MIP-1 α) produced hours later (1-6). This diverse group of proteases, eicosanoids, cytokines, and chemokines performs a variety of immunomodulatory effects, but mainly are inflammatory in nature (1-6).

Mast cells are most known for their roles as primary effector cells in immediate hypersensitivity, and the dreaded histamine release following exposure to allergens. Initial reactions following exposure are responsible for the wheal-and-flare, vasodilatation, rhinitis, and itchiness associated with allergies, and long-term illnesses like asthma can be caused or worsened by repeated exposures to these same allergens (1-7). They are known to play a

prominent role in asthma, where their presence contributes to airway remodeling due to chronic inflammation that is in part dependent on cytokines like IL-4, IL-5, and IL-13 (7). Despite their roles in allergy and asthma that most immunologists know them for, mast cells have been implicated in immunosuppressive roles as well, such as in graft tolerance and protection in acute nephritis (8, 9).

A Disintegrin and Metalloproteinase 10 (ADAM10) is a member of the *A Disintegrin and Metalloproteinase* family, whose members cleave myriad types of transmembrane proteins through ectodomain shedding or regulated intramembrane proteolysis (RIP) (10-12). In an example of the former, TNF- α is released from the membranes of cells by ADAM17 (10). In a common example of RIP, binding of Delta-like 1 (DL1) on one cell to Notch2 on an adjacent cell results in ADAM10 cutting Notch2, followed by γ -secretase activity that allows Notch2 Intracellular Domain (N2ICD) to translocate to the nucleus and transcribe genes such as Hes1 and Hes5 (13). In addition, ADAMs have been proposed to cleave in trans, i.e, an ADAM from one cell cutting cell surface proteins off another cell, but this event has been less noted and studied (14).

ADAMs have pleiotropic effects; for instance, the arguably most studied ADAM, ADAM17 (TACE), can cleave TNF- α , CD44, Delta-like ligand 1 (DL1), and Notch1 (10). Typically, members of the ADAM family, including ADAM10, have been implicated in cancer, development, and Alzheimer's disease (15). ADAM10 has been shown to shed MICA, an MHC-like molecule which binds to NKG2D used by Natural Killer cells (NK cells) and Cytotoxic T cells (CTLs) to identify stressed cells or tumor cells to mediate cell killing (16). MIC molecules are highly expressed on many epithelial tumors and shedding of MICA by ADAM10 can potentially lead to tumor evasion, allowing the tumor to escape the immune system and

eventually metastasize (16). ADAM10 has been implicated in the proper distribution, development, and function of neurons, and is important for proper cleaving and/or signaling of various protein signaling pathways including N-Cadherins, Ephrin, Notch, and γ -Protocadherins (12). In Alzheimer's disease, ADAM10 functioning as an α -secretase is thought to play a neuroprotective role, lessening the accumulation of amyloidogenic β -peptide and bettering the memory loss associated with Alzheimer's (12). For this and other reasons, ADAM10 and the other ADAMs have been researched primarily in cancer, development, and Alzheimer's disease, while their immunological role still requires elucidation.

ADAM10 cleaves many substrates of interest to immunologists, notably Notch 1, Notch 2, DL1, CD23, TNF- α , and IL-6R (11, 17-24). ADAM10-dependent Notch signaling has been demonstrated in recent years to be crucially important for the proper differentiation and distribution of T cells and B cells (18, 25, 26). The critical nature of ADAM10 was demonstrated when gene-deleted mice were found to die at day embryonic 9.5 (27). This lethal phenotype led investigators to develop mice exhibiting cell-specific ADAM10 deletion using the Cre recombinase/loxP system (18, 25). These systems utilize the Cre recombinase/loxP system to make cell specific ADAM10 knockouts, with Cre under the control of various promoters such as CD19 or Lck (18, 25). Gibb et al. showed CD19-Cre ADAM10 KO mice have increased cell-surface, and decreased soluble CD23 in vivo and in vitro, as well as a marked absence of marginal zone B cells due to insufficient Notch2 signaling (18). Similar methods have been used in lck-Cre T-cell specific Cre/loxP systems involving ADAM10 dependent Notch1 signaling (25).

The aforementioned CD23 is the low-affinity Immunoglobulin E (IgE) Receptor (Fc ϵ R2), which plays an inhibitory role in B cell IgE production (28). When cell surface CD23

expression is elevated, IgE production decreases; similarly, when CD23 levels are decreased, IgE production is enhanced (29, 30). Since ADAM10 cleaves CD23, the prevailing thought is that ADAM10 negatively regulates CD23 expression and ergo increases Antigen-specific IgE production by inhibiting this IgE/CD23 feedback mechanism. Evidence supporting this was found by Mathews et al. utilizing ADAM10-specific inhibitors (22). Cumulatively, these results show an integral role for ADAM10 in allergy, asthma, and lymphocyte development; an extension of these experiments in the case of allergy and asthma is to explore the role that ADAM10 plays on mast cells, one of the main allergic effector cells.

In this study we show, to our knowledge, the first description of the role ADAM10 plays on mast cells, though its presence has been noted previously (31,32). ADAM10 is abundantly expressed on mast cells both in vitro and in vivo. Its expression can be inhibited by IL-10, a cytokine known for its suppressive function and produced by T regulatory cells (Treg). Additionally, siRNA depletion of ADAM10 on bone marrow-derived mast cells (BMMC) causes decreased IL-6 production following IgE cross-linking. Loss of ADAM10 also impairs stem cell factor (SCF)-induced migration of BMMC through collagen IV, a component of the basal lamina. In addition, peritoneal mast cells along with T helper cells (Th cells) were found in vastly reduced proportions in an inducible ADAM10 knockout model. ADAM10 KO BMMC produced significantly less of all cytokines measured following IgE cross-linking, including IL-6, TNF- α , IL-13, and MCP-1, when compared to wild type BMMC. Collectively these data show that mast cell ADAM10 can be regulated by a T regulatory cell cytokine, IL-10, and describes key ways in which ADAM10 loss affects prototypical mast cell functions and distribution.

Materials and Methods

Animals

C57BL/6, Mx1CreTg C57BL/6, and ADAM10^{fl/fl} C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and used at a minimum of 6 wk old, with approval from the Virginia Commonwealth University institutional animal care and use committee. STAT3 ^{-/-} mice and controls were kindly provided by John O'Shea (NIAMS/NIH, Bethesda, MD). Mx1CreTg mice were bred with ADAM10^{fl/fl} mice, and through successive breeding developed mice that were Mx1CreTg ADAM10^{fl/fl} (hereafter referred to as ADAM10 KO mice), or Mx1CreTg ADAM10^{+/+} (hereafter referred to as ADAM10 WT mice) as a negative control. All mice used were on a C57BL/6 background.

Genotyping of mice involved lysing tails in a proteinase K lysing buffer. ~5 mm tail portions were placed in eppendorff tubes with 250 μ L of lysing buffer overnight in a 55° C waterbath. In the morning, temperature was increased to 85°C for 45 minutes; tubes were centrifuged at 14000 RPM, and 200 μ L of supernatants were extracted. PCR was performed according to Jackson Laboratory protocols for Mx1Cre expression and ADAM10 expression utilizing primers of as follows: Mx1Cre 5' GCGGAGCCAGCACTATTTA 3' and 5'

CCGGCATCAACGTTTTCTTTT 3', and ADAM10 5' GAGAGGAAAGAAAGTGGCAGA 3' and 5' AGTGGGTGGGTTAATGAGCA 3'.

Mouse Mast Cell Cultures

Mouse bone marrow-derived mast cells (BMMCs) were derived from mice by culture in complete RPMI (cRPMI) 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 1 mM HEPES (all from Biofluids, Rockville, MD), supplemented with IL-3-containing supernatant from WEHI-3 cells and SCF-containing supernatant from BHK-MKL cells. The final concentration of IL-3 and SCF were adjusted to 1 ng/ml and 10 ng/ml, respectively, as measured by ELISA. BMMC were used after 3 weeks of culture.

Cytokines and Reagents

All cytokines, including IL-3, IL-4, IL-5, IL-6, IL-10, IL-12, IL-17 TGF-β, IFN-β, and SCF were purchased from PeproTech (Rocky Hill, NJ). Normal goat IgG and rat-anti mouse ADAM10 primary and secondary antibodies were purchased from R&D (Minneapolis, MN). Rat IgG was kindly provided by Dr. Daniel Conrad (VCU, Richmond, VA). PE-Goat anti-rat IgG was purchased from Southern Biotech (Birmingham, AL). Collagen IV was purchased from BD Biosciences (Bedford, MA). Antibodies recognizing mouse B220, FcεRI, F4/80, Mac1, Gr1, c-Kit, CD3, CD4, and CD8 were purchased from BD Pharmingen (San Diego, CA). Propidium iodide and DNP-HSA were purchased from Sigma-Aldrich (St. Louis, MO).

siRNA Depletion of ADAM10

BMMC were pelleted in groups of 3×10^6 cells, and 10 μL of 20 μM pooled scrambled control siRNA or pooled ADAM10 siRNA (Thermo Fisher Scientific, Waltham, MA) was added to the cell pellet, followed by 100 μL AMAXA Cell Line Nucleofector Kit V buffer (Lonza, Basel, Switzerland). The pellet was resuspended, and pipetted into a cuvette. The cuvette was placed into an AMAXA Nucleofector II device, electroporated on the human monocyte setting, resuspended in 3.5 mL cRPMI + WEHI/BHK, and cultured for 3 days. Efficacy of gene suppression was measured via flow cytometry; only BMMC populations with 50% or greater ADAM10 negative cells were utilized in experiments.

Flow Cytometric Analysis

Surface expression of proteins was measured by flow cytometry on a BD FACScalibur. For 2-step staining, $1\text{--}2 \times 10^5$ cells were pelleted, washed in FACS buffer (PBS, 3% FBS, 0.1% sodium azide), then blocked with 10 μL Goat IgG at 50 $\mu\text{g}/\text{mL}$ in FACS buffer and incubated at 4°C for 10 minutes. 10 μL of 20 $\mu\text{g}/\text{mL}$ rat IgG or rat anti-mouse ADAM10 was added to the cells and incubated at 4°C for 30 minutes. Cells were washed in FACS buffer, resuspended in 10 μL of 2 $\mu\text{g}/\text{mL}$ PE-goat anti-rat IgG diluted in FACS buffer and then incubated at 4°C for 30 minutes. Cells were washed in FACS buffer, resuspended in FACS buffer, and ADAM10 levels were analyzed. For directly-labeled antibody staining, cell pellets were incubated in 10 μL 2.4G2 rat anti-mouse Fc γ RII/III culture supernatant with PE anti-mouse ADAM10 and 10 $\mu\text{g}/\text{mL}$ FITC-labeled B220, Fc ϵ RI, F4/80, Mac1, Gr1, c-Kit, CD3, CD4, or CD8 at 4°C for 30 minutes. Cells were washed in FACS buffer, resuspended in FACS buffer, and analyzed.

Measurement of ADAM10 Levels in Peritoneal Cells

C57BL/6 mice were injected intraperitoneally (i.p.) with 3 mL PBS, agitated, and then PBS was recovered. Cells were assessed by flow cytometric analysis as described above.

