

Transforming Growth Factor- β 1 Antagonizes Interleukin-5 Pro-Survival Signaling by Activating Calpain-1 in Primary Human Eosinophils

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Abstract

Background: Eosinophils rapidly undergo apoptosis unless exposed to prosurvival cytokines such as interleukin 5 (IL-5) or granulocyte-macrophage colony stimulating factor (GM-CSF). *In vivo*, eosinophils are exposed to TGF- β 1 which can induce apoptosis suggesting it may function to counteract the effects of IL-5 or GM-CSF and limit, *in vivo* tissue eosinophilia.

Objective: The objective of this study was to investigate the proapoptotic effects of TGF- β alone and in combination with IL-5 on eosinophils.

Methods: Peripheral blood eosinophil (PBEos) viability was assessed using flow cytometry after exposure to TGF- β 1 and IL-5. Calpain-1 activation was determined in cell extracts by western blot analysis of endogenous substrates and with a fluorogenic α -spectrin substrate. Molecular interactions between calpain1 and calpastatin were assessed by immunoprecipitation and western blotting.

Results: Physiologic concentrations of TGF- β 1 significantly antagonized the prosurvival effects of IL-5. TGF- β 1-induced apoptosis was suppressed by inhibitors of calpain, or its downstream target, caspase 3. TGF- β 1 signaling through Smad3 was unaffected by IL-5 and was required for the pro-apoptotic effects of TGF- β 1. However, IL-5 induced Akt phosphorylation was inhibited by TGF- β 1 and was associated with accelerated calpain cleavage and eosinophil death.

Conclusion: TGF- β 1 induces calpain-1 activation through antagonism of Akt which induces caspase activation and eosinophil apoptosis.

Keywords: TGF- β 1; IL-5; Eosinophils; Calpain-1; Apoptosis; Caspase 3; Intracellular signaling.

Abbreviations: IL-5: Interleukin 5; GM-CSF: Granulocyte-Macrophage colony stimulating factor; TGF- β 1: Transforming growth factor-beta 1; PBEos: Peripheral blood eosinophils; SB: TGF- β receptor kinase I inhibitor, SB431242; PI: Propidium iodine; ERK: Extracellular regulated kinase; PI-3-K: Phosphatidylinositol-3 Kinase; Akt/PKB: Protein kinase B; PARP: Poly ADP ribose polymerase

Key Messages

- TGF- β 1 antagonizes prosurvival signaling but does not accelerate apoptosis in the absence of IL-5.
- TGF- β 1 leads to extracellular calcium flux, triggering activation of calpain-1 and caspase 3.
- IL-5 signaling is antagonized by TGF- β 1 through effects on Akt.

Capsule Summary

The prosurvival effects of IL-5 on eosinophils was negated by TGF- β 1, suggesting a means of reducing eosinophilic inflammation *in vivo*. Mechanistically, TGF- β 1 activated calpain-1 through blockade of Akt signaling and mobilization of extracellular calcium.

Introduction

Peripheral blood eosinophils (PBEos) are terminally differentiated, nondividing cells with a life-span of approximately 3 days in the circulation. Eosinophil production and longevity are positively regulated by IL-5 which induces both bone marrow production as well as increased life-span in the blood and tissues [1]. In addition, IL-5 possesses eosinophil chemotactic activity, increases eosinophil adhesion

to endothelial cells and enhances eosinophil effector functions [2]. Interference with IL-5 signaling by mepolizumab dramatically reduces eosinophil counts in the blood and tissues and shows considerable promise for the treatment of asthma as well as eosinophilic gastritis and esophagitis [3].

