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Transforming Growth Factor-β1 (TGF-β1) Induces Mast Cell Apoptosis

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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

TGF-β1	Transforming Growth Factor-β1
BMP	Bone Morphogenetic protein
MC	Mast cell
BMMC	Bone marrow derived mast cell
Ig	Immunoglobulin
WT	Wild type
SCF	Stem cell factor
IL	Interleukin
$\Delta\Psi m$	Mitochondrial Membrane Potential
Stat5	Signal transducer and activator of transcription 5
pY-Stat5	Tyrosine Phosphorylated Stat5
T_h	Helper T cells
FITC	Fluorescein isothiocyanate
PE	Phycoerythrin
PI	Propidium Iodide
RPA	RNase Protection Assay

Abstract

TRANSFORMING GROWTH FACTOR-β1 (TGF-β1) INDUCES MAST CELL APOPTOSIS

By Farnaz Norozian

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

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Major Director: John J. Ryan, Ph.D., Associate Professor

Mast cells are potent effectors of the inflammatory response, playing an important role in atopy, bacterial immunity, and animal models of arthritis, multiple sclerosis, and heart disease. Hence controlling mast cell numbers and responsiveness is essential for preventing inflammatory disease. This work demonstrated that the cytokine TGF-β1 is a potent inducer of mast cell apoptosis, a finding that was consistent for cultured mouse bone marrow-derived mast cells, peritoneal mast cells, and human mast cells. Cell death appeared to be the result of TGF-mediated repression of IL-3 receptor expression and function, leading to mitochondrial damage and activation of an apoptotic cascade acting

via p53 and caspases. While IL-3 receptor expression was reduced within one day of TGF-β1 stimulation, apoptosis required at least 3 days to occur. This delay in onset is postulated to allow for protective mast cell effector functions, protecting the host from infection while preventing the establishment of chronic inflammation. These studies support the theory that TGF-β1 is an inhibitor of mast cell survival. Because of the widespread expression of TGF-β1, this cytokine may be an ideal candidate for control of mast cell homeostasis.

Introduction

Mast cells are among the key players involved in defense mechanisms against pathogens and signal the initial activities involved in immunity, such as inflammation, that generate a vascular reaction and late phase activities that promote leukocyte accumulation and ultimately wound healing (Groneberg *et al.*, 2005). Mast cells are also critical effector cells in immediate hypersensitivity, observed as a direct result of the various mediators synthesized and secreted by the mast cells (Mekori and Metcalfe, 2000; Williams and Galli, 2000). Mast cell activation results in the release of pre-formed vasoactive amines and de-novo synthesized cytokines, chemokines and prostaglandins that collectively induce a local or systemic inflammatory response (Rivera, 2002). The effects that mast cells have on the body can be traced back to the release of effector molecules stored in granules that are exocytosed upon mast cell activation. Mast cell granules contain a spectrum of molecules such as histamine, which can trigger allergy (Xie and He, 2005; Falus and Meretey, 1992). Mast cells can also be stimulated to synthesize other cytokines and chemokines that have chemotactic effects.

While mast cells are responsible for immediate hypersensitivity, their widespread distribution in the skin and in the respiratory tract suggests a role for these cells as a first-line of defense against invading pathogens, and they have been found to be critical for host resistance to some bacterial infections (Malaviya *et al.*, 1995). Despite this protective role, unregulated mast cell activation can result in deleterious effects. In fact mast cells are associated with human allergic disease and implicated in mouse models of the autoimmune diseases such as multiple sclerosis and rheumatoid arthritis (Brown *et al.*, 2002). Changes in mast cell numbers and immunoregulatory effects have been observed in patients presenting with symptoms ranging from simple cutaneous pruritis, urticaria and skin lesions to severe nausea, vomiting, and tachycardia, as well as patients presenting with delayed hypersensitivity, parasitic or bacterial infections, fibrosis, autoimmune diseases, neoplasia, hyperplasia-induced mastocytosis, rheumatoid arthritis, and inflammatory bowel disease (Ali and Panettieri, 2005; Kanamaru *et al.*, 2005; Xie and He, 2005; Brandt E.B. *et al.*, 2003; Rice *et al.*, 1998; Baram *et al.*, 1997). The immunoregulatory effects of mast cells can even be observed in patients with biomaterial implants, where mast cells and their granular products, especially histamine, have been observed to be important in recruitment of inflammatory cells as a response by the host to biomaterial implants (Tang *et al.*, 1998). This evidence collectively supports the importance of understanding mast cell function in the immune system.

Among the molecules produced by mast cells, many can act in an autocrine manner. Molecules produced by other cells in the environment can affect mast cells in a paracrine fashion (Kitaura *et al.*, 2005). Transforming Growth Factor-beta 1 (TGF-β1) is one such cytokine that is both produced by mast cells and is also available to the mast cell in blood plasma. Our lab recently showed that TGF-β1 inhibits mast cell development and dampens expression and function of mast cell IgE receptors (Kashyap *et al*, 2005; Gomez *et al.*, 2005). Many signaling pathways function to inhibit cell division, survival, and proliferation. TGF-beta is perhaps the best known example of a cytokine-mediated pathway by which proliferation is inhibited (Edlund *et al.*, 2003). These studies raise the possibility that TGF-β1 is a paracrine or autocrine inhibitor of mast cell function.

Transforming Growth Factor-beta

The transforming growth factor-beta (TGF-β) superfamily consists of a large number of structurally related, secreted, dimeric proteins. They act either as hormones, or as local mediators to regulate a wide range of biological functions in all animals. TGF-β function begins in development, where it regulates pattern initiation of embryo, extracellular matrix production, and cell death. In adult life TGF-β functions in tissue repair as well as in immune regulation. The TGF-β family consists of the TGF-β1,-2,-3, as well as activins, and bone morphogenetic proteins (BMPs) (ten Dijke and Hill, 2004; Shi and Massague, 2003; Massague, 1990). TGF-β family proteins have both stimulatory and inhibitory effects on a variety of cellular functions, including differentiation, proliferation, and apoptosis. A number of studies have implicated TGF-β family members in several different physiological processes including inflammation, fibrosis, and angiogenesis. TGF-β proteins have also been linked to autoimmune disease, antherosclerosis, fibrotic disease, and cancer in humans (Blobe *et al.*, 2000; Letterio and Roberts, 1998).

All TGF-β proteins act through enzyme-linked receptors, transmembrane proteins that span the membrane once, with a serine/thereonine kinase domain on the cytosolic side of the plasma membrane (Benning, and Kyprianou, 2002). There are two types of receptors, type I and type II, and each member of the TGF-beta family binds to a specific combination of the receptors where both are required for the proper signal transduction. The most common scenario is that the ligand binds to the type II receptor, which forms homodimers, recruiting, phosphorylating, and activating the typeI receptor homodimer ultimately yielding an activated tetrameric receptor complex. The post tetrameric receptor complex signal is rapidly relayed to the nucleus by way of binding to and phosphorylating Smad, a latent gene regulatory protein (Benning and Kyprianou, 2002). Dependent on the actual ligand, either Smad1, 2, 3, 5, or 8 is activated. Once the specific Smad is phosphorylated, it disassociates and binds to Smad4. This newly activated Smad4 forms a complex with either Smad1, 2, 3, 5 or 8 and moves into the nucleus, where it associates with other gene regulatory proteins, binds to specific target sites on the DNA, and activates a particular set of target genes.

TGF-β1, the prototypic family member and the cytokine of interest for this study, is synthesized as a precursor polypeptide and secreted in a latent form by most cell types (ten Dijke and Hill, 2004; Shi and Massague, 2003; Massague, 1990). Studies showing the inhibitory effects of TGF-β1 on immune cell function support the role of TGF-β1 as a suppressor of immunity and inflammation (reviewed in Letterio and Roberts, 1998). Emphasizing the importance of TGF-β1-mediated immunosuppression is the observation that TGF-β1-null mice develop severe inflammation, wasting syndrome, and organ failure leading to death by 3 weeks of age (Kulkarni *et al.*, 1993; Shull *et al.*, 1992).