Effects of Cytokines on ADAM10 Expression In Vitro

Unless otherwise noted, BMMC were cultured in cRPMI supplemented with 10 ng/mL IL-3 +/- 50 ng/mL of IL-10, TGF- β 1, IL-17, IL-4, IFN- γ , IL-6, IL-5, or IL-12 for 3 days. ADAM10 expression was calculated as fold of control measured by flow cytometry.

mRNA Measurement

Cells were harvested and total RNA was extracted with TRIzol reagent (Life Technologies, Grand Island, NY). cDNA was synthesized using the Verso cDNA Kit (Thermo Scientific, Waltham, MA) following the manufacturer's protocol using oligo dT primers provided in the kit. cDNA was quantified using the Thermo Scientific NanoDrop™ 1000 UV-Vis Spectrophotometer (Thermo Scientific, Waltham, MA) according to manufacturer's recommended protocol. cDNA was either analyzed immediately or stored at -20°C until use. qPCR analysis was performed with the ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) and SYBR® Green detection using a relative standard curve method. Each reaction was performed according to the manufacturer's protocol using 100ng of sample cDNA, 12.5µl of 2X Absolute QPCR SYBR® Green Fluorescein Mix (Thermo Scientific, Waltham, MA) and ADAM10 or GAPDH (housekeeping gene) primers in a final reaction volume of 50µl. Following the addition of all sample DNA or standard DNA and reagents, the plates were sealed with a MicroAmp Optical Adhesive film (Applied Biosystems, Foster City, CA) and centrifuged at 3,000 rpm for approximately 20 seconds. Amplification conditions for all reactions consisted of a heat-activation step at 95°C for 15 minute followed by 40 cycles of 95°C for 15s, 53°C for

30s and 72°C for 34s. Fluorescence data was collected during the extension step of the reaction. The instrument was set to run in 9600 emulation mode, with auto ramping. Resulting data were analyzed with ABI's SDS v1.2 software package using a manual C_t of 0.20 and the auto baseline setting.

For each gene amplified, a standard curve from serial dilutions of a known concentration of cDNA was achieved. The standard curve consisted of eight 2-fold dilutions of a 400ng/reaction cDNA sample taken from unstimulated B6 x 129 wild-type cells. The standard curve dynamic range used in this study was selected such that a reliable standard curve encompassing most unknown C_t values was generated. For each gene being analyzed, the input (concentration) of each unknown sample was calculated by comparing its C_t value with the standard curve using the formula $10^{[(C_t - b)/m]}$ where b is the y-intercept of the standard curve and m is the slope of the standard curve. ADAM10 relative expression values were normalized by dividing by the amount of GAPDH.

Cytokine Measurement

BMMC treated with ADAM10- depleting or scrambled siRNA were cultured in cRPMI with 10 ng/mL IL-3 +/- 0.5 µg/mL IgE overnight at a concentration of 5×10^5 cells/mL. BMMC were washed in PBS, resuspended at 1×10^6 cells/mL in cRPMI with 10 ng/mL IL-3 +/- 50 ng/mL DNP-HSA for 16 hours, after which supernatants were taken. IL-6, IL-13, TNF- α , and MCP-1 supernatant levels were measured by ELISA kits from PeproTech (Rocky Hill, NJ). These experiments were repeated with ADAM10 KO mice using 10 ng/mL of DNP-HSA.

Migration Assay

8 μm polycarbonate 24 well transwell inserts from Corning were coated with 1 $\mu\text{g}/\text{cm}^2$ mouse collagen IV resuspended in 0.05 M HCl. Plates were incubated for 1 hour at room temperature to allow the collagen to solidify. ADAM10 siRNA-depleted or control scrambled siRNA-treated BMMC were resuspended at 2×10^6 cells/mL and starved in FBS-free cRPMI containing 10mg/ml bovine serum albumin (BSA) for 2 hours. A chemotactic gradient was created by adding 700 μL of FBS-free cRPMI/BSA with IL-3 at 0.5 ng/mL +/- 100 ng/mL SCF in the bottom well and 200 μL of the previously starved BMMC supplemented with 0.5 ng/mL IL-3 in the upper well. Cells were incubated for 16 hours at 37° C and live cells that had migrated to the bottom chamber were enumerated via timed counting by flow cytometry, using propidium-iodide exclusion staining. Fold of negative control (media lacking SCF) for each population was calculated for all groups.

To assess IgE-induced migration, ADAM10 siRNA-depleted or control scrambled siRNA-treated BMMC pre-incubated +/- IgE were resuspended at 2×10^6 cells/mL in FBS-free cRPMI/BSA for 2 hours. A chemotactic gradient was created by adding 700 μL FBS-free cRPMI/BSA with IL-3 at 10 ng/mL in the bottom well +/- 50 ng/mL dinitrophenyl-coupled human serum albumin (DNP-HSA), and 200 μL of the previously starved BMMC supplemented with 10 ng/mL IL-3 in the upper well. Live cell numbers were determined by timed flow cytometry counting as above.

Results

ADAM10 Is Expressed by Many Peritoneal Cells Including Mast Cells

Since ADAM10 has been shown to exist on various immunological cells, including mast cells, we decided to expand on the small amount of data regarding mast cell ADAM10 expression by harvesting mouse peritoneal lavage cells. We measured surface ADAM10 on several cell types via lineage markers with flow cytometry (Figure 1). We corroborated work done by other groups in determining that many cell types express surface ADAM10, including peritoneal mast cells (Figure 1A) (31,32). A clear majority of peritoneal mast cells (c-Kit+) extracted, ~85%, were surface ADAM10-positive. This was significantly greater than all other cell types examined, which had minor ADAM10-positive subpopulations, ranging from 10-45%. These included B cells (B220+), Th cells (CD4+), CTL (CD8+), and Macrophages (CD11bhi) (Figure 1A). In addition, peritoneal mast cells expressed ADAM10 at levels that were 2-3 times higher than all other cell types examined, suggesting that ADAM10 is of potential importance in mast cell biology (Figure 1B). These mast cell data encouraged us to investigate ADAM10 regulation and its role in mast cell function.

IL-10 Reduces ADAM10 Expression

Building on our previous experiments showing ADAM10 expression on mast cells, we examined whether different cytokines had positive or negative effects on BMMC ADAM10 surface expression as measured by flow cytometry. We chose cytokines representing the functional responses of Treg, Th1, Th2, and Th17 cells, which are known to orchestrate immune responses. BMMC were cultured for 3 days with several cytokines, including IL-10, TGF- β 1, IL-12, IFN- γ , IL-4, IL-5, IL-6, and IL-17, in order to determine the effects on ADAM10 surface levels. Only IL-10 had any effect on BMMC ADAM10 surface expression, decreasing ADAM10 surface levels (Figure 2). IL-10 is available from many cell sources, including Treg, Th1, and Th2 cells, and known to elicit immunosuppression. These data suggest that ADAM10 expression can be limited by extrinsic factors, including IL-10.

To further characterize the nature of ADAM10 regulation by IL-10, we determined the concentration and time dependence of IL-10 effects on mast cell ADAM10 expression (Figure 3). Increasing the IL-10 concentration reduced ADAM10 surface expression by approximately 70% at 100ng/ml IL-10, with half-maximal inhibition at approximately 0.5ng/ml (Figure 3A). Surface ADAM10 decreased over the course of 4 days in response to IL-10 (Figure 3B). Surface ADAM10 levels eventually returned to base levels after 7 days (data not shown). In order to determine if changes in ADAM10 surface levels were matched by altered mRNA expression, we measured ADAM10 expression by qPCR over the 4-day time course. We observed that decreases in surface ADAM10 levels were preceded by reduced ADAM10 mRNA levels after 1 day of IL-10 stimulation and that this mRNA reduction eventually rebounded after reaching its nadir on day 2 (Figure 3C). Because many IL-10 effects, especially those requiring mRNA alterations, involve the transcription factor STAT3, we assessed the effects of IL-10 on wild type and STAT3 KO BMMC. As shown in Figure 3D, IL-10 down regulation of ADAM10 surface

expression was effectively abolished in BMMC lacking the STAT3 transcription factor, even when high concentrations of IL-10 were added to cultures.

ADAM10 Depletion by siRNA Abrogates IgE Stimulated BMMC Cytokine Signaling

In order to determine the importance of ADAM10 to mast cell function, we transiently depleted ADAM10 by siRNA transfection. BMMC were only used if transfection resulted in at least 50% reduction in ADAM10 positive cells as measured by flow cytometry (Figure 4). IgE-induced cytokine secretion was assessed, including IL-6, IL-13, TNF- α , and MCP-1. While complete depletion of ADAM10 was unobtainable by this method, 50% reduction in ADAM10 was sufficient to reduced IgE-mediated IL-6 production was reduced to approximately 50% of its levels in control BMMC (Figure 5A). Intriguingly, only IL-6 production was affected, whereas IL-13, TNF- α , and MCP-1, showed no significant difference (Figure 5B-D). It was interesting that this fold reduction was noted even in basal IL-6 released from unstimulated BMMC (Figure 5A). This suggests that ADAM10 plays a role upstream of IL-6 production, regardless of stimulation by IgE+Antigen.

ADAM10 Depletion by siRNA Abrogates BMMC Migration to SCF

Since ADAM10 has been shown to cleave many substrates involved in adhesion and migration, we hypothesized that the loss of ADAM10 would impair the ability of BMMC to migrate through the ADAM10 substrate collagen IV to known BMMC chemotactic factors such as SCF and Ag (11). SCF is both chemotactic and mitogenic for mast cells, whereas collagen IV is widely known as an integral part of the basal lamina (33). Using collagen IV-coated transwells, we showed that ADAM10 knockdown via siRNA significantly inhibited SCF-induced migration (Figure 6A). We expected this trend to be consistent in IgE+Antigen-induced

migration, but in fact, IgE+Antigen induced migration was not affected by ADAM10 knockdown (Figure 6B). ADAM17 has been shown to shed mast cell c-Kit, and due to the ability of ADAM10 and ADAM17 to cleave some of the same substrates as indicated previously, there was some suspicion that ADAM10 may be cleaving c-Kit as well. However, we did not observe any differences in surface c-Kit levels, in addition to surface FcεRI levels, on ADAM10 knockdown BMMC and control BMMC (data not shown).

ADAM10 KO Mast Cells Mirror the Effects of ADAM10 siRNA Depletion

In order to better study the expression and importance of ADAM10, we developed an inducible Cre/loxP mouse with systemic ADAM10 deletion, since traditional ADAM10 KO mice die during embryogenesis by day 9.5 (27, 34). Cre was induced under control of the Mx1 promoter, which is elicited by type I interferons produced during viral infection. After i.p. injection of poly I:C, which mimics double-stranded RNA, type I interferons are produced in an anti-viral reaction, which activates the Mx1 promoter (35). This activates Cre recombinase, which excises exon 3 of the ADAM10 gene, rendering it non-functional. The phenotypic characterization of the very similar inducible ADAM10KO of Yoda et al. was described in a recent article, in which they found splenomegaly and myeloproliferation in mice 4 weeks after poly I:C injection (34). Our initial findings mirror theirs with enlarged spleens present 2 weeks after administration of poly I:C, in addition to accumulation of Gr1+CD11b+ cells in the spleen (data not shown). BMMC derived from ADAM10 KO mice had undetectable surface ADAM10 levels (Figure 7).

IgE crosslinkage demonstrated reduced production of IL-6, TNF- α , IL-13, and MCP-1 when comparing ADAM10 KO BMMC to those derived from wild type littermates (Figure 8A-

D). Additionally, repetition of the collagen IV BMMC migration experiment mirrored results found with siRNA ADAM10 depletion; SCF-induced migration through collagen IV is abrogated in ADAM10 KO BMMC (Figure 9).