IL-5 induces a variety of signaling cascades including Jak-STAT, Ras-Extracellular Signal Regulated Kinase (ERK) and Phosphatidylinositol-3 Kinase (PI3K)/Akt (protein kinase B, PKB) pathways. All have been implicated in IL-5-dependent PBEos survival, proliferation and differentiation *in vitro* and *in vivo* [4-6]. Activation of these cascades antagonizes Bax activation, prevents mitochondrial disruption and blocks caspase cleavage and activation [7,8]. Much less clear is how the function and longevity of IL-5 activated PBEos or tissue eosinophils are suppressed *in vivo* upon cessation of pro-inflammatory stimuli.

One possible mechanism invokes pro-apoptotic co-signaling by

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anti-inflammatory cytokines such as TGF- β 1 [9]. Tissue eosinophils are exposed to autocrine and paracrine sources of TGF- β 1, whose expression increases at the sites of allergic inflammation including asthmatic airways [10]. Human eosinophils have intact Smad signaling and respond to the anti-inflammatory properties of TGF- β including acceleration of apoptosis [10-14]. How TGF- β 1 antagonizes eosinophil survival in the context of IL-5 as seen *in vivo* remains incompletely understood.

Calpains are non-lysosomal cysteine proteases that are selectively activated in response to calcium signals [15] and thereby control cellular functions such as cytoskeletal remodeling, cell-cycle progression, gene expression and apoptotic cell death [16-18]. In mammals, calpain-1 (calpain I, μ -calpain) and calpain-2 (calpain II, m-calpain) are ubiquitously expressed and distinguished by their *in vitro* calcium requirements. Both calpain-1 and calpain-2 are heterodimers consisting of an 80KD, calcium-binding catalytic subunit and a 30-kDa regulatory subunit. The activity of calpains is tightly controlled by the endogenous inhibitor calpastatin. Calpastatin is an intrinsically unstructured protein that in the presence of calcium, reversibly binds to and inhibits four molecules of calpain [19,20]. Calpains have been reported to regulate neutrophil apoptosis [21,22]. Recently we showed that calpain inhibitors reduced PBEos death likely by preventing the cleavage and activation of pro-apoptotic Bax [7].

In this study, we demonstrate that calpain-1 is activated during spontaneous eosinophil apoptosis which is antagonized by IL-5. TGF- β 1 can overcome IL-5 mediated suppression of calpain by inducing extracellular calcium entry, blocking Akt signaling and accelerating the cleavage of calpastatin and procalpain-1. These results suggest that TGF- β 1 can induce PBEos apoptosis by modulating IL-5 signaling through Akt and calpain dependent mechanisms.

Methods

Reagents

Recombinant human IL-5, purified human TGF- β 1 and caspase-3 inhibitor (Z-DEVD-FMK) were purchased from R&D Inc. (Minneapolis, Minn., USA). Calpeptin, LY-294002 (phosphatidylinositol 3-kinase inhibitor), Akt inhibitor IV, BAPTA/AM, fluorogenic alpha-spectrin, CytoBuster™ protein extraction reagent, cocktail set III were purchased from Calbiochem (La Jolla, CA, USA). TGF- β 1/activin type I receptor kinase inhibitor (SB431542) was purchased from Tocris (Ellisville, Mo., USA). Antibody to phospho-Erk1/2 and total Erk1/2 were purchased from Promega (Madison, WI, USA). Anti-calpain1/2, anti-phospho-JNK (Thr183/Tyr185), anti-phospho-p38 (Thr180/Tyr182), anti-Akt, anti-phospho-Akt (Ser473), anti-phospho-Smad2 (Ser465/467) and anti-phospho-Smad 3 (Ser423/425) were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-Smad2 and Smad3 were purchased from Abcam (Cambridge, MA, USA). Anti-calpastatin was purchased from Chemicon International (Temecula, CA, USA). Anti- α -spectrin was purchased from Novus Biologicals (Littleton, CO, USA). Monoclonal anti- β -actin (Ab-1) was purchased from Oncogene Research Products (San Diego, CA, USA). Horseradish peroxidase-conjugated anti-rabbit (secondary antibody; NA934V) and the enhanced chemiluminescence ECL immunoblot detection system were from Amersham Pharmacia Biotech (Piscataway, NJ, USA).