It is consistently evident that a key strategy of multicellular control is to balance cellular growth and proliferation with cellular death. Therefore when mutations occur in genes that regulate apoptosis, there is an increase in net cell numbers, a key hallmark of malignant cells (Edlund *et al.*, 2003; Greenlee *et al.*, 2000). Furthermore it has been shown that disrupting the genes with products regulating cellular growth, proliferation and or apoptosis, such as TGF-β1, can lead to excessive inflammatory responses (Kulkarni *et al.*, 1993). Such a mutation in TGF-β, its receptors, or any of the components of the pathway by which it signals a cell to undergo apoptosis could result in tumor cell development, fibrosis, angiogenesis, and range of autoimmune diseases listed above.

The term apoptosis, also known as programmed cell death or cellular suicide, comes from the Greek word for "falling off," as in leaves from a tree, suggesting a natural event in the life of living things. Apoptosis was first defined by J.F.R. Kerr in 1972 as a systematic sequence of structural changes that a cell undergoes to ultimately achieve programmed cell death (Kerr *et al.*, 1972). The cells of multicellular organisms are not only highly organized but also strictly regulated. Cellular regulation via apoptosis is not only a mechanism controlling when a cell needs to undergo programmed cell death, but also the rate at which the process occurs.

Under normal conditions in a eukaryotic cell, there is an astonishing amount of apoptosis that occurs in both developing and adult animal tissues. For example, during embryonic development, cells undergo apoptosis when they are no longer needed such as the webbing between fingers. Apoptosis also occurs in adults as billions of cells can undergo apoptosis every hour in the bone marrow. This rate of cell death is balanced by the division of cells with in the tissue (Tomei and Cope, 1991). Of course the above examples are a mere glimpse into the world of cellular regulation via apoptosis. The role of apoptosis may vary from tissue to tissue and cell-to-cell, however, the molecular mechanisms of apoptosis and its control described below share common themes. These also work co-ordinately among various systems with cell proliferation to regulate cell numbers in the multicellular world.

Intracellular & Extracellular System of Apoptosis

Apoptosis is a critical component of cellular regulation in normal cells. It is then fitting that apoptosis is a well organized process with many intricate pathways still being studied and elaborated. Generally when a cell undergoes apoptosis, it shrinks and the chromatin condenses. The cytoskeleton collapses, the nuclear envelope disassembles, and the nuclear DNA breaks up into fragments. The cell surface displays properties allowing it to be recognized by target cells, which serve as signals for the apoptotic cell to be phagocytosed rapidly. The common intercellular machinery responsible for apoptosis seems to be similar across all animal cellular systems (Wyllie *et al.* 1990; Kerr *et al.* 1972).

The caspases are proteins with a key role in apoptotic pathways. Caspases are a family of proteases with a cysteine in their active site and cleave their target proteins at specific aspartic acids. They are synthesized in the cell as inactive precursors and are activated upon proteolytic cleavage. Ultimately amplification of a proteolytic cascade results in the cleavage of other key proteins such as the nuclear lamin, or a DNA-degrading enzyme. This protease cascade is not only destructive and self-amplifying but also irreversible (Wyllie *et al.* 1990).

These procaspase activations can be triggered from either the outside or inside of the cell, for example, through killer lymphocytes, or via the mitochondrial pathway by cytochrome C release, respectively. There are extracellular survival factors that suppress apoptosis. These function in cellular growth control. When these survival factors are removed the cell activates its programmed cell death mechanisms. A key player in the mechanism by which a cell's survival is controlled is the state of the cell surface receptor expression. A given ligand requires a functional receptor and functional pathway-associated proteins to signal a cell to survive or undergo apoptosis.

Thus far the mechanisms explained by which extracellular factors control a multicellular organism have been positive regulators. There are equally important inhibitory extracellular signal proteins that oppose the positive regulators and thereby inhibit proliferation. One of the best understood inhibitory signal proteins belongs to the TGF-β family. TGF-β is known to stimulate apoptosis as well as inhibit the proliferation of all most all cell types, with the one exception being fibroblasts.

TGF-β1 is produced by mast cells and may be stored in their granules (Pennington *et al.*, 1991). TGF-β1 is found in the cell matrix and kept in its latent form, which is cleaved to the active form when needed (Lindstedt *et al.*, 2001). TGF-β1 can be observed in circulating blood at concentrations exceeding 30 ng/ml (Young *et al.*, 1999). In vivo studies have shown that TGF-β1 inhibits murine hypersensitivity (Meade *et al.* 1992). However work done in the past in an attempt to elucidate the effect of TGF-β1 contradicts the preliminary finding in our lab where TGF-beta has been observed to induce mast cell death by apoptosis. Broide *et al.* (1989) reported that TGF-β1 selectively inhibited IL-3 dependent proliferation of bone marrow-derived mast cells without affecting their overall function or differentiation. However, the work reported by Broide *et al.* (1989) was performed using cellular proliferation assays administering TGF-β1 at a concentration of 0.1 ng/ml. In the same study cells were observed for only a short period of time, within 72 hours after TGF-β1 treatment, and cell viability determined after trypan blue staining. Although trypan blue is a useful, time efficient technique for determining viable cell numbers, there are more sensitive techniques by which cellular proliferation, survival and ultimately apoptosis can be measured. Furthermore, another study reported that TGF-β1 prevents the Stem Cell Factor (SCF) rescue of IL-3 deprived IL-3-dependent mast cells. SCF, the ligand for Kit, is known to rescue mast cells from an apoptotic fate (Mekori and Metcalfe, 1994). This same study reported that no substantial effects were observed for IL-3 dependent mast cells treated with TGF-β1. Perhaps these studies did not find the link between TGF-β1 and mast cell apoptosis however, they did find the inhibition of receptor expression and or signaling that we suspect causes apoptosis. Inconsistencies between studies presented in the past and the observations in our lab, provided an opportunity to explore and elucidate the functions and immunoregulatory abilities of cytokines such as TGF-β1 on mast cells. The objective of this study was to elucidate the effects of TGF-β1 on the survival of mast cells.

Experimental Designs and Methods

Cytokines and reagents

Murine IL-3, stem cell factor (SCF), and TGF-β1 were purchased from R&D System (Minneapolis, MN). Human SCF was the kind gift of Amgen Corp. Mouse IgE, fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse Kit, phycoerythrin (PE)-conjugated anti mouse IL-3Rβ, and PE-conjugated anti-human Kit were purchased from BD PharMingen (San Diego, CA). FITC-conjugated rat anti-mouse IgE was purchased from Southern Biotechnology Associates (Birmingham, AL). Stat5 antibodies were purchased from Upstate Biotechnology (Lake Placid, NY).

Mouse bone marrow-derived mast cells (BMMC)

Bone marrow cells were obtained from C57BL/6J female mice (3-5 weeks old) (Jackson labs., Bar Harbor, ME) by with a 22 gauge needle, flushing femurs and tibias containing complete RPMI media (RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, 100 μ/ml streptomycin, and 1 mM sodium pyruvate (all from Biofluids, Rockville, MD). Red blood cells were removed with a ammonium chloride potassium (ACK) lysis buffer. Cells were counted following trypan blue staining (trypan blue solution, 0.4%, SIGMA, St. Louis, MO) and plated at 3 x 10^5 cells/ml in cRPMI +

Interleukin 3 (IL-3) + Stem Cell Factor (SCF) (30 ng/ml each) (Peprotech, Rocky Hill, NJ) and incubated at 37˚C for the duration of cultures (ThermoForma, Marietta, OH). Non-adherent cells were transferred to new plates every 4 days to select against adherent cells. This ensured good recovery of mature mast cells after 21 days of culture.