ADAM10 KO Mice have Decreased Peritoneal Mast Cell Numbers

We measured the relative abundance of different cell lineages in the peritoneal cavity of ADAM10 KO mice. ADAM10 was undetectable on the surface of B cells (B220+), CTL (CD8+), macrophages (CD11b^{hi}), and neutrophils (SSC^{hi}, Gr1+) from ADAM10 KO mice. All of these cell types were present in equal proportions between wild type and ADAM10 KO mice (Figure 8). In contrast, CD4 Th cell and mast cell populations were decreased to approximately ~40% of wild type levels (Figure 10).

Discussion

While ADAM10 is well known for its role in development, cancer, and Alzheimer's disease, a groundswell of papers reporting on its immunologic functions has arisen in recent years (10-12, 15). The role of ADAM10 in addition to TACE/ADAM17 as a sheddase of TNF- α has been debated, but recent work makes clear that both are capable of cleaving TNF- α and that cleavage events seem to be context dependent (17, 23). ADAM10, effective in site 2 cleavage in Notch1 and 2 signaling, is critically important to proper development of T cells, in addition to marginal zone B cells (13, 18, 25). In addition to being important in lymphocyte development, several papers by the Conrad group in recent years have shown ADAM10 regulating B cell Ig production, including being the primary sheddase of CD23, a negative regulator of IgE production (13, 17-22, 26). Importantly for our work, Mathews et al. showed in an IgE/mast cell dependent airway hyperresponsiveness model (AHR) that B cell ADAM10 deficiency decreased specific IgE in bronchoalveolar lavage fluid (BALF), and reduced eosinophils and IL-5 production, usually attributed to Th2 cells or mast cells (22). Due to the connections of ADAM10 with allergies and asthma established by these studies, the extension of this work was to investigate the effects of ADAM10 expression on one of the effector cells of these conditions, mast cells.

The presence of ADAM10 on mast cells explanted from human lung tissue and HMC-1, a human cell line, has been reported as one of many mast cell proteases (31, 32). Accordingly, our work provides the first specific exploration of the role of ADAM10 with regard to mast cell function. Additionally, the nearly ubiquitous presence and relative amount of mast cell surface ADAM10 compared to other cell types implies this sheddase plays an important part in mast cell biology, as our work demonstrates. ADAM10 certainly can be considered proinflammatory considering it is important in shedding of TNF- α (17, 23). The probable proinflammatory role of mast cell ADAM10 is reinforced by our data indicating IL-10-mediated downregulation. We and others have shown that IL-10 serves as an autocrine and paracrine suppressor of the mast cell response, reducing IgE-mediated cytokine secretion and degranulation. The suppressive effects of IL-10 require approximately 3 days to manifest, fitting the same time course of ADAM10 inhibition (36-44). We postulate that ADAM10 blockade is one of several anti-inflammatory effects of IL-10, designed to limit mast cell-mediated pathology. This could have implications in contact hypersensitivity, asthma, and other mast cell mediated diseases

The role of mast cell cytokines in diseases such as allergy, asthma, and parasitic infections has been well studied (1-7). Our data indicate that ADAM10 works in a proinflammatory manner, and is important for the production of IL-6, TNF- α , MCP-1, and IL-13 following IgE cross-linking (Figures 5A-D, 8A-D). Mast cell IL-13 can contribute to airway remodeling and mucus production in asthma, as mast cells are found throughout the smooth muscle of bronchial passages (7). IL-6 and TNF- α are hallmarks of inflammation, inciting the egress of other cells to sites of inflammation; in this capacity, mast cells can attract and activate other immune cells following exposure to allergens in diseases like atopic dermatitis (45). Mast cells produce many chemokines, such as MCP-1 and MIP-1 α , which can assist in chronic

allergic reactions (1). Collectively, any chronic disease involving IgE activation of mast cells could potentially be helped through loss or perhaps inhibition of ADAM10; Mathews et al., have demonstrated this to be so in mast cell dependent AHR (22).

TACE/ADAM17 has been shown to be important in murine embryonic stem cell-derived mast cell survival because of its role in c-Kit shedding (31). The overlap of ADAM10/ADAM17 substrates suggests that ADAM10 could regulate c-Kit shedding as well; however, none of our experiments showed any differences in mast cell c-Kit levels, in either siRNA ADAM10 knockdown populations or ADAM10 KO mice compared. Importantly, our data indicate a potential role for ADAM10 in Kit signaling, since SCF-mediated migration through collagen IV was decreased in the absence of ADAM10 despite no effect on cell surface expression (Figures 6A, 9). Migration of mast cells to other stimuli could be impacted as well, though we found that IgE-mediated movement towards antigen was unaffected (Figure 6B). In tandem with our cytokine data, these results indicate that ADAM10 has pleiotropic effects on mast cell function, including migration and effector functions, warranting further study.

As studies have indicated a role for ADAM17 in SCF-mediated survival of mast cells, future efforts should determine whether ADAM10 affects this as well (31). This is intriguing, as we noted a >50% decrease in mast cell numbers among ADAM10 KO mice (Figure 10). The lack of mast cells found within the peritoneum of ADAM10 KO mice is potentially very important; our data indicate that in some cases, mast cell migration is dependent on ADAM10 (Figure 6A-B, 9). This however, does not prove that mast cell infiltration of the peritoneum is impaired because the possibility remains that ADAM10 is crucially important for the normal differentiation and growth of mast cells. Indeed, ADAM10 has been implicated in aberrant growth and differentiation of marginal zone B cells, and famously, T cells (18, 25). It is probable

that ADAM10 deficiency negatively impacts mast cell development, as Notch2 signaling and CD44 have been implicated in the differentiation of mast cells previously (46-48). To date, we have found no differences regarding proliferation and survival of ADAM10 KO BMMC.

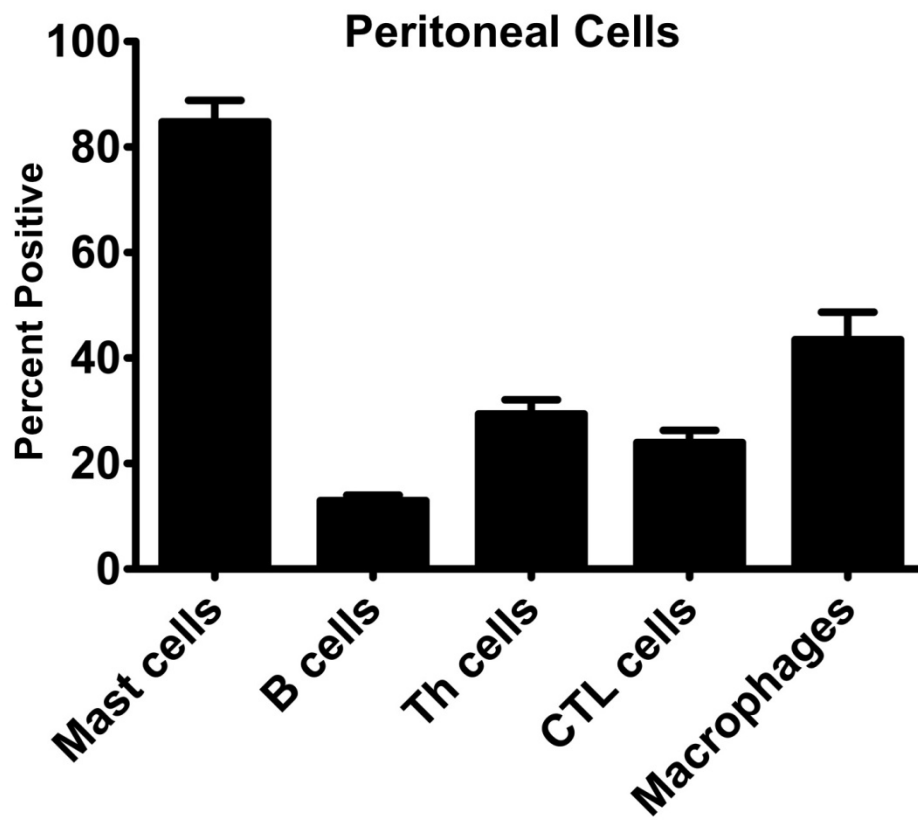
Mast cells have been widely noted for their role in many immune responses, including allergies, asthma, helminth infections, and also allograft tolerance (49,50). Several reports have described important mechanisms involving ADAM10 substrates on mast cells that could potentially rely on proper ADAM10 function for complete or partial occurrence (47, 48 50-52). For instance, CD44, which binds hyaluronic acid and undergoes ectodomain shedding by ADAM10, is important for the distribution of mast cells in mice throughout the body, including the peritoneum (48, 54). Nakano et al. showed that DL1-dependent Notch1 signaling confers Antigen-presenting capabilities to BMMC (51, 52). Along the same line, Sakata et al. have shown that Notch2 signaling is important in mast cell fate determination, but also that Notch2 expression is required for proper distribution of intestinal mast cells and eradication of *Strongyloides venezuelensis* (47, 48). These responses could depend on ADAM10, and merit further study. Important for future studies examining the role of mast cell ADAM10, human mast cells have been shown to associate with bronchial smooth muscle cells in vitro via CD44 and collagen I interactions (53). Our results show that ADAM10 has important roles in mast cell function, including migration and cytokine secretion. The mechanisms by which these functions are accomplished, and the potential for targeting ADAM10 in mast cell-associated diseases warrants further study.

Figures

Figure 1A-B

Figure 1: Peritoneal lavage was performed on C57BL/6 mice. Cells were then stained with the indicated lineage markers and Mean Fluorescence Intensity (MFI) was measured by flow cytometry. A . Percent of each lineage marker that was ADAM10 positive. B. MFI of ADAM10 staining. Data shown are representative of 3 experiments with n=3-5 each experiment. Bars are mean +/- Standard Error (SE).

A



B

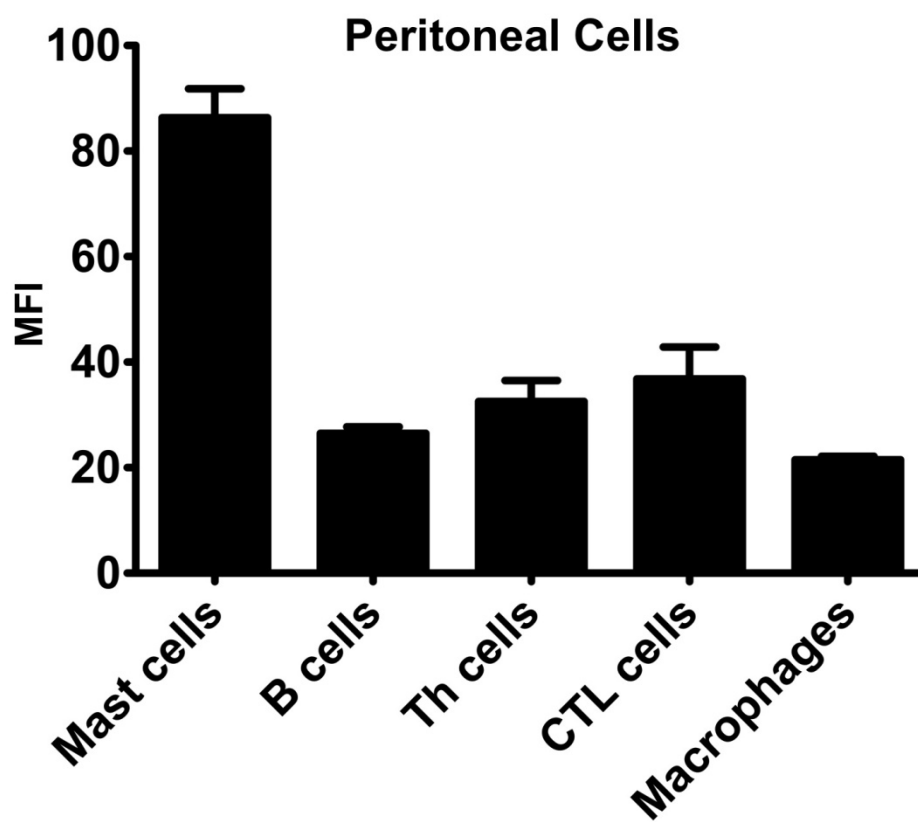


Figure 2

Figure 2: BMMC were cultured for 3 days in the presence of IL-3 alone or IL-3 and one of each of the indicated cytokines at 50 ng/mL, after which ADAM10 levels were measured by Flow Cytometry. Data shown are representative of 3 experiments with at least 6 samples. Bars are mean \pm SE. *, $p < 0.05$.

