**TGF-β1 enhances mouse mast cell release of IL-6 and IL-13**

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Abstract

For immune cells, TGF-β1 can enhance or repress effector functions. Here, we characterize the effects of TGF-β1 on IgE-mediated activation of primary murine mast cells derived from hematopoietic stem cells (BMMC). We also investigated potential interaction between TGF-β1 and stem-cell factor (SCF).

Resting IL-6 production was increased with TGF-β1 but significance was lost following BMMC activation via IgE receptor (FcεRI) crosslinking. SCF also enhanced resting levels of IL-6, but there was no difference from control once FcεRI was engaged. SCF had no effect on IL-13 production; however, TGF-β1 treatment enhanced release of IL-13 upon FcεRI activation. Lastly, percent colocalization of SCF receptor (CD117) and FcεRI were unaffected by TGF-β1 treatment. These data reveal a novel positive effect of soluble TGF-β1 on mast cell activation.

Introduction

Transforming Growth Factor Beta (TGF-β1) is a widely expressed cytokine. The TGF-β1 signaling pathway evolved approximately one billion years ago as an immune regulatory mechanism among vertebrates. TGF-β1 modulates cellular responses starting with binding to TGF-β receptor II (TGF-βRII).

TGF-βRII then aggregates with TGF-βRI at the cell surface inducing the phosphorylation of Smad proteins intracellularly. Ultimately, this cascade reaches the nucleus for transcriptional regulation. TGF-β1 is documented as generally inhibitory starting in the 1990s: as an anti-inflammatory, anti-autoimmune cytokine [1].

Mast cells are myeloid lineage cells of hematopoietic origin. They are present in the skin and along mucosal membranes, especially the gut where they combat helminth parasites. Well known for their roles in allergic pathologies, mast cells also are key to physiology at mucosal barriers. Mast cells are capable of collecting and presenting antigen to other cells [2]. They are also central in driving a Th2 response inducing B cells to class switch to IgE via IL-4 and IL-13 [3]. Canonical activation of mast cells starts with the priming of their high affinity IgE receptor, FcεRI. In the body, mast cells are stably coated
with IgE bound to FcεRI. Upon multivalent antigen binding of these IgE-FcεRI complexes, the receptors cluster – crosslink, and internalize, triggering signaling cascades resulting in degranulation of the cell and activation of transcription factors, such as STAT5, to upregulate cytokine production [3]. Hours to days later these cytokines (e.g., IL-6, IL-13) are secreted [2]. They also exhibit an alternative pathway to activation through IL-33 and its receptor (ST2), independent of FcεRI [4].

Recent evidence suggests that the interaction between mast cells and typical immunosuppressive cytokines varies from other immune cells. A typical inhibitory cytokine, IL-10, was shown to behave as an immunostimulant when given to mast cells and in the development of mucosal food allergies [5]. At the post-transcriptional level, IL-10 was found to regulate microRNAs, which enhanced skin mast cell secretion of IL-6 and IL-13 [6]. These interactions necessitate closer examination of the behavior of mast cells regarding common stimulatory and inhibitory molecules. Herein, we describe effects of TGF-β1 on IgE-mediated mast cell activation by observing the production of two cytokines commonly associated with this process: IL-6 (an inflammatory Th1/Th17 cytokine) and IL-13 (a chemotactic cytokine that drives a Th2 response). We demonstrate that soluble TGF-β1 amplifies mast cell release of these cytokines. This effect opposes SCF exposure, depending on the cytokine; and it is unlikely due to receptor interference at the membrane.

**Methods**

**Mice**

C57/BL6 mice were housed and humanely euthanized according to University of Northern Colorado IACUC protocol #1702C-NP-M-20. When possible, tissue was obtained from control mice scheduled for other experiments.

**BMMC Differentiation**
BMMC were cultured in RPMI 1640 (Thermo Fisher) supplemented with: 10% FBS (VWR), 1% pen/strep, 2mM L-Glutamine, 1mM Sodium Pyruvate, 10mM HEPES (Thermo Fisher), 30ng/mL IL-3 (Peprotech), and 0.05mM β-mercaptoethanol (Bio-Rad). Bone marrow was flushed from mouse femurs and tibias, then cleared of erythrocytes with ACK lysis buffer (Quality Biological). Cultures were initiated at 500,000 cells/mL in T75 flasks (VWR); individual mouse cultures were kept separate. Cultures were observed daily, if adherent cells were present, desired suspension cells were transferred to a new flask. Cultures were maintained between 250,000 and 1,000,000 cells/mL for 4-6 weeks to achieve pure BMMCs. All cultures were randomly divided into SCF-treated and untreated groups during differentiation. SCF-treated cell populations were given 10ng/mL SCF (Peprotech).

TGF-β1 Treatment

For TGF-β1 treated groups, SCF-treated and untreated cells were distributed in 6-well plates at a density between 250,000 and 1,000,000 cells/mL. Cells were treated with 2ng/mL murine TGF-β1 (Cell Signaling) and maintained for two days before IgE priming.

