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EFFECTS OF TGF-β1 AND IL-33 ON MAST CELL FUNCTION

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EFFECTS OF TGF-β1 AND IL-33 ON MAST CELL FUNCTION

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

by

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List of Abbreviations

Akt: Protein kinase B, serine/threonine-specific protein kinase

AMH: Anti-müllerian hormones

BMMC: Bone marrow-derived mast cell

BMP: Bone morphogenic proteins

ANOVA: Analysis of Variance

BHK: Supernatant from SCF-producing BHK-MKL, cell line

ERK: Extracellular-signal-regulated kinase

IgE: Immunoglobulin E

IgG: Immunoglobulin G

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

IL-6: Interleukin 6, a pro-inflammatory cytokine

IL-33: Interleukin 33, binds ST2

NF-κB: Nuclear factor κB

P38: a mitogen-activated protein kinase

IκB: Inhibitor of NF-κB
SCF: Stem cell factor

ST2: IL-33 receptor

IC$_{50}$: Half maximal inhibitory concentration

MCP-1: Monocyte chemotactic protein 1

PBS: Phosphate buffered saline

P65: a subunit of NF-$\kappa$B

TNF: Tumor necrosis factor $\alpha$

P50: a subunit of NF-$\kappa$B

c-Kit: SCF receptor

gMFI: Geometric mean fluorescent intensity

SNP: Single Nucleotide Polymorphism

TBS: Tris-Buffered Saline

TBS-T: TBS + 0.1% Tween-20

TGF$\beta$: Transforming growth factor beta

WEHI: Supernatant from IL-3 producing cell
Abstract

THE EFFECTS OF TGF-β1 AND IL-33 ON MAST CELL FUNCTION

By Victor S. Ndaw, B.S.

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

Virginia Commonwealth University, 2015

Major Advisor: John J. Ryan, Ph.D., Professor of Biology

TGFβ is involved in many pathological conditions, including autoimmune disorders, cancer, and cardiovascular and allergic diseases. We have previously found that TGFβ can suppress IgE-mediated mast cell activation in human and mouse mast cells in vitro. IL-33 is a recently discovered member of the IL-1 family capable of inducing mast cell responses and enhancing IgE-mediated activation. In this study, we investigated the effects of TGFβ on IL-33-mediated mast cell activation. Bone marrow-derived mast cells cultured in TGFβ -1, -2, or -3 showed reduced IL-33-mediated production of TNF, IL-6, IL-13 and MCP-1, in a concentration-dependent manner. Furthermore, TGFβ also reduced expression of the T1/ST2 receptor as well as IL-33-mediated TAK1 and ERK phosphorylation. TGF-β1 injection suppressed IL-33-mediated production of systemic inflammatory cytokines in vivo. The role of IL-33 in the pathogenesis of allergic diseases is incompletely understood. These findings, consistent with our previously reported effects of TGFβ on IgE-mediated activation, demonstrate that TGFβ can provide broad and substantial inhibitory signals to activated mast cells.
**Introduction**

The past two decades in developed countries have been characterized by major health concerns due to an increased prevalence of atopic and autoimmune diseases caused by environmental or genetic factors. Transforming Growth Factor beta (TGFβ), a pleiotropic cytokine, has been implicated in the development of autoimmune disorders, chronic inflammatory conditions and atopic diseases such as asthma and atopic dermatitis (1-5). The TGFβ superfamily is comprised of more than 30 members including activins, inhibins, bone morphogenic proteins (BMP) and anti-müllerian hormones (AMH) that play a critical role in regulating tissue repair, embryogenesis, cartilage homeostasis, cell growth, proliferation and cancer (6). Furthermore, TGF-β1 has recently been shown to be involved in hematopoiesis and Treg and Th17 differentiation (7-9). Thus, clinical therapies based on modulation of this cytokine represent an important new approach to the treatment of immune disorders.