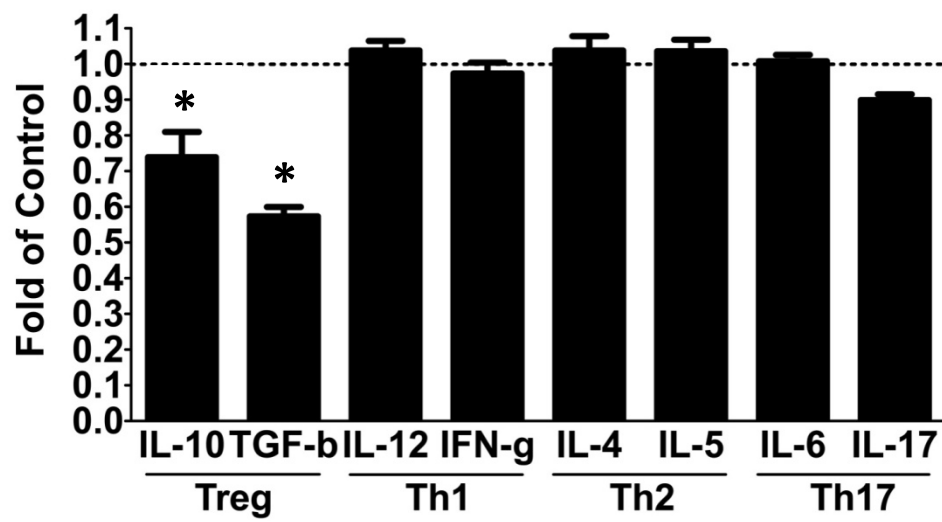
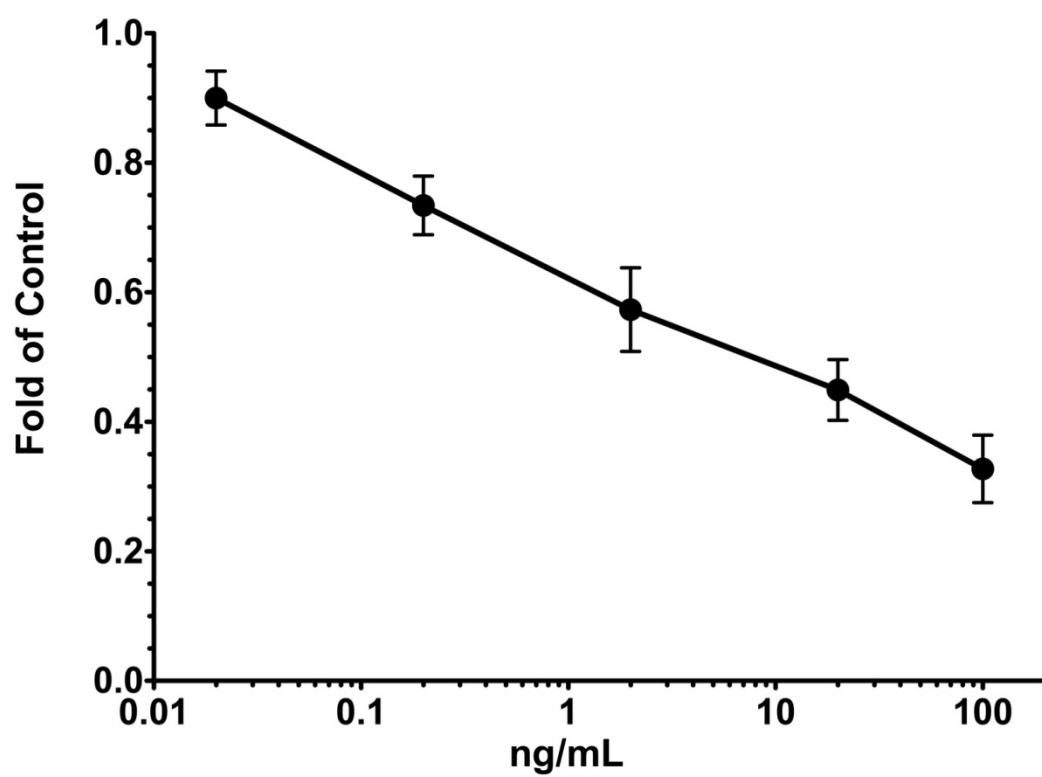


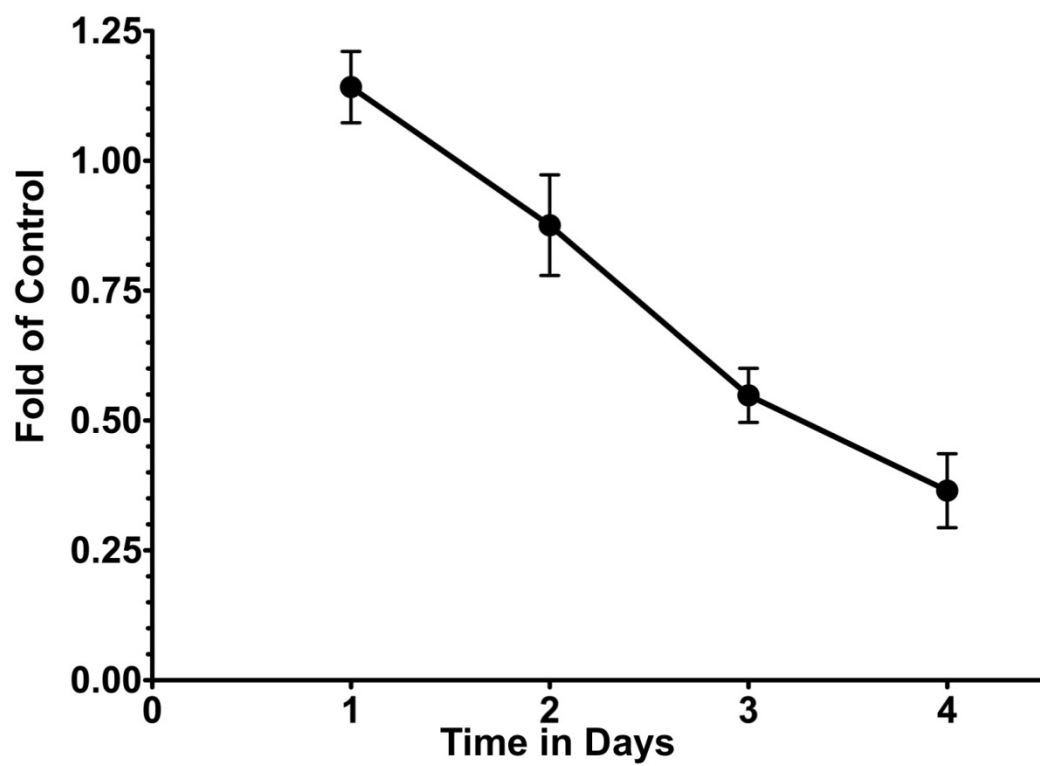
Figure 3A-D

Figure 3: BMMC were cultured in the presence of IL-10. In A and D, x-axes are ng/mL, and in B and C, x-axes are time in days. Y-axes are fold of control. A. BMMC were cultured for 3 days in 10 ng/mL IL-3 +/- IL-10 at 0.2, 2, 20, or 100 ng/mL. ADAM10 surface expression was measured by flow cytometry. B. BMMC were cultured for 1-4 days in IL-3 +/- IL-10 at 10 ng/mL. ADAM10 mRNA was measured by qPCR. C. BMMC were cultured for 1-4 days in IL-3 +/- IL-10 at 10 ng/mL. ADAM10 surface expression was measured by flow cytometry. D. WT BMMC or STAT3^{-/-} BMMC were cultured for 3 days in 10 ng/mL IL-3 +/- IL-10 at 0.2, 2, 10, or 100 ng/mL. ADAM10 surface expression was measured by flow cytometry. Data shown are means +/- SE of at least 3 experiments with 6 or more samples each. *, $p < 0.05$, ***, $p < 0.0005$.