After cells reached maturation, which was constituted by a population of >95% with surface expression of Kit and T1/ST2 and FcεRI (data not shown), they were maintained in 20% WEHI conditioned cRPMI. FcεRI expression was monitored by with mouse IgE (Pharmingen, San Diego, CA) and secondary staining with FITC-rat anti-mouse IgE (Southern Biotechnology, Birmingham, AL). Antibody labeled data was acquired with a BD-Pharmingn FACScan flow cytometer. BMMC were used within three months of their maturation.

Peritoneal cells were harvested from euthanized mice by lavage of the peritoneum with 5 ml of cRPMI injected into the peritoneal cavity. These cells were cultured in cRPMI and IL-3+SCF. Human skin-derived mast cell populations, were derived as described previously (Kepley, 2005) and provided by Dr. Kepley.

Culture conditions

Cells were plated at 3 x 10^5 cells/ml in cRPMI. After 4 hours of starvation, cytokines were added at the following final concentrations: IL-3 at 5 ng/ml; TGF-β1 at 5 ng/ml or 10 ng/ml as noted. Treatment conditions were as follows: treated with IL-3, IL-3 + Vehicle, or IL-3 + TGF-β1. IL-3 and TGF-β1 were purchased from R&D (Minneapolis, MN). A solution of 4 mM HCl + 1 mg/ml bovine serum albumin (BSA) was used as a vehicle to resuspend lyophilized TGF-β1. Cells were incubated at 37°C for indicated times. Feeding of cultures was maintained every 4th day of culture, with half of the medium and cytokines removed and replaced.

Mast cell viability and apoptosis

Cells were washed and re-plated at $3x10^5$ cells/ml in 200 μl cRPMI/well in 96-well flat-bottom plates. IL-3 was added at 5 ng/ml, with indicated concentrations of TGF-β1. Cultures were incubated for the indicated times. Every 4 days half the media and cytokines were replaced. A volume of 200 μl was harvested from culture conditions described above, centrifuged in a 96-well v-bottom plate form 5 minutes and then washed with PBS, twice. Cells were then fixed by resuspending in 150 μl PI fixation buffer (35% 1X phosphate buffer solution (PBS), 52% EtOH, and 12.5% FCS), and stored at 4°C for 4 hrs-7 days. After fixation, cells were washed with PBS and resuspended in 200 μl of PI-DNA staining buffer (1ml stock PI (1 mg/ml (SIGMA, St. Louis, MO)), 18.8 mls 1X PBS, 200 μl of RNAse A (1 ng/ml (Boehringer Mannheim, Indianapolis, IN)), and 4 μl 0.5 M EDTA (Quality Biological Inc., Gaithersburg, MD)), incubated at room temperature in the dark for2-3hrs. Cells were assessed for ≥ diploid (viable) or sub-diploid (apoptotic) DNA content by propidium iodide (PI) staining following cell fixation and permeabilization (PI-DNA staining) by flow cytometry. Samples were analyzed by flow cytometry to determine the percentage of the population in sub-diploid DNA state.

Tissue culture conditions for cell viability and surface receptor expression

Cells were washed and re-plated at $3x10^5$ cells/ml in 200 µl cRPMI/well in 96-well flat-bottom plates. IL-3 was added at 5 ng/ml, with indicated concentrations of TGF-β1. Cultures were incubated for the indicated times. Every 4 days half the media and cytokines were replaced. Cells were harvested, and washed twice with FACS buffer (500 ml 1X PBS, 15 ml FBS, and 5 ml 10% Na Azide (SIGMA, St. Louis, MO), blocked with an nonspecific binding antibody and then IL-3 Receptor-α, and IL-3 Receptor-β, and c-kit Receptor expression was monitored with labeled or unlabeled antibodies for the receptors (Purified IL-3R α; PE anti-mouse β_{IL-3}/βc; PE anti-mouse CD117; respectively, BD Bioscience, San Jose, CA), and data was acquired with a BD-Pharmingen FACScan flow cytometer.

RNase Protection Assay (RPA):

Cells were cultured and treated by cytokines as previously indicated. To establish whether the regulation of receptor expression was controlled at either the RNA level or at the protein level, cells were cultured as described above and harvested at times before apoptosis was observed with PI-DNA analysis. Whole cell RNA was isolated from 5×10^6 cells, and mRNA expression of the receptors of interest or their subunits were checked by RPA. RNA extraction was done with Trizol (Life Technologies, Gaithersburg, MD). The Riboquant system kit (BD Pharmingen, San Diego, CA) was used to synthesize ^{32}P-UTP (Uridine 5'-Triphosphate, [α-^{32}P], IMP Biomedicals, Aurora, OH) labeled probes containing the IL-3 receptor β mast cells gene and the control genes L32 and GAPDH, following the manufacturer's protocol. The expression of the genes of interest was then determined with using polyacrylaminde gel electrophoresis. Results were visualized with radiography, and quantitative analysis was obtained with phosphorimaging. The ratio of the pixel intensity for each band of interest to the sum of the pixel intensities for the housekeeping genes (L32+GAPDH) in that lane were determined. Calculations of percent change in expression relative to control conditions were determined by comparing these ratios.

Western blot analysis

Cells were cultured and treated by cytokines as previously indicated. Tyrosine phosphorylated Stat5, total Stat5, and actin were detected by western blotting of total cell lysates (approximately 50 μg/lane). Anti-Stat 5 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA.). Cells were washed twice with RPMI, replated at $5x10^6$ cells/ml, incubated over night without IL-3, then restimulated with IL-3 at 100 ng/ml for fifteen minutes. Cell lysates were harvested and protein levels analyzed by western blot. To determine the percent change in expression, band intensity was measured by densitometry. The ratio of tyrosine phosphorylated Stat5 to total Stat5 was determined, and ratios were compared between lanes to determine percent decrease in the presence of TGF-β1.

Caspase activation analysis:

Cells were cultured and treated by cytokines as previously indicated. Caspase activation was assessed with the Caspatag assay (Intergen, Purchase, NY) specific for caspase 3, 8, and 9 activation following the manufacturer's instructions.

Mitochondrial membrane potential analysis:

Cells were cultured and treated by cytokines as previously indicated. Cells were harvested, washed twice with 1X PBS. A $Di(OC_6)_3$ (3,3'-Dihexyloxacarbocyanine, iodide) was added to 200 μl of cells at a final concentration of 1 nM (Molecular Probes, Eugene, OR). Cultures were incubated for 15 minutes at 37°C in a CO_2 incubator. Cells were washed twice with 1X PBS and resuspended in 200 μl PBS for flow cytometric analysis. Mitochondrial membrane potential was observed, and data collected with the BD-Pharmingen FACScan flow cytometer.

Results

TGF-β1 induces mast cell apoptosis

To determine the effects of TGF on mast cell survival, mouse BMMC were cultured with IL-3 +/- TGF-β1. BMMC are primary, IL-3 dependent mast cells that function as a reliable model system for mucosal mast cells (Rottem *et al.*, 1992), hence the effects of TGF-β1 on this population are likely to be representative of connective tissue mast cells. As shown in Figure 1A, BMMC cultured with TGF-β1 for 6 days exhibited an increase in sub-diploid DNA content, indicating DNA fragmentation that is consistent with apoptosis. Apoptosis occurred consistently after 3 days of culture, with a peak of 40-50% cell death after 4 days of culture (Figure 1B). This effect was dose-dependent, requiring approximately 2 ng/ml TGF-β1, with maximum apoptosis observed at 10 ng/ml. As stated, this is well with in the range of TGF-β1 available in normal human serum (Young *et al.*, 1999).