TGFβ-1, -2, and -3 are produced as inactive precursors in the blood and connective tissue, bound to latency associated protein. Mast cell proteases, often released after antigen-mediated IgE crosslinkage, can cleave and activate latent TGFβ proteins. This may serve as a critical feedback regulator of the mast cells response. IgE-mediated mast cell activation elicits release of preformed mediators such as histamine and the production of arachidonic acid metabolites, cytokines, and chemokines that collectively increase vascular permeability, constrict airways, and recruit leukocytes to inflammatory sites (10).

IL-33, one of the newest members of the IL-1 family is often referred to as an alarmin because it is released upon endothelial or epithelial cell damage. IL-33 activates mast cells via
receptors ST2 (IL-1RL1) and the IL-1 receptor accessory protein (IL-1RAcP) to induce secretion of Th2 cytokines implicated in anaphylaxis, asthma and atopic dermatitis(11).

Our data demonstrate that TGFβ-1, -2, and -3 can inhibit IL-33-mediated mast cell activation, in vitro and in vivo. Additionally we show that ST2 receptor expression is suppressed by TGF-β1, and investigate IL-33 receptor signaling events altered by TGF-β1.
Materials and Methods

Animals

C57BL/6J, BALB/cJ, C3HeJ and 129S1/SvImJ (hence referred to as 129/Sv) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). They were maintained in a pathogen-free facility at Virginia Commonwealth University (VCU). Protocols and studies involving animals were performed in accordance with the VCU Institutional Animal Care and Use Committee guidelines.

Mouse mast cell cultures

Bone marrow-derived mast cell cultures (BMMC) were derived from mouse femurs 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 10 mM HEPES (Biofluids, Rockville, MD), supplemented with IL-3-containing supernatant from WEHI-3 cells and SCF-containing supernatant from BHK-MKL cells for 21 days. The final concentration of IL-3 and SCF was adjusted to 1 and 10 ng/ml, respectively, as measured by ELISA. Peritoneal cells were isolated in PBS, and then expanded in culture for 7 days as described for BMMCs to generate peritoneal mast cells. For in vitro analyses, BMMCs were washed and incubated at 37°C for 4 h in cRPMI 1640 without cytokines to synchronize cell cycle staging. Cells were then plated at $5 \times 10^5$ cells/ml and incubated at 37°C for the indicated times in cRPMI 1640 with IL-3 + SCF (10 ng/ml each) with or without TGF-β1.
(10 ng/ml unless otherwise noted). Cells were activated by culturing overnight with 50 ng/ml IL-33 for 16-24 hours, after which culture supernatants were harvested for ELISA analysis.

**Cytokines and reagents**

Purified DNP-specific mouse IgE was purchased from BD Pharmingen (San Diego, CA). Dinitophenyl-coupled human serum albumin (DNP-HSA) was purchased from Sigma Fine Chemicals (St. Louis, MO). Murine IL-3 and SCF were purchased from PeproTech (Rocky Hill, NJ). Human TGF-β1, TGF-β2 TGF-β3 and IL-33 were purchased from BioLegend (San Diego, CA). Antibodies recognizing actin were bought from Sigma-Aldrich (St. Louis, MO). Rat anti-mouse FcγRII/RIII (2.4G2), purified mouse IgE, purified anti-mouse IgE, FITC-conjugated rat IgG isotype control, and FITC-conjugated anti-mouse CD117 (c-Kit) were purchased from BD Pharmingen. Mouse T1/ST2 (IL-33 R) monoclonal antibody (clone DJ8) FITC-conjugated or PE-conjugated rat IgG2b isotype control and PE-conjugated anti-mouse IgE were purchased from eBioscience (San Diego, CA). Anti-Akt, TAK1, ERK, IKB, p38 and JNK Abs were purchased from Cell Signaling (Danvers, MA).