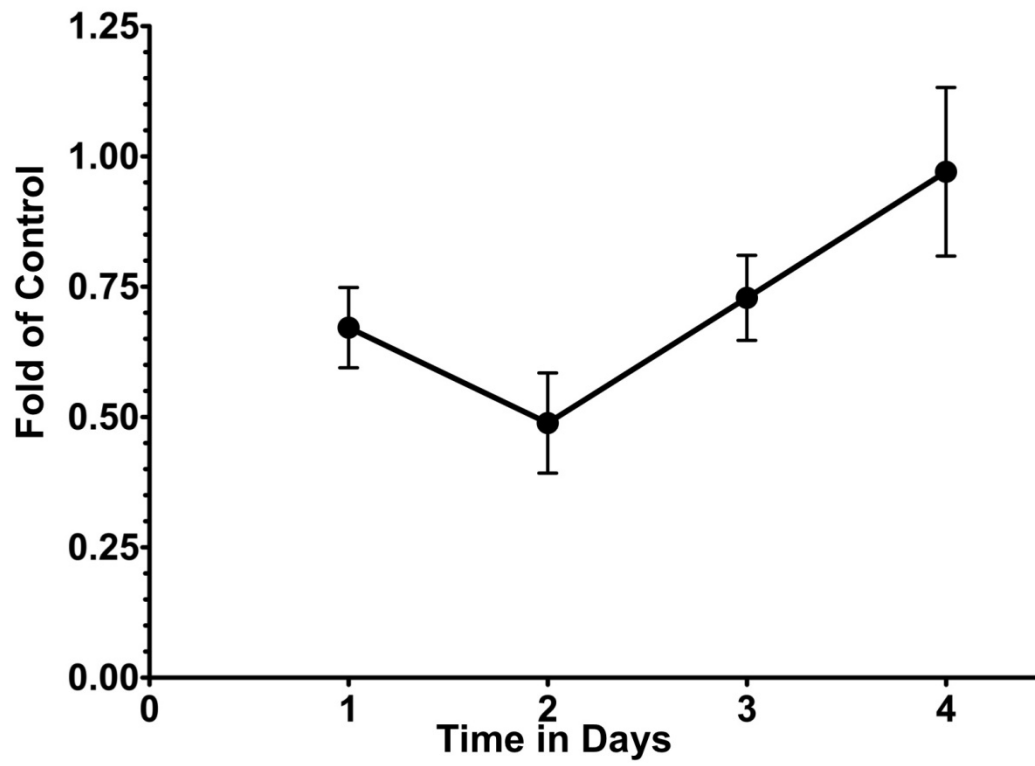
A



B



C



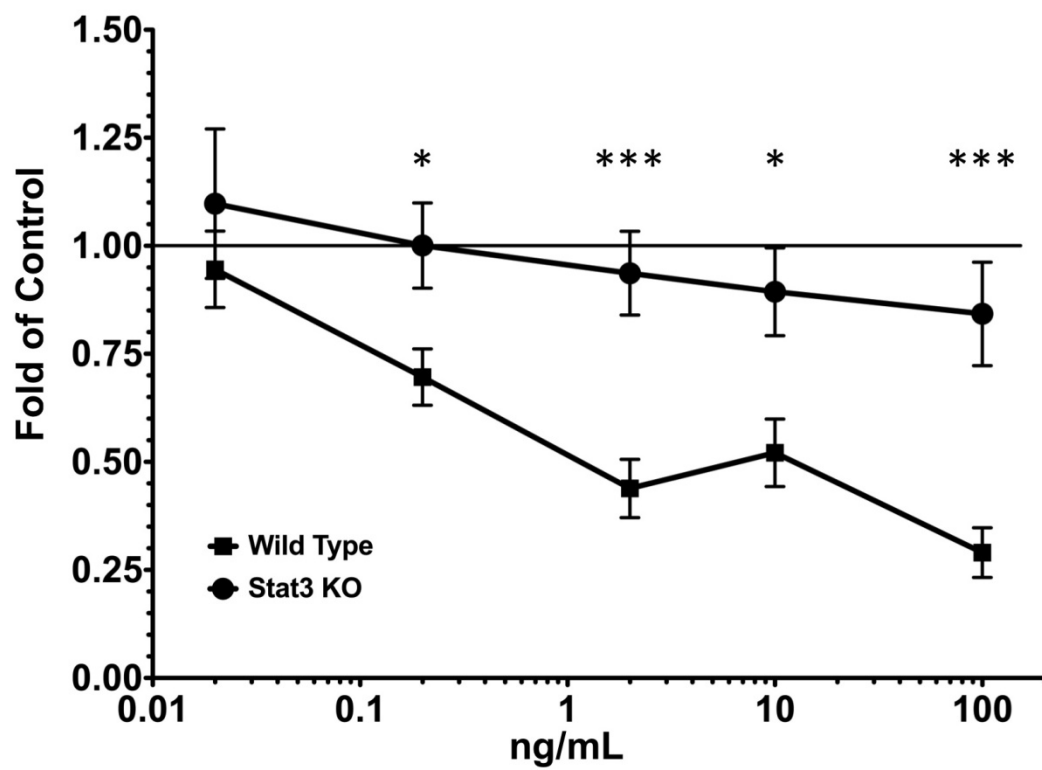


Figure 4

Figure 4: BMMC from control scrambled siRNA (red) or ADAM10 siRNA depleted (purple) were stained for ADAM10 by flow cytometry. Isotype is blue. X-axis is MFI. Data shown are representative of 1 experiment, n=3.

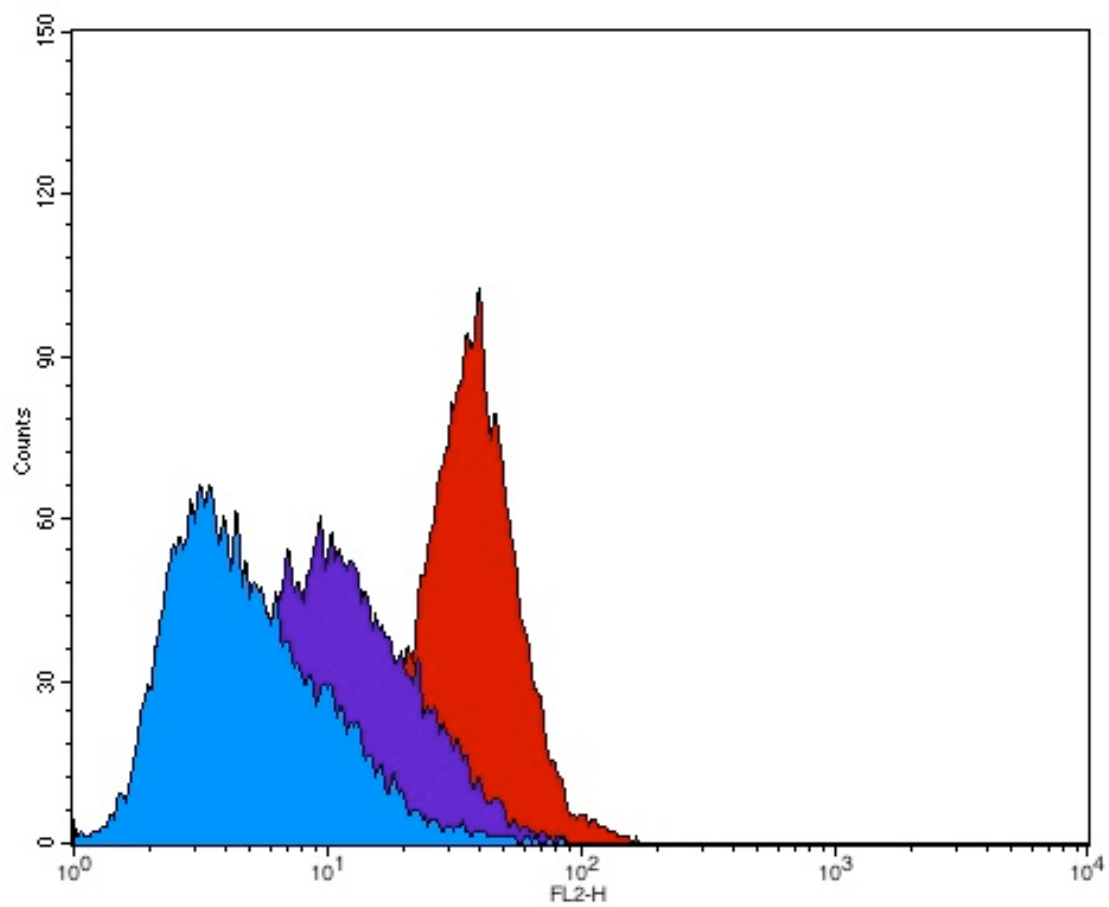
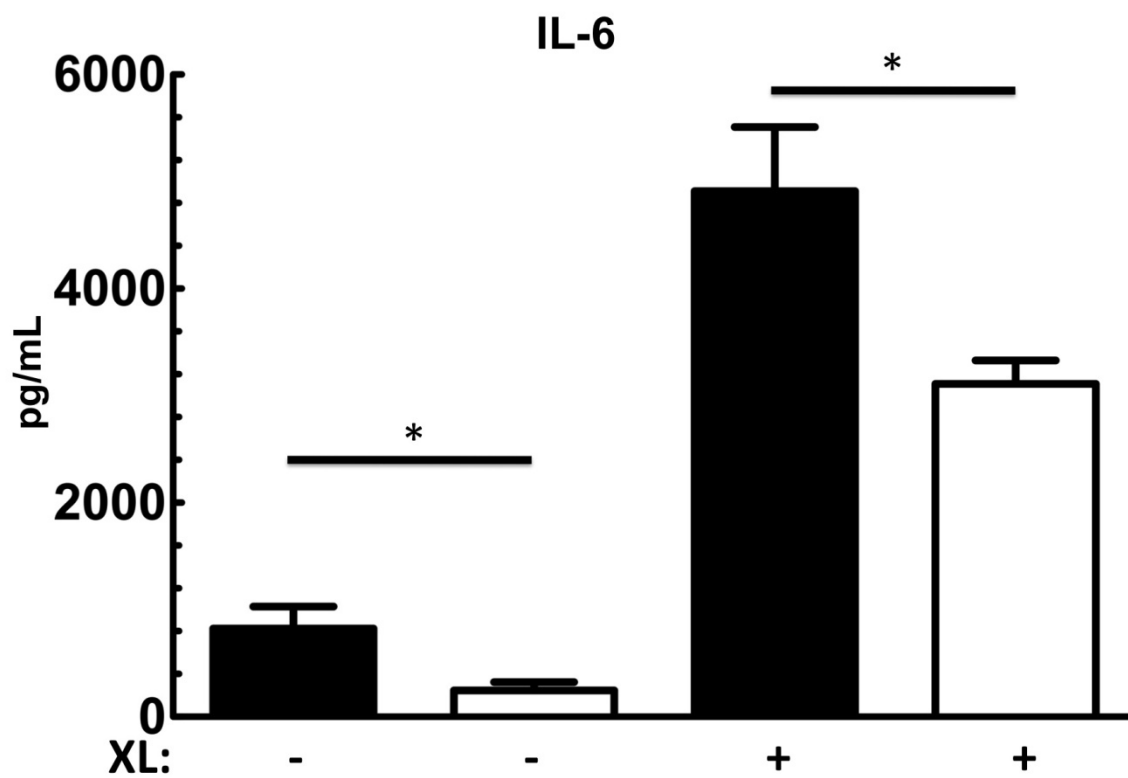


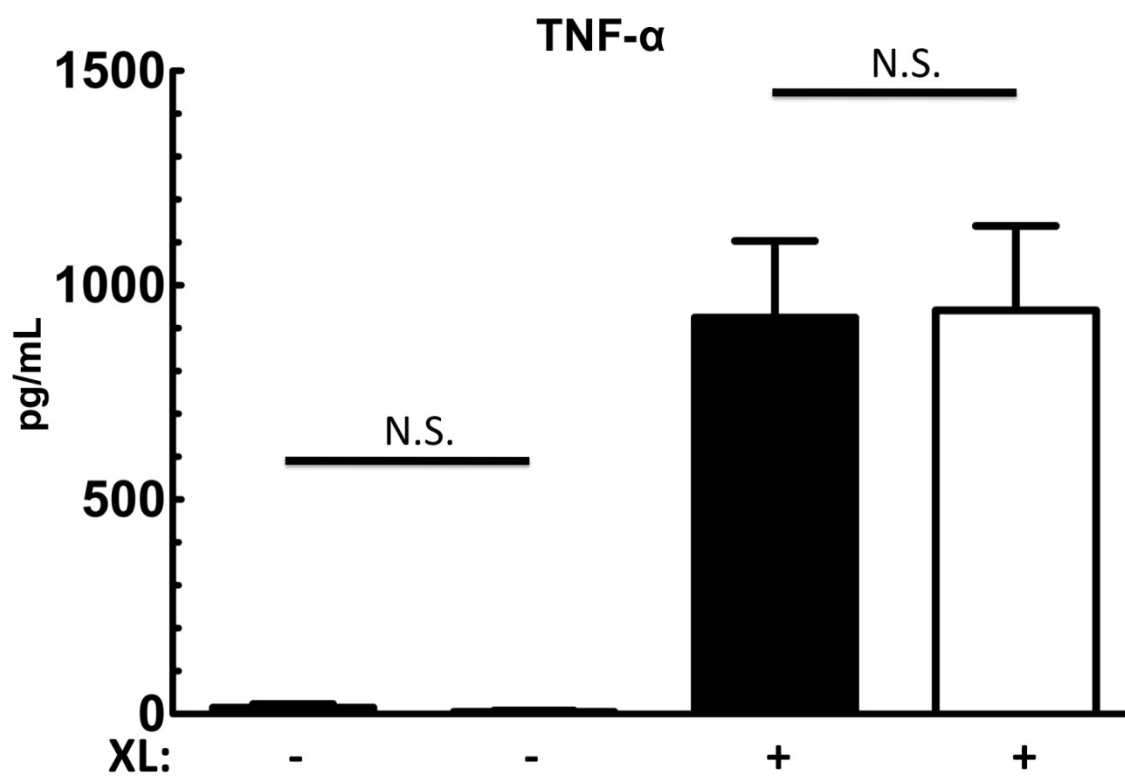
Figure 5A-D

Figure 5: BMMC were transfected with control scrambled siRNA (black bars) or ADAM10 siRNA (white bars). BMMC were then cultured in IL-3 +/- .5 $\mu\text{g/mL}$ IgE overnight, washed, and cross-linked with 50 ng/mL DNP-HSA for 16 hours. Supernatants were collected to measure cytokine levels by ELISA. A. IL-6. B. TNF- α . C. IL-13. D. MCP-1. All data are means +/- SE of 3 experiments, n=2 each experiment. *, p < 0.05