IgE-mediated activation and quantification of BMMC cytokines

Cells were incubated overnight with 500ng/mL of IgE (BD Biosciences; 557079) specific to trinitrophenyl keyhole limpet hemocyanin (TNP-KLH; Santa Cruz Biotechnologies). Treatments were rinsed with RPMI and re-suspended in complete medium. The IgE-FcεRI complexes were crosslinked by adding 300ng/mL TNP-KLH and incubating overnight (IgE-exposed, no TNP-KLH controls for each group included). ELISAs were performed on conditioned media according to the manufacturer’s protocols for IL-6 (PeproTech; 900-T50) and IL-13 (PeproTech; 900-K207).

Microscopy

Cells were treated (TGF-β or IgE-XL) as stated above. Following treatment, cells were rinsed with 1× PBS, fixed (4% PFA), and blocked (5% normal goat serum).
Primary antibodies (FITC-CD117 (mouse-mAB; 1:50; Biolegend #105805), Alexa647 FcεR1 (mouse-mAB; 1:50; Biolegend #134309), and TGFβRII (rabbit-mAB; 1:50; ABClonal #A11765)) were incubated at 4°C for 24 h. Following incubation, cells were rinsed and anti-rabbit Alexa568 (1:500; Life Technologies #A11011) was incubated for 1h at room temperature. Cells were mounted onto microscope slide using 10µL SlowFade-Gold DAPI mounting medium (Life Technologies #S36938) and No. 1 cover slip.

Images were collected using a Zeiss confocal microscope equipped with a 100× oil objective (N.A. = 1.4), acquisition image size 512×512 pixels (33.3µm×33.3µm), light collection 450nm-1000nm. Thirty cells were randomly selected from each slide for analysis. The JACoP plug-in for ImageJ was used to determine the Manders’ coefficient for each image. Coefficients ranged from 0 to 1, which were then transformed into percent colocalization by multiplying by 100.

Statistics

Cytokine secretion was measured in pg/mL per 10^6 cells. Cytokine levels were also quantified based on the fold change compared to the untreated controls. Data was tested for normality using the Shapiro-Wilks test and transformed accordingly.

Cytokine release data were analyzed via Welch’s t-test. Significance was noted at p≤0.05.

Fluoresence microscopy data are reported as least square means±standard error of the mean with significance at p<0.05. The effects of TGF-β1 and FcεRI crosslinking on colocalization of BMMC surface receptors were analyzed using one-way analysis of variance.

Results

TGF-β1 enhances IL-6 secretion by BMMCs

BMMCs were treated with TGF-β1, SCF, or both, and then activated by crosslinking FcεRI. The following IL-6 secretion data were collected from at least 4 separate BMMC populations (i.e., bone marrow differentiated from different mice). IL-6 was increased following IgE-mediated activation (Figure 1 a), but IL-6 was also increased in cells treated with either SCF or TGF-β1 alone independent of IgE-mediated
activation; cells treated with both ligands exhibited higher levels compared to untreated controls (Figure 1 c). When cross-linked, only TGF-β1 appeared to enhance IL-6 secretion but the difference was not significant (Data not shown). These data show that soluble TGF-β1 directly enhances mast cell IL-6 production independent of IgE-mediated activation.

**TGF-β1, but not SCF, enhances IL-13 secretion from BMMCs.**

Concentrations of IL-13 were also measured as the fold change when crosslinked. Un-crosslinked populations secreted little to no detectable IL-13. Figure 1 b shows the raw IL-13 production based on treatment. Unlike IL-6, IL-13 production prior to IgE activation was not affected based on cytokine treatment. However, treatment with TGF-β1 resulted in higher levels of IL-13 upon IgE-mediated activation – this effect persisted when co-treated with SCF, but not with SCF alone (Figure 1 d). These data show that soluble TGF-β1 enhances IL-13 production upon FcεRI engagement.

**Crosslinking decreases FcεRI-CD117 (c-kit) percent colocalization, independent of TGF-β1 treatment.**

Percent colocalization of FcεRI-CD117, TGF-βRII-CD117, and TGF-βRII-FcεRI were determined using immunofluorescence microscopy (Figure 2). Crosslinking (+XL) BMMCs decreased percent colocalization of FcεRI and CD117 in the presence and absence of TFG-β (Figure 2 b). Additionally, the decreased percent colocalization was proportional between treatment groups, indicating TGF-β1 does not directly affect surface receptor function on mast cells (Figure 2 c).