**Enzyme–linked immunosorbent assay (ELISA)**

Cytokine levels were detected by standard ELISA Kits purchased from BioLegend (San Diego, CA) using the manufacturer’s protocol.

**Flow cytometric analysis**
Surface expression and intracellular staining (ICS) analysis were performed using a standard flow cytometry protocol described previously (BD FACSCalibur, BD Biosciences, Franklin Lakes, NJ). To assess the percentage of peritoneal populations, we identified Macrophages as Mac1⁺/Gr1⁻, neutrophils as Mac1⁻/Gr1⁺, and MDSC as Mac1⁺/Gr1⁺. Mast cells were identified as FcεRI⁺/Kit⁺. For cytokine measurements, cells were first activated for 90 min with IL-33, and then cultured for 6-8 h in the presence of 5 μM monensin at 37°C, before fixation in PBS with 4% paraformaldehyde, and staining in the presence of 0.5% saponin with PE-conjugated anti- IL-13 or -Mip-1α. TNF (clone MP6-XT22) and IL-6 (clone MP5-20F3) were detected using anti-mouse Ab (BioLegend).

**Western blot analysis**

Western blotting was performed using 30-50 μg total cellular protein per sample. Protein was loaded and separated over 4–20% gradient SDS polyacrylamide gels (Bio-Rad, Hercules, CA). Proteins were transferred to nitrocellulose (Pall Corporation, Ann Arbor, MI), and blocked for 60 min in 0.1% casein in TBS. Blots were incubated in 0.1% casein/TBS-T with a 1:1000 dilution of anti-Akt, -TAK1, -ERK, -IKB, -GAPDH or –β-actin overnight at 4°C with gentle rocking. Blots were washed six times for 5 min each in TBS-T, followed by incubation in 0.1% casein/TBS-T with a 1:15000 dilution of HRP-linked anti-IgG (matched to the appropriate primary Ab source species, from Cell Signaling, Danvers, MA). Size estimates for proteins were obtained using m.w. standards from Bio-Rad (Hercules, CA).
**TGF-β1 Injection**

Mice (C57BL/6J, 8–12 wk old, n = 5/group) were injected i.p. with 0.5 μg TGF-β1 or PBS twice daily for 3 days and once on day 4, IL-33 (1 μg) was injected for 4 hours before peritoneal cells were harvested. Peritoneal lavage cells were counted with trypan blue and stained for flow analysis. The mean mast cell number from each mouse was then averaged from all five mice. Cardiac puncture was done to collect serum and cytokine levels were quantified by ELISA.

**Statistics**

Data shown in each figure are the mean and standard errors of the indicated number of samples. For comparisons of two samples, Student’s t–Test was used. For comparisons of multiple samples to a control group, one–way analysis of variance (ANOVA) was employed.
Results

TGFB1 suppresses IL-33-mediated cytokine production from mouse mast cells across multiple genetic strains

We previously found that TGFB1selectively suppresses development, survival, and IgE-mediated cytokine production of bone marrow derived mast cells (BMMC), with some indication that genetic backgrounds can influence TGFB1 responsiveness (12). In this work we investigated these effects on mast cells stimulated with IL-33. Mice BMMC were cultured for 4 days in the presence or absence of TGFB1 prior to IL-33 stimulation. As shown in Figure 1, TGFB1significantly suppressed production of IL-6 and TNF among C57BL/6J, 129/SvJ, C3H/HeJ and BALB/cJ BMMC. Concentration response analysis was performed on C57BL/6J and 129/SvJ BMMC to determine IC50 of each genetic background (Figure 2). Although TGFB1reduced cytokine production in both genetic strains, we found that C57BL/6J MCs were more sensitive to its effect than 129/Sv. We also tested the effects of other TGF isoforms (TGFB2 and TGFB3) on BMMC. As shown in Figure 3, IL-6 and TNF levels were also reduced upon TGFB2 and TGFB3 treatment of C57BL/6J BMMC. Finally, we investigated the effects of TGFB1 on peritoneal mast cells. We found that TGFB1significantly diminished IL-6 production in C57BL/6J peritoneal mast cells. However, IL-6 production by BALB/cJ and 129/SvJ peritoneal mast cells was unchanged (data not shown).