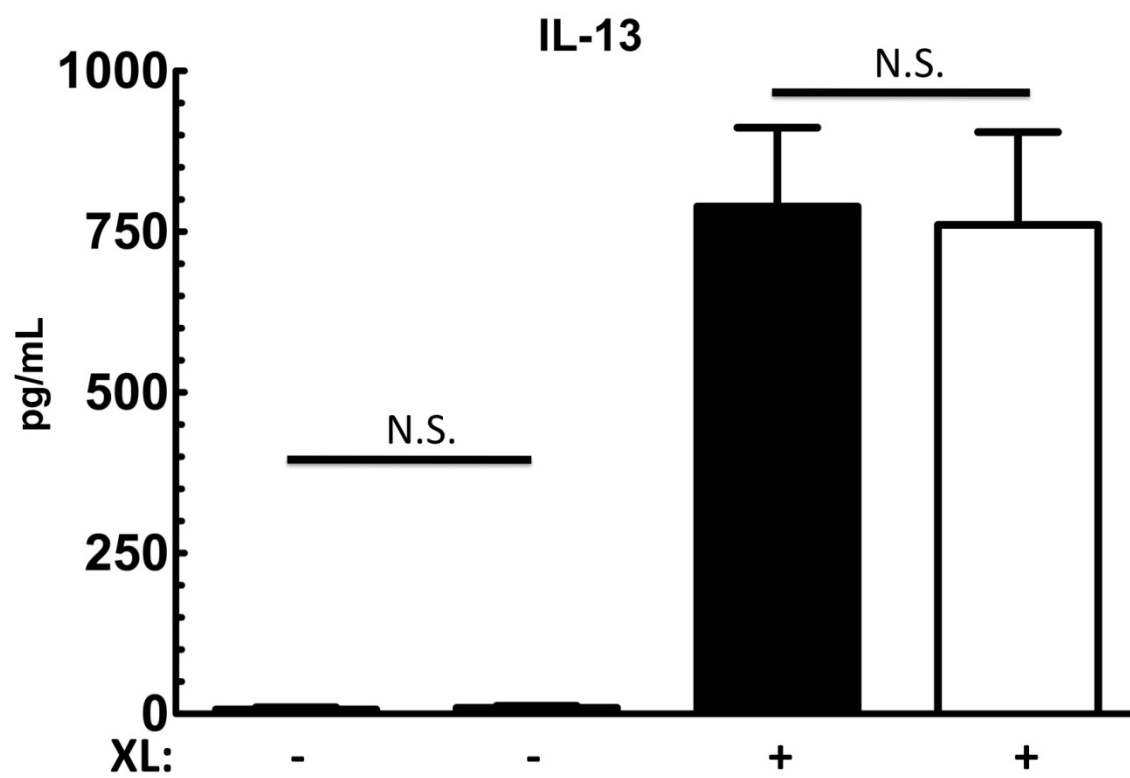
A



B



C



D

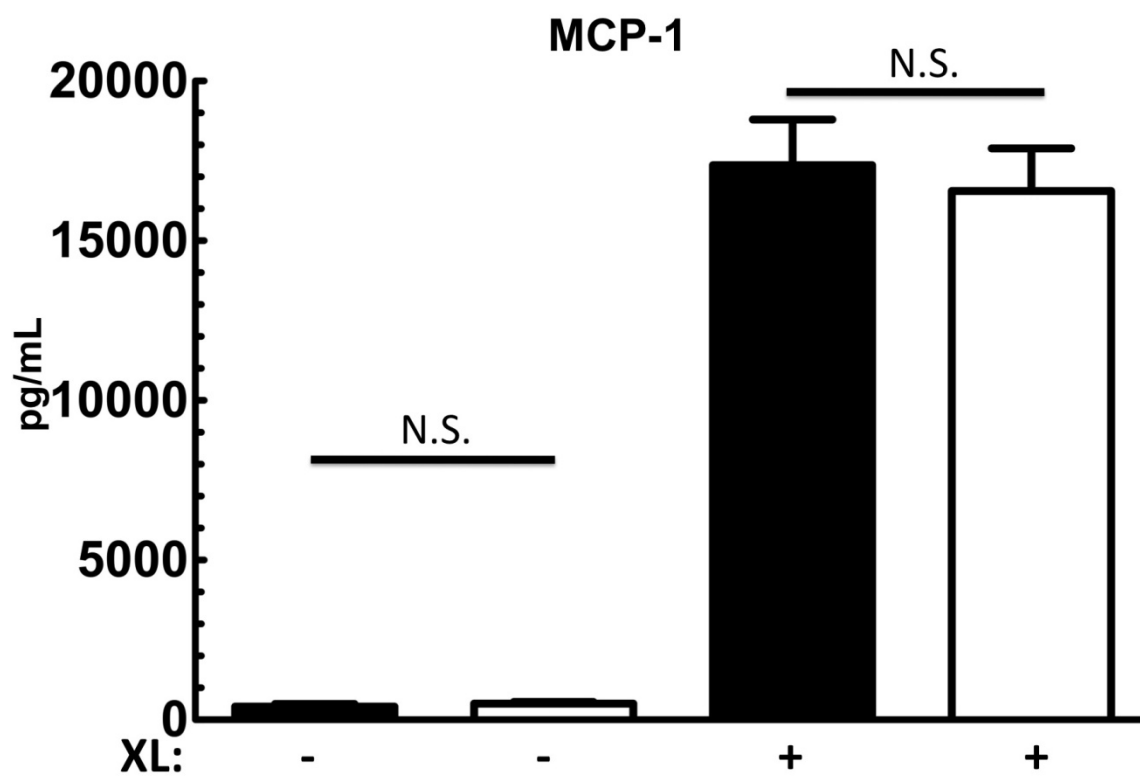
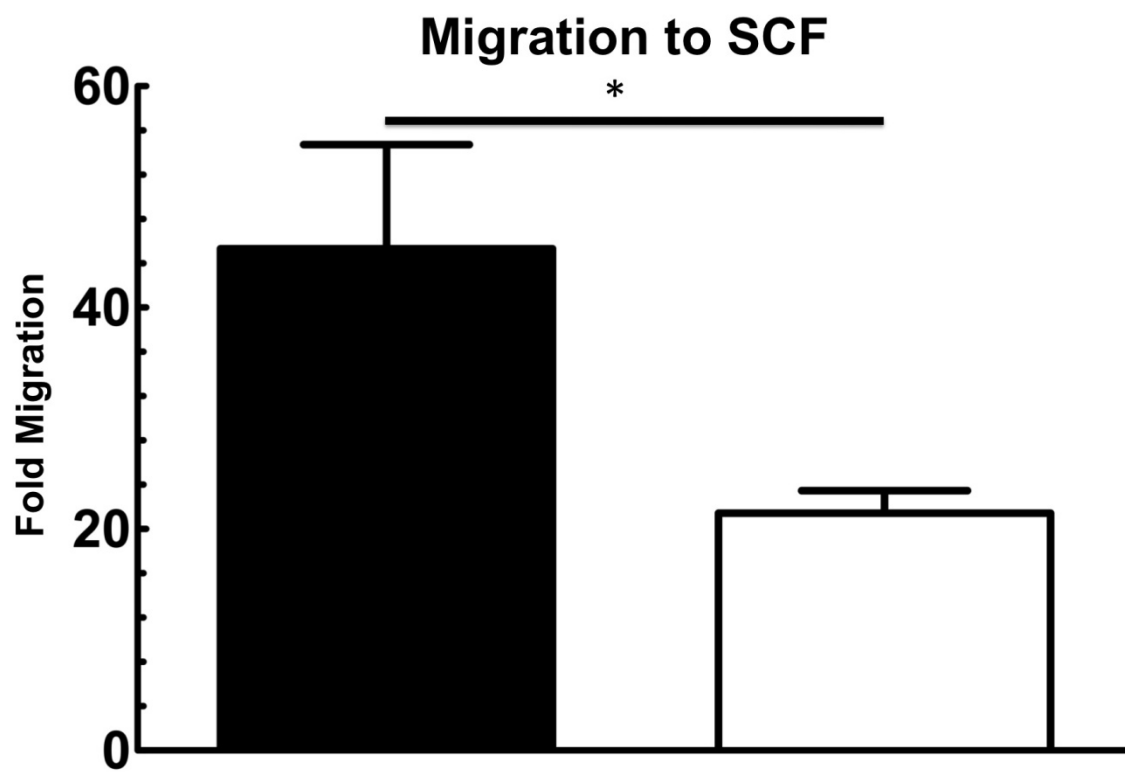


Figure 6A-B

Figure 6: BMMC were transfected with scrambled control siRNA (black bars) or ADAM10 siRNA (white bars) and migration through collagen IV to Antigen (Ag) and SCF was assessed. A. Migration of each BMMC group to 100 ng/mL SCF was assessed. B. Migration of each BMMC group preloaded with IgE or not to 50 ng/mL DNP-HSA was assessed. Data shown are means \pm SE of 2 experiments, total n=3 each treatment. *, $p < 0.05$.