TGF-β1-induced apoptosis correlates with reduced IL-3 receptor expression

TGF-β1 is known to block expression of the mast cell survival receptor Kit and also inhibits Kit-mediated rescue from growth factor withdrawal-induced apoptosis (de Vos *et al.*, 1993; Dubois *et al.* 1994; Mekori and Metcalfe, 1994). Since IL-3 is the survival factor used in these assays, loss of its survival function seemed a plausible means by which TGF-β1 could elicit programmed cell death. To address this possibility, we measured the effects of TGF-β1 on BMMC IL-3 receptor expression with flow cytometry. The IL-3R is composed of an alpha (α) chain paired with either an IL-3-specific beta (β) chain or a common (βc) chain shared with IL-5 and GM-CSF (Kitamura and Miyajima, 1992). IL-3Rα expression was found to be below the level of detection (data not shown). In contrast, an antibody that recognizes both IL-3Rβ chains demonstrated robust expression that was significantly inhibited by TGF-β1 (Figure 2A). Importantly, the reduction of IL-3Rβ expression preceded the onset of cell death, with 30% inhibition after 8 hours of TGF-β1 stimulation, peaking at 65% inhibition by day 3 of culture, when apoptosis was first detected (Figure 2B). Since BMMC die approximately 3 days after IL-3 withdrawal (our unpublished findings), the timing of TGF-β1-mediated inhibition of IL-3R expression fits well with the onset of apoptosis.

TGF-β1 was found to blocked mast cell FcεRI expression by reducing translational efficiency, with little effect on mRNA expression (Gomez *et al.*, 2005). Like FcεRI, we found that TGF-β1 had no effect on IL-3Rβ mRNA expression, and reduced βc message by only 20% at time points preceding or after the onset of IL-3R repression (Figure 2C and D). It appears that TGF-β1 most likely dampens IL-3R expression through post-translational effects that occur with the appropriate timing to explain the onset of mast cell apoptosis.

TGF-β1-mediated IL-3R repression inhibits Stat5 activation and maintenance of mitochondrial membrane potential

If the reduction in IL-3R expression is functionally significant, it should prevent proper activation of the Stat5 pathway, which we have shown to be essential for mast cell survival (Shelburne *et al.*, 2003). To test the effects of TGF-β1 on IL-3-mediated Stat5 signaling, BMMC were cultured in IL-3 +/- TGF-β1 for 3 days, the point at which IL-3R expression reached its nadir and apoptosis was initiated. After a starvation period to remove any residual IL-3 signaling, these cells were re-stimulated with IL-3, and Stat5 phosphorylation was measured by western blotting. As shown in Figure 3A, TGF-β1 reduced Stat5 phosphorylation by 45-50%. Since we have found that Stat5 expression is necessary for maintaining mitochondrial membrane potential ($\Delta\Psi m$) (Shelburne *et al.*, 2003), we determined the effect of TGF-β1 on $\Delta\Psi m$ via $Di(OC_6)_3$ staining. As shown in Figure 3B, TGF-β1-stimulated cells exhibited reduced $Di(OC_6)_3$ staining, shifting toward the spectrum displayed by BMMC cultured without IL-3, a condition known to induce mitochondrial damage (Bojes *et al.*, 1999). The results suggest that TGF-β1-mediated repression of the IL-3 receptor is biologically relevant, reducing IL-3 signaling to an extent that induces mitochondrial damage.

TGF-β1-induced apoptosis requires p53 expression

Loss of IL-3 signaling has been shown to induce a p53-dependent apoptotic cascade that occurs with mitochondrial damage (Blandino *et al.*, 1995). The effects of TGF-β1 mirror IL-3 deprivation, and hence may employ the p53 pathway for programmed cell death. In fact, we found that p53-deficient (KO) BMMC exhibited little apoptosis after culture with TGF-β1 (Figure 4A). This p53 dependency was confirmed by a substantial reduction in TGF-β1-mediated activation of effector caspases-3 and -7 (Figure 4B). These data support the theory that TGF-β1 induces mast cell apoptosis by sufficiently repressing IL-3 receptor expression to mimic IL-3 withdrawal and the p53-dependent/mitochondrial pathway that ensues from this deprivation.

Factor-independent mastocytoma cells are resistant to TGF-β1-induced apoptosis

If the effects of TGF-β1 are mediated via its blockade of the IL-3 receptor, cells not requiring this growth factor should be resistant to TGF-β1. To test this, we cultured factor-independent P815 mastocytoma cells in IL-3 +/- TGF-β1. These cells possess a mutant, constitutively active Kit receptor that drives their continual proliferation. As shown in Figure 5A, TGF-β1 stimulation for 6 days had little effect on the viability of P815 cells. We detected no significant change in sub-diploid DNA content and no increase in Caspase-3/7 activation (Figure 5B). Thus these IL-3-independent mastocytoma cells are completely resistant to TGF-β1 induced cell death, supporting our hypothesis that TGF-β1 acts through repression of IL-3R expression and signaling.

TGF-β1 represses Kit expression and induces apoptosis in mouse peritoneal mast cells and cultured human mast cells

While mouse BMMC are a reliable model system for studying mast cell biology, their IL-3 dependency is distinct from the importance of SCF in vivo. To confirm that our findings with BMMC cultures were consistent in other mast cell populations, we measure the effects of TGF-β1 on freshly isolated mouse peritoneal mast cells and cultured human mast cells, which rely upon SCF for survival and proliferation signals. Peritoneal cells were cultured for 6 days in IL-3 +SCF +/- TGF-β1. As shown in Figures 6A and B, TGF-β1 reduced the expression of both IL-3Rβ and SCF receptor, Kit, nearly 60%. Reduction in number of mast cells in theses cultures was also observed at 80.5% (SD = 14.2, n = 6). Human skin-derived mast cell populations (HSMC) also confirmed the inhibitory effects of TGF-β1. By day 3 of culture in SCF+ TGF-β1, HSMC showed greatly reduced Kit expression (Figure 6C). This effect mimicked our observation with the mouse IL-3R. Further, TGF-β1 induced human mast cell apoptosis, as judged by the presence of sub-diploid DNA (Figure 6D). These results were consistent in HSMC derived from 3 individuals, with apoptosis increasing from 18.2% to 36.1% after the addition of TGF-β1 ($p = 0.02$). Hence the apoptotic effects of TGF-β1 are consistent in murine and human mast cells cultured ex vivo.

Discussion

Mast cell activation is a central facet of atopic diseases such as allergic asthma. The incidence of these diseases has risen dramatically in recent years, emphasizing the importance of understanding and controlling mast cell function. Our efforts have focused on mast cell homeostasis, regulating cell numbers and function. Since mast cells provide critical resistance to bacterial and parasitic infections, but also elicit inflammation related atopy, arthritis, multiple sclerosis, and heart disease, this cellular homeostasis maybe the fulcrum balancing health and disease. Mast cells are responsive to many cytokines that can provide homeostatic control. It has been shown that the Th2 cytokines IL-4 an IL-10 repress mast cell development, activation, and survival (Bouton *et al.*, 2004; Gillespie *et al..*, 2004, Bailey *et al.*, 2004; Yeatman *et al.*, 2000; Mirmonsef *et al.*, 1999; Ryan *et al.*, 1998). While Th2 cells are closely tied to mast cell activity, the presence of TGF-β1 in tissues where mast cells reside, and the high level of serum TGF-β1 available during inflammation-induced vasodilation drew our attention to this cytokine. In testing the effects of TGF-β1 on mast cells, we found it capable of suppressing mast cell development and inhibiting IgE receptor expression and function (Gomez *et al.*, 2005; Kashyap *et al.*,2005). It was during these experiments that we noted the apoptotic effect of TGF-β1.

This work shows that TGF-β1 elicits mast cell apoptosis by repressing IL-3R expression, resulting in a factor-withdrawal response occurring with p53 activation, mitochondrial damage and caspase activation. These effects were consistent in cultured mouse mast cells, peritoneal mouse mast cells, and human mast cells. Thus it is unlikely that our data are related to culture artifacts or species differences. This inhibitory signaling was sensitive, occurring at 1-2 ng/ml, which is well below the physiological concentration of TGF-β1.