TGFB1 suppresses T1/ST2 expression
Given the sensitivity variance of 129/SvJ, BALB/cJ and C57BL/6J BMMC to TGF-β1, we sought to determine its effects on the expression of the ST2 receptor through which IL-33 mediates its function. BMMCs of each strain were cultured for 3 days in the presence or absence of TGF-β1 and stained with anti-ST2. FACS analysis showed that TGF-β1 suppressed mast cell ST2 surface expression to a similar extent across the different backgrounds (Figure 4). We then assessed TGF-mediated cytokine suppression intracellularly upon IL-33 stimulation. As shown in Figure 6, IL-6, IL-13, TNF and CCL3 levels were significantly decreased in 129/SvJ, BALB/cJ and C57BL/6J BMMC. To determine if the decrease in cytokine production correlated with reduced ST2, we compared this relationship by flow cytometry. As depicted in Figure 5, we used intracellular staining to examine groups of cells with similar ST2 expression levels. Among TNF-positive cells, those receiving TGF-β1 produced less TNF than untreated cells at each ST2 receptor level measured (Figure 5). Furthermore, TNF production was generally correlated with ST2 staining intensity in the absence of TGF-β1. However, this curve reached its plateau quickly in the presence of TGF-β1, widening the gap between the two groups. This observation indicated that TNF production was no longer dependent on ST2 expression levels, suggesting that ST2 signaling may be compromised.

**TGF-β1 suppresses TAK1 and ERK activation, but maintains IκB expression downstream of IL-33 signaling**

ST2 triggering by IL-33 activates a signaling cascade that includes the MAP3K TAK1, subsequent activation of MAPK family members, and NF-κB function (13). We investigated the
ability of TGF-β1 to suppress expression and activation of these proteins. C57BL/6J BMMC treated with TGF-β1 for four days showed no change in p38 or JNK phosphorylation, but had a significant reduction in ERK-1/2 and TAK1 activity (Figure 7 & 8). Additionally, expression of the NF-κB inhibitor IκB was elevated at 5 and 15 minutes after IL-33 activation in TGF-β1 treated cells compared to the control group.

**TGF-β1 suppresses IL-33-mediated cytokine production in vivo**

To determine the effects of TGF-β1 in vivo, we i.p. injected C57BL/6J mice with TGF-β1 twice daily for 3 days and once on the 4th day. IL-33 was then administered through i.p. injection, 6 hours before cardiac puncture was done to assess plasma cytokine levels by ELISA. IL-33 injection increased plasma cytokines, as anticipated. By contrast, IL-6, IL-13 and CCL2 levels were significantly reduced in mice that received TGF-β1 relative to those that were injected with PBS alone (Figure 9). These in vivo data support our in vitro findings that TGF-β1 is a potent inhibitor of IL-33-mediated signaling.
Discussion

Mast cells are known as key regulators implicated in allergic responses. IL-33 mediated stimulation can induce secretion of Th2 cytokines from mast cells that are implicated in anaphylaxis, asthma and atopic dermatitis (11, 14). Therefore, a better understanding of this alarmin can represent a potential way to reduce inflammation associated with this cytokine.