A



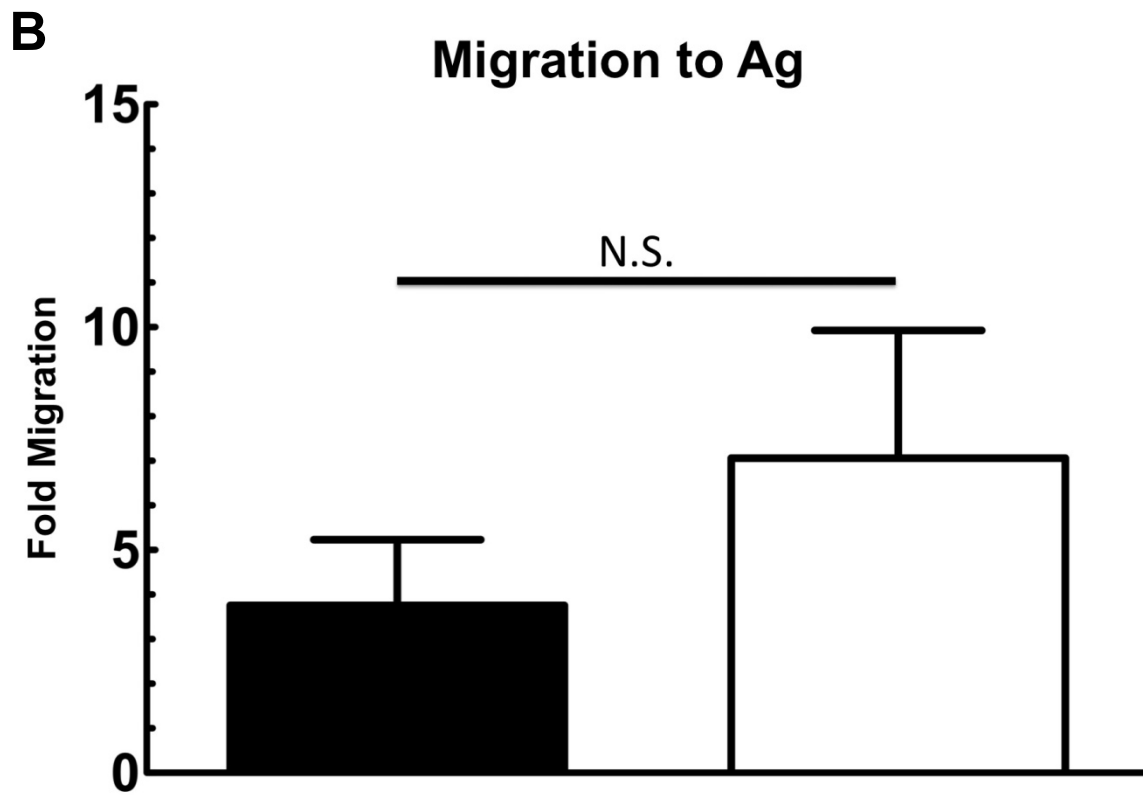


Figure 7

Figure 7: BMMC from WT (red) and ADAM10 KO (purple) were stained for ADAM10 by flow cytometry. Isotype is blue. X-axis is MFI. Data shown are representative of 1 experiment, n=3.

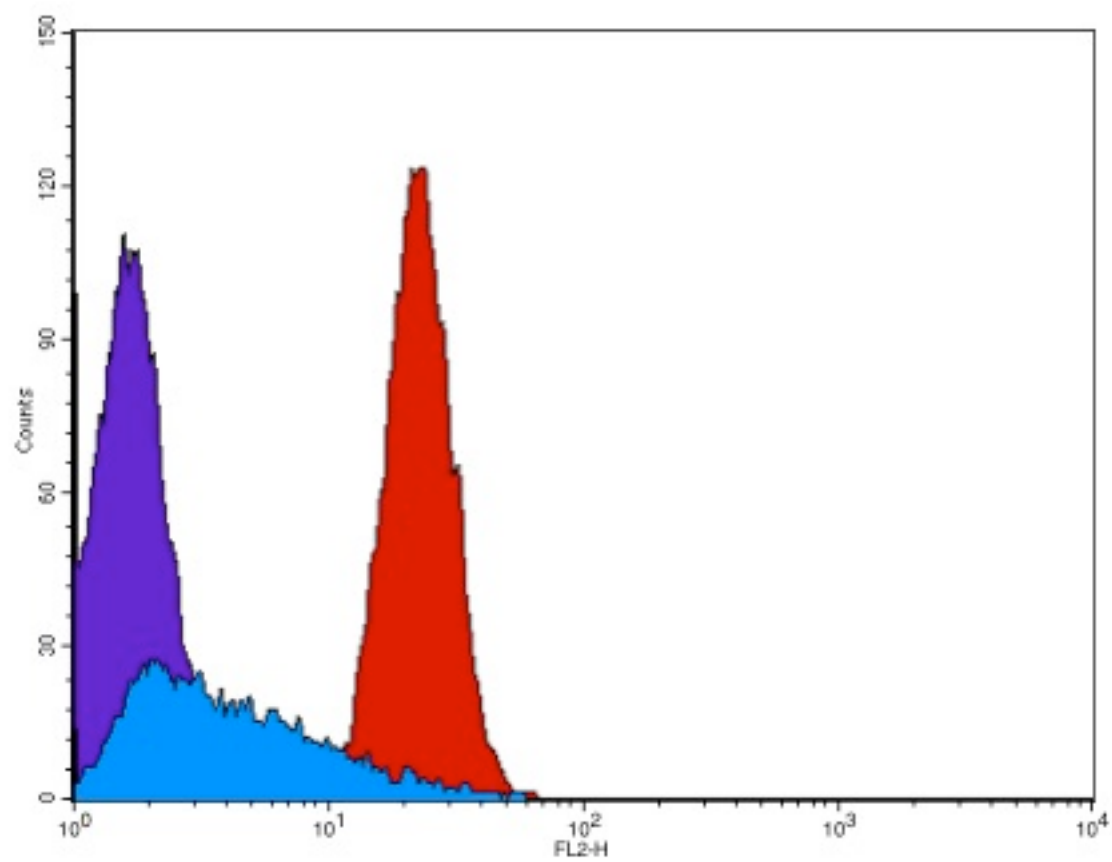
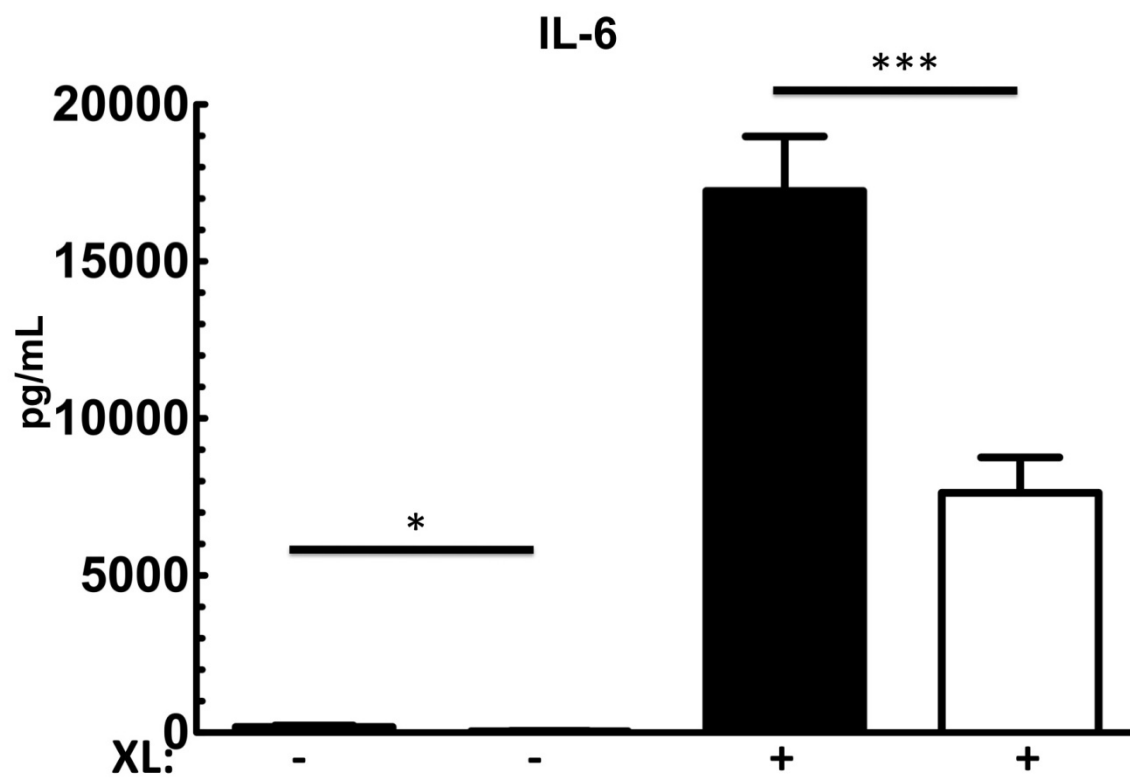


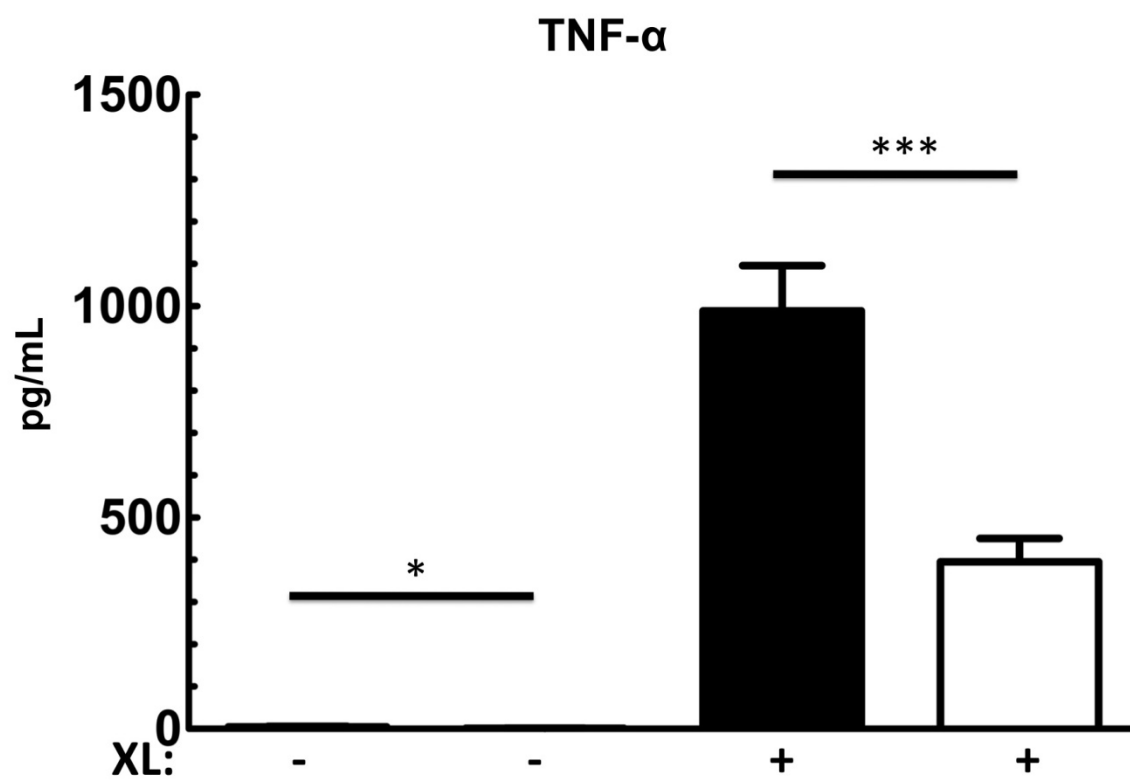
Figure 8A-D

Figure 8: Wild type (black bars) or ADAM10KO (white bars) BMMC were cultured in 10 ng/mL IL-3 + .5 μ g/mL IgE for 24 hours, washed, and cross-linked with 10 ng/mL DNP-HSA for 16 hours. Supernatants were collected to measure cytokine levels by ELISA. A. IL-6. B. TNF- α . C. IL-13 D. MCP-1. Data shown are means \pm SE from 1 experiment, n=3 each treatment. *, $p < 0.05$, ***, $p < 0.0005$.