Our studies with TGF-β1 demonstrate its role as a potent inhibitor of mast cell, supporting the hypothesis that it can contribute to mast cell homeostatsis. This theory is bolstered by the work of several other labs. For example, TGF-β1 diminished IgE-mediated histamine release of TNF alpha production in vitro (Bissonnette *et al.*, 1997), and inhibited in vivo mast cell responses (Meade *et al.*,1992). TGF-β1 has been shown to inhibit IL-3, IL-4, and SCF-mediated signaling of mast cells, decreasing proliferation or rescue from apoptosis (Toyota *et al.*, 1995; Mekori and Metcalfe, 1994; Broide *et al.*, 1989).

The timing of TGF-β1-mediated inhibitory effects mirrors very much the work of this lab with IL-4 and IL-10, which diminishes mast cell function and survival after 3-6 days of culture (Bouton *et al.*, 2004; Gillespie *et al.*., 2004, Bailey *et al.*, 2004; Yeatman *et al.*, 2000; Mirmonsef *et al.*, 1999; Ryan *et al.*, 1998). We have postulated that this delay in inhibitory signaling may frame an “inflammatory window” during which mast cell responses elicit inflammation to control infection, but after which mast cells are repressed to prevent tissue damage. The effects of TGF-β1 fit well with this theory. For example, TGF-β1 has been reported to elicit mast cell migration (Olsson *et al.*, 2001; Olsson *et al.*, 2000). There is also evidence that mast cell proteases activate latent TGF-β1 (Lindstedt *et al*, 2001; Taipale *et al.*, 1995). Thus it is plausible that TGF-β1 acts to draw mast cells to an area of inflammation, where they serve a protective role. Subsequent to prolonged (3 day) stimulation with TGF-β1, in part mediated by latent TGF-β1 activation by mast cell proteases, mast cell function and survival would be repressed. This feedback system would restore homeostasis and prevent chronic inflammatory disease.

This is the first evidence that TGF-β1 directly induces apoptosis of mouse and human mast cells. Our results support the concept that TGF-β1 and other inhibitory cytokines normally function in a homeostatic fashion controlling the mast cell inflammatory response. It is plausible that loss of these control mechanisms contributes to inflammatory and autoimmune diseases, emphasizing the importance of understanding the molecular mechanisms controlling mast cell homeostasis.

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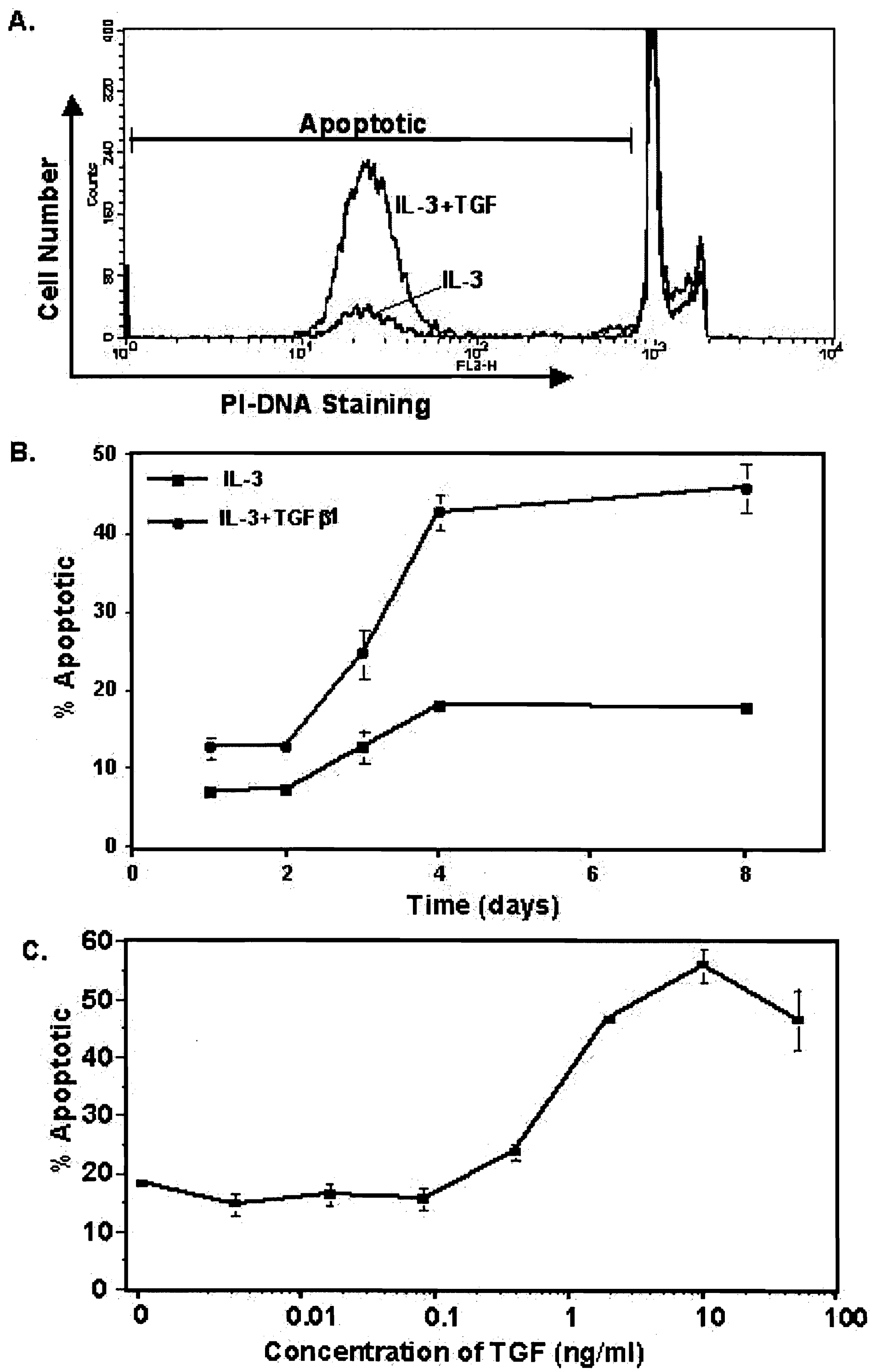
Figure 1: TGF-β1 Induces Mast Cell Apoptosis

(A) The Effect of TGF-β1 on Murine Mast Cells. Mouse BMMC were cultured for 6 days in IL-3 +/- 10 ng/ml TGF-β1. Apoptosis was measured by the presence of sub-diploid DNA content after PI-DNA staining, as indicated by marked region in histogram.

(B) The Effect of TGF-β1 Over Time. BMMC were cultured as in part (A) for the indicated days, and apoptosis was measured by PI-DNA staining. Data shown are means and standard errors from at least 6 samples/point.

(C) The Effect of TGF-β1: A Dose Response. Concentration response for TGF-β1-induced apoptosis. BMMC were cultured in Il-3 +/- the indicated concentrations of TGF-β1 for 7 days. Apoptosis was measure by PI-DNA staining. Data shown are means and standard errors of 6 samples/point.



A.
Apoptotic
IL-3+TGF
IL-3
Cell Number
Counts
FL3-H
PI-DNA Staining
B.
IL-3
IL-3+TGF β1
% Apoptotic
0
10
20
30
40
50
0
2
4
6
8
Time (days)
C.
% Apoptotic
0
10
20
30
40
50
60
0
0.01
0.1
1
10
100
Concentration of TGF (ng/ml)

Figure 2: TGF-β1 Inhibits Mast Cell IL-3R

(A) **The Effect of TGF-β1 on IL-3Rβ Expression.** Mouse BMMC were cultured for 3 days with IL-3 +/- TGF-β1 (10 ng/ml). Cells were analyzed for surface IL-3Rβ expression via flow cytometry. Control stain was performed with PE-coupled IgG.

(B) **The Effect of TGF-β1 on IL-3Rβ Expression Over Time.** Time course of TGF-β1-mediated inhibitory effects of IL-3Rβ expression. Cells were cultured for the indicated times, and percent decrease in IL-3Rβ expression was calculated by comparing mean fluorescence intensity of IL-3Rβ staining from cells cultured +/- TGF-β1, as measured by flow cytometry. Data shown are means and standard deviation of at least 3 samples/point.