**Discussion**

In our experiments soluble TGF-β1 stimulates IL-6 secretion independent of IgE-mediated activation. TGF-β1 has been shown to promote mast cell IL-6 production in the context of lung inflammation; this promotes neutrophil apoptosis and clearance [7]. However, this mechanism involved T<sub>reg</sub> cell surface sequestered TGF-β1 [8]. Our findings suggest that TGF-β1 plays a directly stimulatory role on IL-6 production, potentially independent of immunosuppressive T<sub>reg</sub>, which could elicit acute phase inflammatory responses. Characterizing the mechanism(s) involved and carefully testing the direct
effect(s) of soluble TGF-β1 on other myeloid cells is warranted. Alternatively, myeloid-derived suppressor cells (MDSC) utilize IL-6 to support tumor progression [9]. MDSCs also enhance IL-6 and IL-13 secretion by activated mast cells [10]. The careful study of interactions between TGF-β1, IL-6, T_{reg}, and mast cells will be insightful in chronic inflammatory settings, such as high-grade solid cancers.

Here, TGF-β1 also enhances production of IL-13 following mast cell activation via FcεRI. IL-13 is a major cytokine in T_{H}2-related immune responses, likely responsible for the clearing of large extracellular insults, such as gut parasites [11]. However, unmitigated IL-13 release will drive B-lymphocyte class switching to IgE, which in turn coats naïve mast cells via FcεRI, thus prompting a vicious T_{H}2 cycle [12]. Our experiments show that TGF-β1 directly enhances IL-13 production, which propagates such pathologies.

Through fluorescence microscopy we observed any effects TGF-β1, and its receptor TGFBRII, might have at the signaling apex of the definitive mast cell surface receptors FcεRI and CD117 (SCF receptor). FcεRI and CD117 colocalization is lost upon crosslinking of FcεRI with IgE-antigen complexes. This is not surprising as FcεRI internalization after cross-linking is known [13]. However, this decline in colocalization is unchanged with TGF-β1 treatment, suggesting there is no apparent surface cross-talk occurring among these receptors. It has been shown that TGF-β1 transcriptionally represses FcεRI and CD117 through regulation of Etf homologous factor (Ehf) [14]. This inhibition was not observed in our studies, suggesting reduced receptor expression of CD117 through flow cytometric analysis does not directly correspond to reduced receptor expression.

We demonstrate that TGF-β1 plays a direct stimulatory role on primary mast cells in vitro. The specific molecular mechanism underlying this effect is still unknown. TGF-β1 might modulate targets outside of the canonical TGF-β1 signaling cascade (Smads) as preliminary experiments using a TGF-βR1 inhibitor resulted in no significant differences in cytokine expression (data not shown). IL-6 secretion was increased independent of IgE-mediated activation, suggesting that TGF-β1 non-canonically targets the
151 MAP kinase or Akt pathways to enhance IL-6 production [15]. Future research into the phosphorylation
152 patterns of this pathway will reveal the specific effects of TGF-β1. Additionally, IL-13 production was
153 altered only after IgE activation, suggesting that TGF-β1 modulates the FcεRI signaling pathway (e.g.,
154 STAT5). Altogether these data necessitate careful examination of soluble TGF-β1 with respect to mast
155 cell effector functions.

156 Authors’ Contributions

157 DL undertook experiments, design and data analysis, and drafted the manuscript; MP performed
158 microscopic analyses; NP conceived, designed, and coordinated the study, and assisted manuscript
159 drafting. All authors gave final approval for publication.

160 Competing Interests

161 The authors declare no competing financial interests.

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**Figure Legends**

**Figure 1:** IL-6 and IL-13 secretion by mast cells based on treatment. (a and b) Raw cytokine production for IL-6 (a) and IL-13 (b) corrected for the number of cells assayed. (c) IL-6 production in resting (un-crosslinked) BMMCs. (d) Fold change in IL-13 production following mast cell activation.

**Figure 2:** Colocalization of TGF-βRII, FcεRI and CD117. (a) Representative micrographs of TFG-βRII with FcεRI or CD117. (b) Representative micrographs of FcεRI and CD117; corresponding colocalization shown below. (c) Quantitative analysis of colocalization within treatment. Arrow represent regions of colocalization. Micron bar represents 10μm. Means with different letters abc differ significantly within treatment (P < 0.05).
Figure 1

The graphs illustrate the production of cytokines IL-6 and IL-13 under different conditions.

**a)** IL-6 levels in cell cultures with varying treatments of IgE-XL, TGF-β, and SCF.

**b)** IL-13 levels under the same treatments as in (a).

**c)** IL-6 levels in unactivated cells with treatments of TGF-β and SCF.

**d)** Fold change in IL-13 after activation with TGF-β and SCF.

Significant changes are indicated by asterisks (*) in the graphs.
Figure 2

a) TGF-β - - + + +
    IgE-XL - + - + +

b) TGF-β - - + + +
    IgE-XL - + - + +

c) FcεR1-CD117 □ TGFβR2-CD117 □ TGFβR2-FcεR1

Percent Colocalization

- TGFβ-XL - TGFβ-XL + TGFβ-XL + TGFβ-XL

a b c a b a b

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