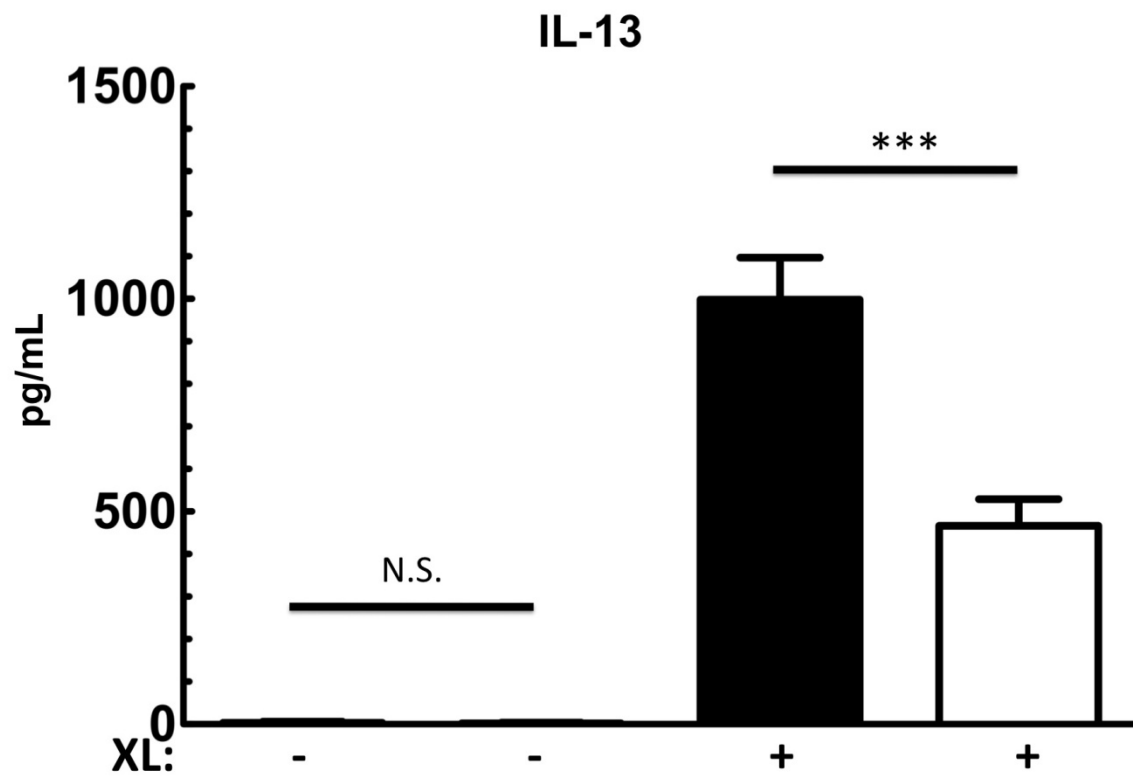
A



B



C



D

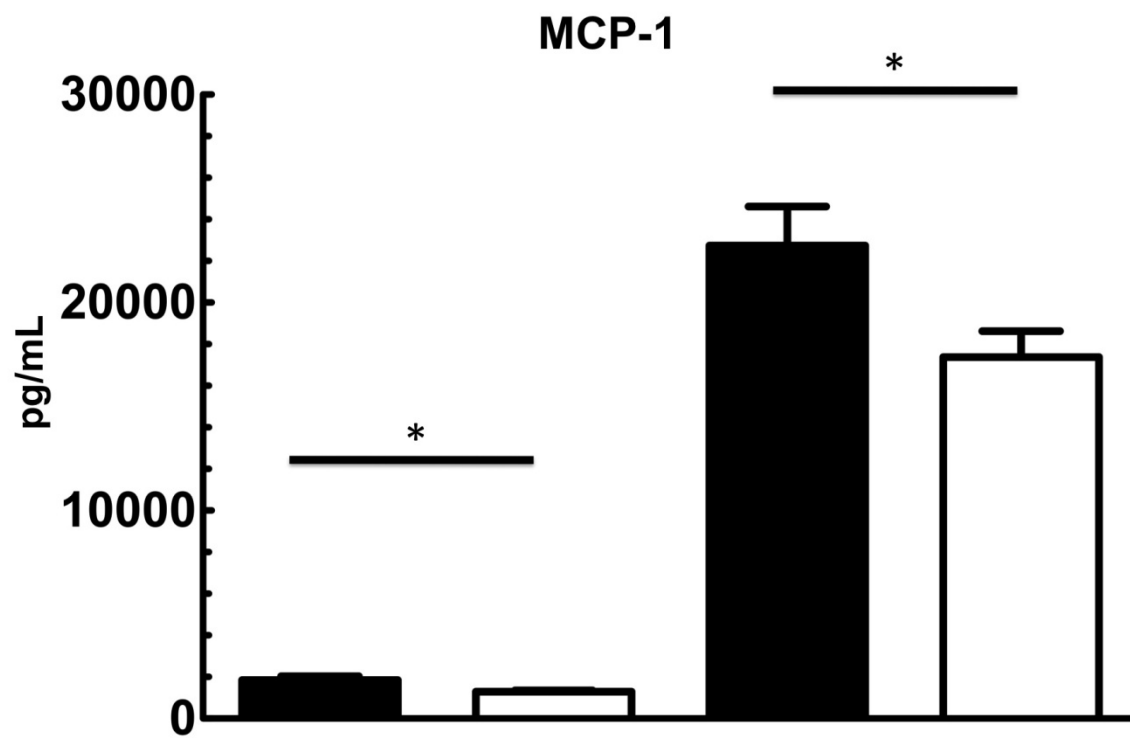


Figure 9

Figure 9: Migration through collagen IV to 100 ng/mL SCF was assessed in WT BMMC (black bars) and ADAM10KO BMMC (white bars) Data shown are means \pm SE of 1 experiments, $n=3$ ***, $p < 0.0005$.

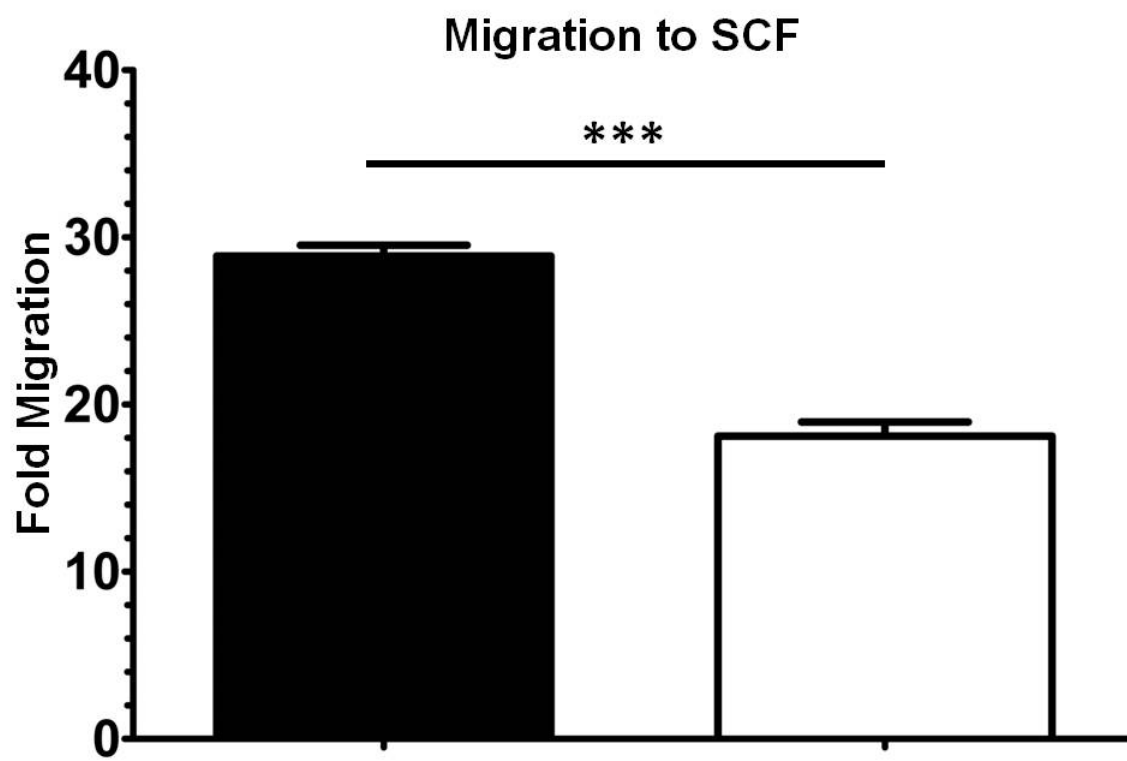
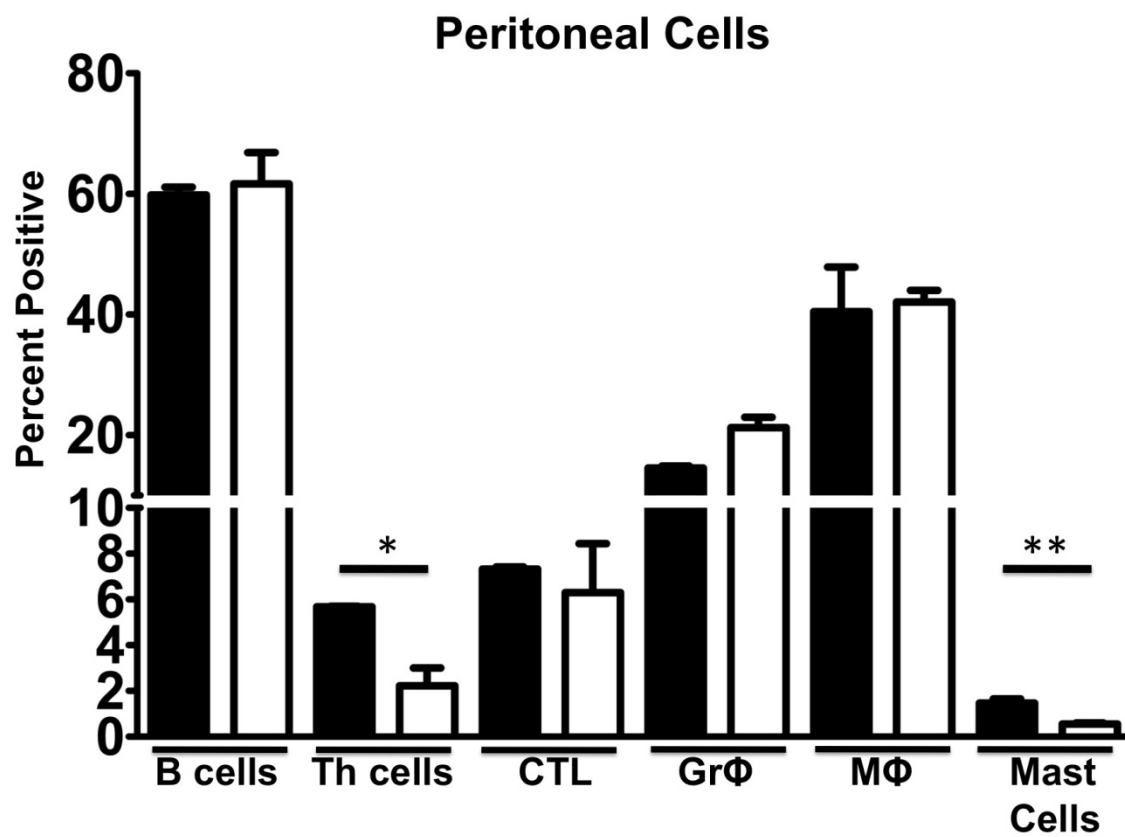


Figure 10

Figure 10: Peritoneal lavage was performed on Wild type (black bars) and ADAM10 KO (white bars) mice. Cells were then stained with the indicated lineage markers and Mean Fluorescence Intensity was measured by flow cytometry. Data shown are means \pm SE of 1 experiment with n=2-3 mice each. *, $p < 0.05$, **, $p < 0.005$.



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Vita

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