Previous studies noted that polymorphisms in the TGF-β1 promoter region have been shown to segregate with asthmatic families that have elevated serum IgE (15). This suspicion that TGF-β1 might contribute to allergic disease prompted our lab and others to investigate its effects on IgE-mediated responses. We found variable TGF-β1 effects on IgE-mediated cytokine production, dependent of genetic background (12). While TGF-β1 suppressed IgE-mediated inflammatory cytokine secretion among C57BL/6J mast cells, mast cells from 129/SvJ mice were resistant. Additionally, we and others have previously noted other suppressive effects of TGF-β1 on mast cells, including decreased IgE-mediated cytokine production in vitro and in vivo (16, 17), diminished proliferation and survival (18, 19), and reduced cKit and FceRI expression (16, 17). However, TGF-β1 is not entirely suppressive to mast cells, as it elicits their migration (20, 21). Importantly, these effects might be mediated by mast cells in an autocrine fashion, since mast cell proteases released during degranulation can cleave and activate latent TGF-β1 in the tissue (10, 22, 23). Therefore, it is logical to deduct that TGF-β1 can serve as a feedback regulator of mast cell function.

These data encouraged our interest in TGF-β1 as a mediator of mast cell homeostasis during IL-33 activation. In this work it was surprising to see that TGF-β1 suppresses IL-33-
mediated cytokine production from mouse mast cells across multiple genetic strains (*Figure 1*). Additionally, on a per cell basis, TGF-β1 reduced the surface expression levels of ST2, with peak effects after 3 days (*Figure 4*). Furthermore, the relationship between ST2 levels and TNF secretion (*Figure 5*) suggested that ST2 signaling may be compromised, since TNF levels were no longer strongly correlated with ST2 expression. Taken altogether, these findings allow us to postulate that the mechanism of control, through which TGF-β1 exerts its IL-33-mediated function of reducing cytokine production across multiple genetic backgrounds, differs from FcεRI signaling.

TGF β-activated kinase 1 (TAK1), a MAP3K, is a key regulator of innate immunity and pro-inflammatory signaling pathways. Some studies show that in response to interleukin-1, TNF, and toll-like receptor agonists, it mediates the activation of NF-κB(24). Due to the implication of this protein downstream of the ST2 receptor, we sought to investigate the effects of TGF-β1 on TAK1, ERK and IκB phosphorylation, proven to be essential for ST2-mediated cytokine production(25). Supporting the theory that TGF-β1 alters ST2 signaling, it was interesting to observe that TGF-β1 suppressed TAK1 and ERK phosphorylation but increased total levels of IκB. Additionally, we were encouraged by the finding that TGF-β1 injection suppressed IL-33-mediated inflammatory responses *in vivo*, similar to its *in vitro* effects. Nilsson’s(14) group recently reported that IL-33 injection elicits a peritoneal neutrophil recruitment that requires mast cell-derived TNF production. Thus while our in vivo data do not allow us to state categorically that the effects of TGF-β1 targeted the mast cell lineage, they are in keeping with the current understanding of how mast cell contribute to this response. Moreover, these data support the
hypothesis that TGF-β1 can mitigate IL-33 effects systemically in an in vivo environment. A better understanding of these effects could reveal why some individuals prone to more robust mast cell responses common in Th2 diseases such as allergy and asthma.

Overall, TGF-β1 remains an enigmatic factor on mast cells homeostasis. Although IC₅₀ in Figure 1 between Th1 and Th2 mice varied, more extensive research should be completed to better comprehend the effect of genetic influence of TGF-β1 on mast cell activated with IL-33. Furthermore the use of TAK1, ERK and IKB inhibitors can be investigated in our future studies, both in vivo and in vitro, to better understand the role of TGF-β1 in the IL-33 pathway of mast cells in order to design therapeutics that will be beneficial in a variety of autoimmune diseases.
Figure 1. TGF-β1 suppresses IL-33-mediated cytokine production from mouse mast cells across multiple genetic strains