(C) **RPA of IL-3Rβ Chains. Effect of TGF-β1 on IL-3Rβ mRNA expression.** RNase protection assay was used to measure IL-3Rβ mRNAs from BMMC cultured for the indicated time +/- TGF-β1.

(D) **The Effect of TGF-β1 on IL-3Rβ mRNA Expression Over Time.** Summary of TGF-β1 effects on IL-3Rβ mRNA expression. After normalizing to L32+GAPDH loading controls, percent decrease in mRNA expression was calculated as described in Materials and Methods. Data shown are means and standard deviation of 3 samples/point.

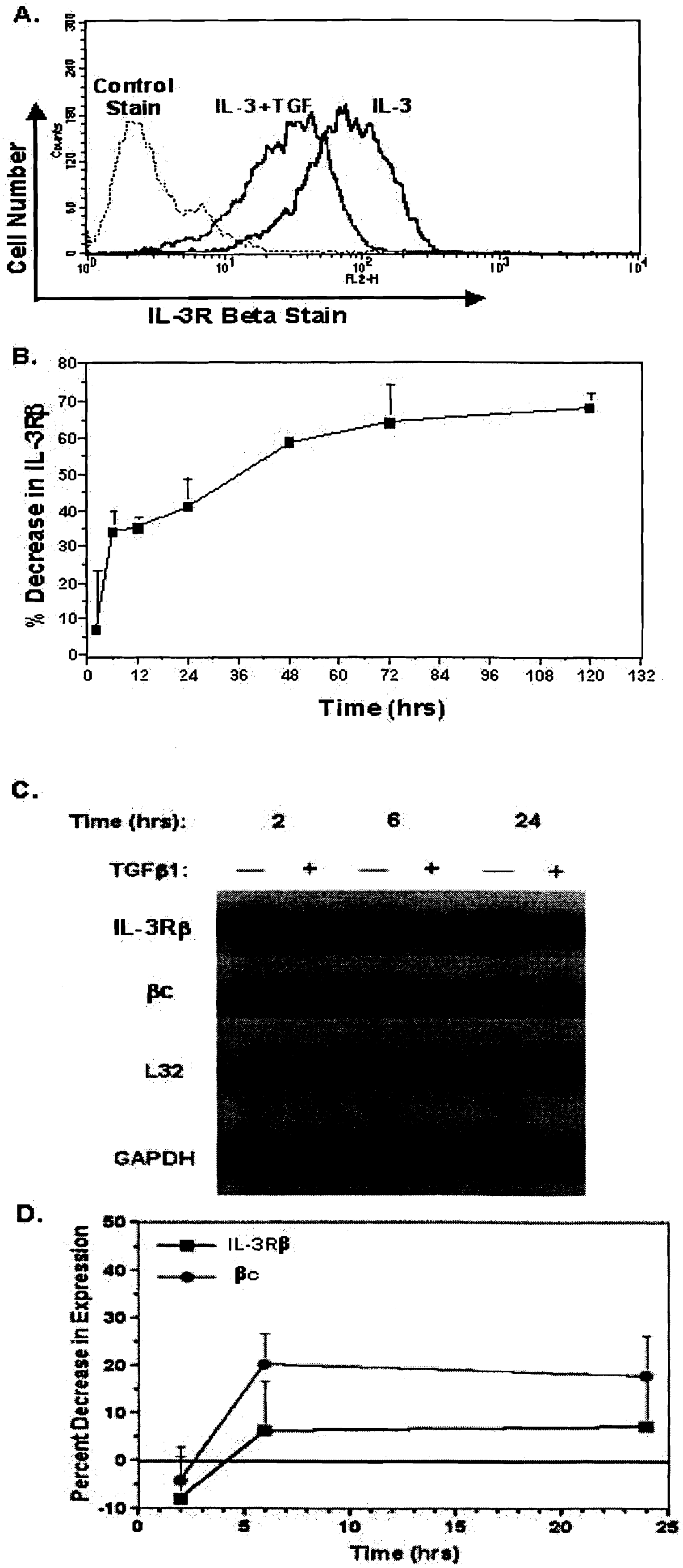

A.
Control Stain
IL-3+TGF
IL-3
Cell Number
Counts
FL2-H
IL-3R Beta Stain
B.
% Decrease in IL-3Rβ
Time (hrs)
C.
Time (hrs):
2
6
24
TGFβ1:
IL-3Rβ
βc
L32
GAPDH
D.
IL-3Rβ
βc
Percent Decrease in Expression
Time (hrs)

Figure 3: TGF-β1 Inhibits IL-3R Signaling.

(A) **TGF-β1 on Stat5 and p-STAT5 Expression Over Time.** BMMC were cultured for 3 days in IL-3 +/- TGF-β1, washed and incubated overnight in the same medium lacking IL-3, then restimulated with IL-3 (100 ng/ml) for 15 minutes. Total cell lysates were subjected to western blot analysis with phosphotyrosine-specific (pY) anti-Stat5. Membrane was stripped and re-probed with anti-Stat5 and anti-actin. After normalizing to Stat5 expression via densitometry, pY-Stat5 expression was found to be reduced by 44.6% in samples receiving TGF-β1. Similar results were found in two experiments.

(B) **ΔΨm of Mast Cells Treated With TGF-β1.** BMMC were cultured for 4 days in IL-3 +/- TGF-β1 or in media lacking cytokines. $Di(OC_6)_3$ staining was used to measure changes in ΔΨm, as detected by flow cytometry. Numbers in parentheses indicate the percentage of each population demonstrating reduced ΔΨm.

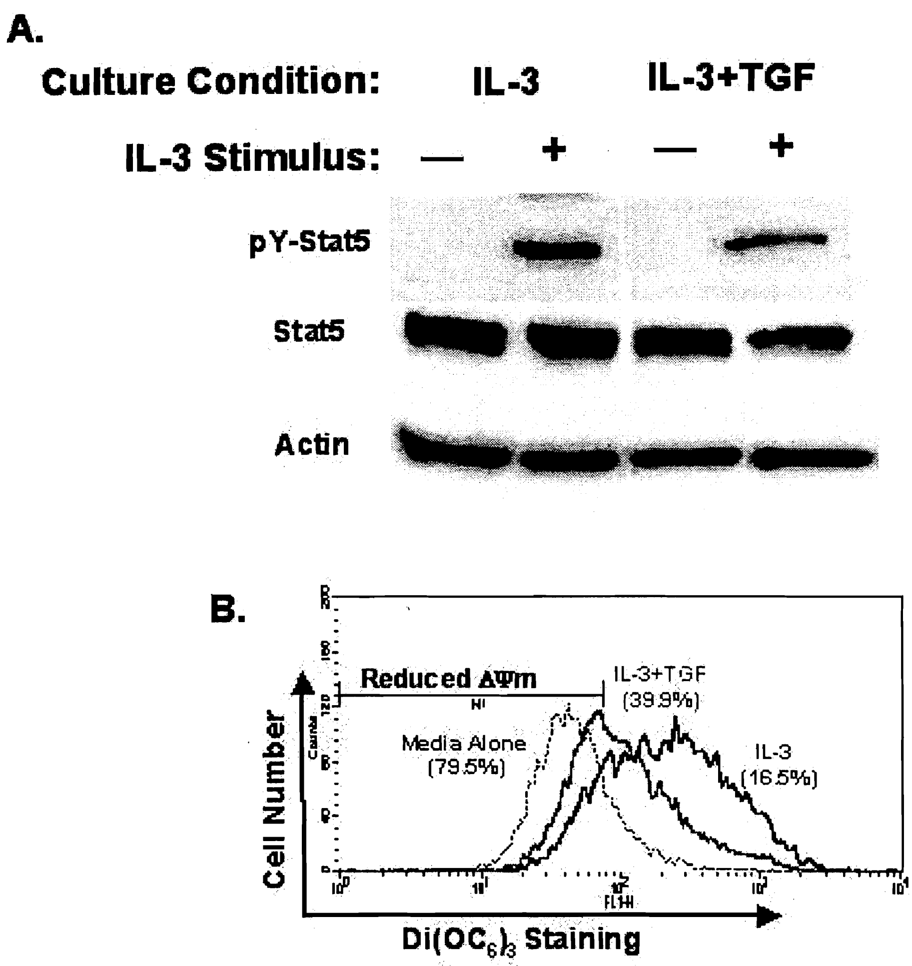

A.
Culture Condition:
IL-3
IL-3+TGF
IL-3 Stimulus:
—
+
—
+
pY-Stat5
Stat5
Actin
B.
Reduced ΔΨm
IL-3+TGF
(39.9%)
Media Alone
(79.5%)
IL-3
(16.5%)
Cell Number
Di(OC6)3 Staining