BMMC were cultured for 4 days in IL-3 and SCF, ±TGF-β1 (all cytokines at 10ng/ml) and stimulated with IL-33 (50ng/mL) for 16 hours. Cytokine levels were determined by ELISA. Data shown are mean±SE from triplicate samples of at least 2 separate experiments. *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001.
Figure 2. Variable IC$_{50}$ of TGF-$\beta$1 for each genetic background
BMMC were cultured for 4 days in IL-3 and SCF (10ng/ml), ±TGF-$\beta$1 (0.1-10ng/mL) and stimulated with IL-33 (50ng/mL) for 16 hours. Cytokine levels were determined by ELISA. Data shown are mean±SE from triplicate samples of at least 3 separate experiments. *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001.
Figure 3. Comparison of other TGF isoforms (TGF-β2 and TGF-β3)
BMMC were cultured for 4 days in IL-3 and SCF (10ng/ml), ±TGF-β1, -β2 and -β3 (0.1-10ng/mL) and stimulated with IL-33 (50ng/mL) for 16 hours. Cytokine levels were determined by ELISA. Data shown are mean±SE from triplicate samples of at least 3 separate experiments. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.
Figure 4. TGF-β1 suppresses ST2 expression on mast cells from various genetic strains

BMMC were cultured for three days in IL-3 and SCF (10ng/ml) ±TGF-β1 (10ng/ml), cells were stained with anti-ST2 for FACS analysis. Data shown are mean±SE of ST2 gMFI from triplicate samples of at least 2 separate experiments. *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001
Figure 5. The effects of TGF-β1 on cytokines and ST2 expression on mast cells

BMMC were cultured for three days in IL-3 and SCF (10ng/ml) ±TGF-β1 (10ng/ml), cells were stained with anti-ST2 for FACS analysis IL-33 (50ng/mL) stimulation for 90 minutes. Correlation of gMFI of TNF and T1/ST2 levels of expression. Data shown are mean±SE from triplicate samples of at least 2 separate experiments. *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001.
Figure 6. The effects of TGF-β1 on IL-33-mediated cytokine production of BMMCs
BMMC were cultured for three days in IL–3 and SCF (10ng/ml) ± TGF-β1 (10ng/ml), IL-33 (50ng/mL) stimulation for 90 minutes changes in IL-6, IL-13, TNF and CCL3 levels on a per cell basis were determined using intracellular staining cells were stained for FACS analysis. Data shown are mean±SE of %positive cells from triplicate samples. *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001.
Figure 7. Effects of TGF-β1 downstream IL-33 signaling on TAK1 and IκB
BMMC were cultured for three days in IL-3 and SCF (10ng/ml) ± TGF-β1 (10ng/ml), as described in Materials and Methods. Cells were activated with IL-33 (200ng/mL) for 5-30 minutes. The lysates were blotted for pTAK1, and IκB. Data are representative of three populations of at least 2 separate experiments, with mean±SE, after normalizing to GAPDH loading. *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001
Figure 8. Effects of TGF-β1 downstream IL-33 signaling on ERK 1/2
BMMC were cultured for three days in IL–3 and SCF (10ng/ml) ±TGF-β1 (10ng/ml), as described in Materials and Methods. Cells were activated with IL-33 (200ng/mL) for 5-30 minutes. The lysates were blotted for ERK 1/2. Data are representative of three populations with mean±SE, after normalizing to total protein loading. *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001
Figure 9. TGF-β1 suppresses IL-33-mediated cytokine production in vivo. TGF-β1 (0.5μg) or PBS was injected intraperitoneally twice daily for 3 days and once on the fourth as described in Materials and Methods. Cytokine profile of plasma post-cardiac puncture was determined by ELISA. Data shown is representative of 5 animals per group with mean±SE. *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001.
Reference List


VITA

Victor Stephane Ndaw was born on July 21st, 1989 in Dakar, Senegal. He is a dual citizen of the United States of America and Brazil. In 2008, he graduated from Lake Braddock Secondary School in Burke, Virginia and went to pursue a degree in biology and minor in chemistry at Virginia Commonwealth University. Following the completion of the Bachelors of Science in 2013, he stayed at Virginia Commonwealth University to begin the Master of Science program in Biology.