Figure 4: TGF-β1-mediated Apoptosis Proceeds Via The Mitochondrion and p53

(A) **The Effect of TGF-β1 on p53KO Survival.** Wild type (WT) and p53-deficient (KO) BMMC were cultured in IL-3 +/- TGF-β1 for 6 days, and apoptosis was measured by PI-DNA staining. Data shown are means and standard errors of at least 9 samples/point. *$p < 0.001$ by Student's t test.

(B) **The Effect of TGF-β1 on WT and p53 KO Mast Cell Caspase 3/7 Activity.** Effects of TGF-β1 on caspase 3/7 activation in WT and p53 KO BMMC. Cells were cultured for 6 days in IL-3 +/- TGF-β1, and caspase activation was measured by flow cytometry as described in Materials and Methods.

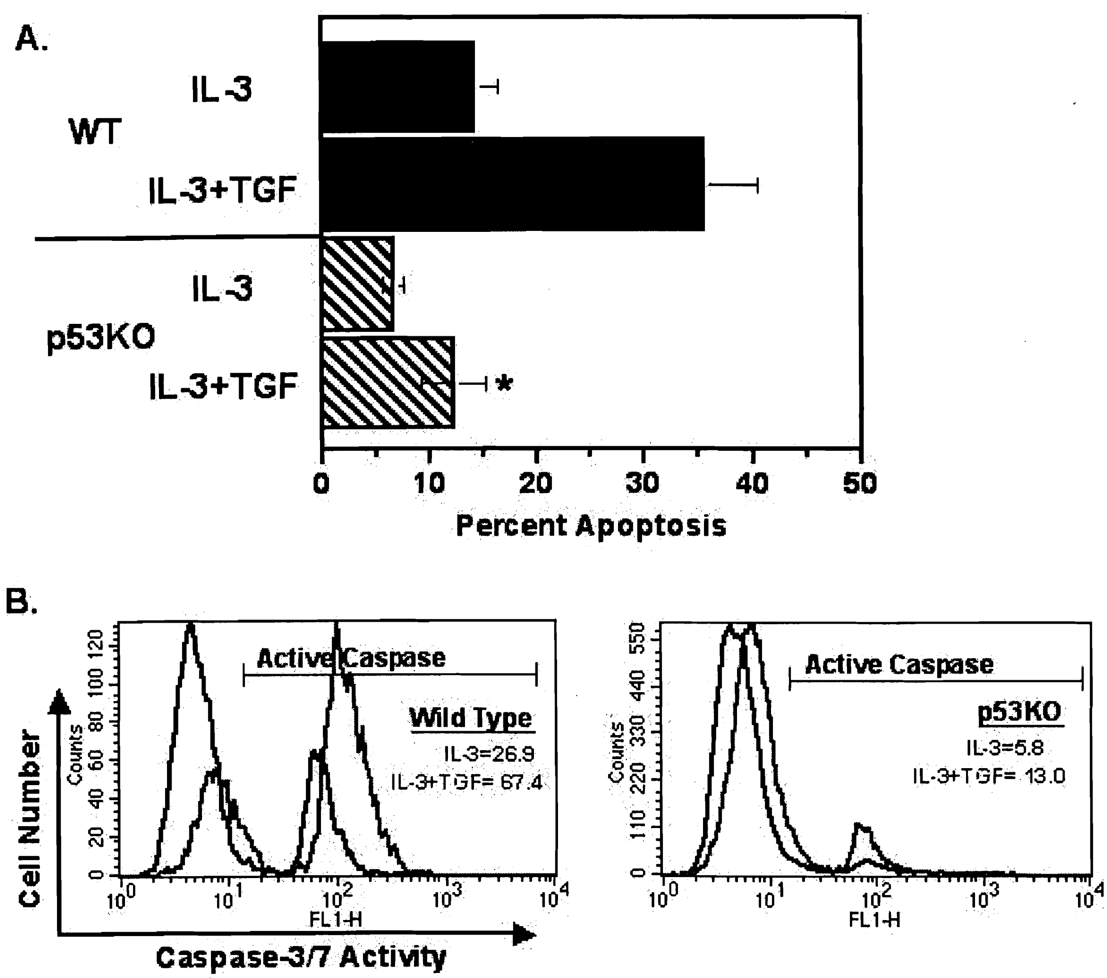

A.
WT
IL-3
IL-3+TGF
p53KO
IL-3
IL-3+TGF
*
0
10
20
30
40
50
Percent Apoptosis
B.
Active Caspase
Wild Type
IL-3=26.9
IL-3+TGF= 67.4
Counts
FL1-H
Cell Number
Caspase-3/7 Activity
Active Caspase
p53KO
IL-3=5.8
IL-3+TGF= 13.0
Counts
FL1-H

Figure 5: Effect of TGF-β1 On Mastocytoma Cells.

(A) % Apoptosis of WT vs Mastocytoma Mast Cells Treated With TGF-β1. BMMC and P815 mastocytoma cells were cultured in IL-3 +/- TGF-β1 for 6 days. Apoptosis was measured by PI-DNA staining. Percentage of the population demonstrating sub-diploid DNA content is indicated.

(B The Effect of TGF-β1 on WT vs Mastocytoma Mast Cell Caspase 3/7 Activity. BMMC and P815 mastocytoma cells were cultured in IL-3 +/- TGF-β1 for 6 days. Caspase 3/7 activation was measured by flow cytometry. Percentage of each population with active caspase is indicated.

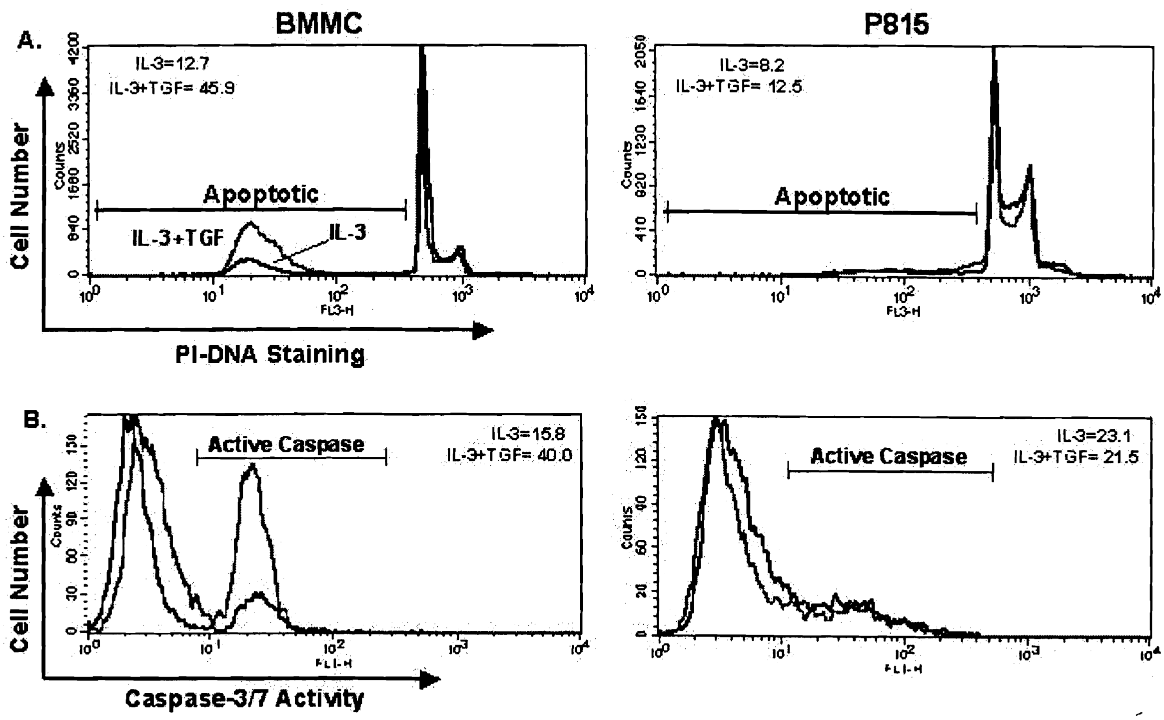

A.
BMMC
P815
Cell Number
Counts
IL-3=12.7
IL-3+TGF= 45.9
Apoptotic
IL-3+TGF
IL-3
FL3-H
PI-DNA Staining
IL-3=8.2
IL-3+TGF= 12.5
B.
Active Caspase
IL-3=15.8
IL-3+TGF= 40.0
FL1-H
Caspase-3/7 Activity
IL-3=23.1
IL-3+TGF= 21.5

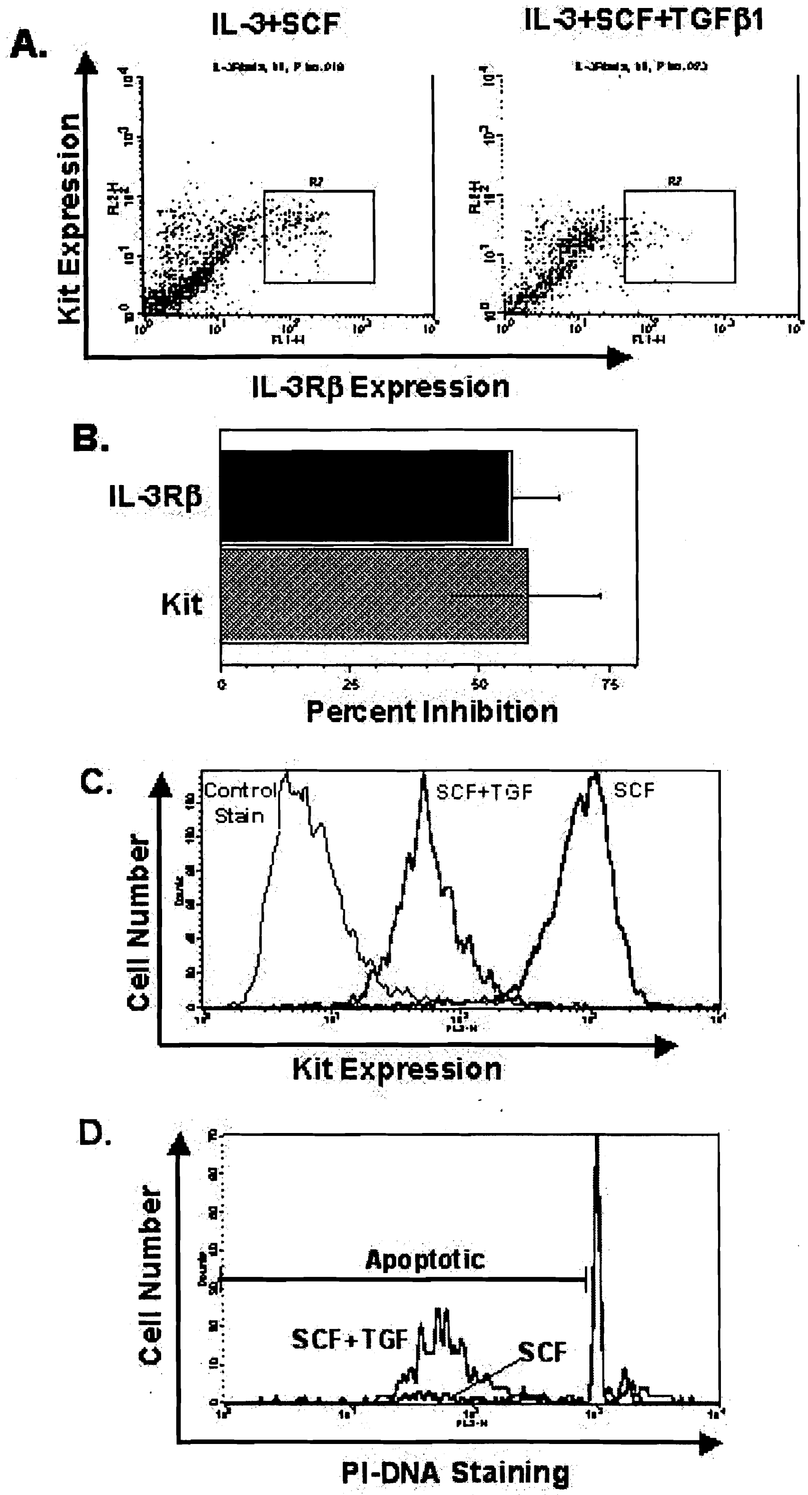
Figure 6: Effects of TGF-β1 On Mouse Peritoneal and Human Mast Cells.

(A) **The Effect of TGF-β1 on Mouse Peritoneal Mast Cell Kit Expression.** Mouse peritoneal cells were cultured in IL-3+SCF +/- TGF-β1 for 6 days, followed by flow cytometric analysis with anti-Kit and anti-IL-3Rβ. Dot plot is a representative 1 of 6 sample sets.

(B) **Percent Inhibiton of Mouse Peritoneal Mast Cell IL-3Rβ and Kit Expression After Treatment with TGF-β1.** Mouse peritoneal cells were cultured in IL-3+SCF +/- TGF-β1 for 6 days, followed by flow cytometric analysis with anit-Kit and anti-IL-3Rβ. The average decrease in Kit and IL-3Rβ expression +/- SD shown.

(C) **The Effect of TGF-β1 on Human Mast Cell Kit Expression.** Skin-derived human mast cells were cultured in SCF +/- TGF-β1. On day 3, surface Kit expression was measured by flow cytometry.

(D) **The Effect of TGF-β1 on Human Mast Cell Survival.** Apoptosis was measured in cultures described in part (C) on day 7, by PI-DNA staining. Data shown are representative of 3 independent mast cell cultures that yield similar results.


A.
IL-3+SCF
IL-3+SCF+TGFβ1
Kit Expression
IL-3Rβ Expression
R2
B.
IL-3Rβ
Kit
0
25
50
75
Percent Inhibition
C.
Control
Stain
SCF+TGF
SCF
Cell Number
Kit Expression
D.
Apoptotic
SCF+TGF
SCF
Cell Number
PI-DNA Staining

Vita

Farnaz Norozian was born in Tehran, Iran on December 31st, 1977. When she was eight years old she traveled with her family to Vienna, Austria, where she lived before moving to the United States. She attended the Islamic Saudi Academy, in Alexandria, Virginia, and later graduated from Herndon High School in 1995. Farnaz spent the better half of the next decade traveling extensively abroad, while studying biology, psychology, criminal justice, and aviation, at Northern Virginia Community College. In 2002 she moved to Richmond, Virginia to obtain her B.S. in Biology at Virginia Commonwealth University, which she completed in 2004. In the fall of that year she began to work towards her Master of Science in biology, which would provide her the opportunity to complete the research she had started as a junior in the Ryan Laboratory of Molecular Immunology. The fall of the following year she successfully defended her thesis six months earlier than expected of her graduating class. Farnaz will complete her degree at VCU in the Spring of 2006 and will venture off, yet again, to medical school.