Calpain activity assay

Calpain activity was determined in cytosolic extracts, prepared with CytoBuster Protein Extraction Reagent. Protein concentrations

were determined by the Bradford method. Protease activity was determined for 15 μ g protein after the addition of 100 μ l reaction buffer (50 mM Tris/HCl, pH 7.5, 50 mM NaCl, 1mM EDTA, 1mM EGTA, 5mM 2-mercaptoethanol, 5 mM CaCl₂) and 50 μ l of the fluorogenic calpain-1 substrate (10 μ M). The change in fluorescence was measured in a SpectraMax Gemini XPS spectrofluorometer (Molecular Devices Corporation, USA.) at an excitation wavelength of 490 nm and an emission wavelength of 518 nm.

Subjects and eosinophil preparation

Peripheral blood was obtained by venipuncture from healthy or mildly atopic donors. Peripheral blood eosinophils were purified by a negative immunomagnetic procedure as described [23]. Only eosinophil preparations >97% pure were used. After isolation, eosinophils were cultured at 37°C in a humidified atmosphere of 5% CO₂ and 95% air at a density of 1x10⁶ cells per ml in RPMI-1640 medium, 10% (vol/vol) FBS and gentamycin (50 mg/ml) all from Life Technologies. All participants have a clinical record at the University of Wisconsin Hospital and informed consent was obtained according to an approved protocol of the University of Wisconsin Hospital Institutional Review Board.

Cell viability and flow cytometry

Eosinophils (1x10⁶ cells per ml) were cultured in 96-well tissue culture plates (BD Biosciences). As needed, cells (1x10⁵) were stained with trypan blue and counted manually or annexin V-fluorescein isothiocyanate and propidium iodide (BD Bioscience) and analyzed by flow cytometry.

Immunoblot and Immunoprecipitation

For immunoblot, equal numbers of untreated or treated eosinophils were pelleted, suspended in cold water plus SDS-PAGE loading buffer and boiled for 5 minutes prior to SDS-PAGE and western blotting. For immunoprecipitation, cells were lysed in NP-40 buffer (50 mM Tris/HCl, 150 mM NaCl, 2 mM EDTA, 1% NP-40, pH 8.0). Protein concentrations were determined by the Bradford method. 2–5 μ g antibody was added to each supernatant of cell lysates, followed by incubation for 2–4 h at 4°C. Protein G-agarose beads (Sigma-Aldrich) were added and incubation was continued overnight with shaking. Pellets were washed five times with lysis buffer and beads were dissolved in SDS-PAGE loading buffer prior to immunoblot analysis.

Statistical analysis

Data sets were analyzed with statistics package provided in GraphPad Prism 5 (La Jolla, CA). One way ANOVA was used to compare groups. All data are reported as the mean $\pm$ the standard error of the mean (SEM); n = 3–5. p < 0.05 was considered significant.

Results

Calpain is involved in spontaneous or TGF- β mediated apoptosis

Eosinophils rapidly undergo spontaneous apoptosis in culture in the absence of IL-5 or GM-CSF. Autocrine secretion of TGF- β 1 has been reported to reduce eosinophil survival possibly through the activation of protein kinase R (PKR) [12,14]. However, the upstream effectors necessary for TGF- β 1 to antagonize IL-5 prosurvival signaling remains poorly understood. Therefore, pure PBEos were cultured with or without TGF- β 1 and/or IL-5. After 3 days, cell viability was assessed by propidium iodide (PI) and annexin-V staining by flow

cytometry (Figure 1A, 1B) or by western blotting for cleaved PARP and caspase 3 (Figure 1C). We confirmed previous observations that TGF- β 1 attenuates the pro-survival effects of IL-5 in a dose-dependent manner (Figure 1A). However, even supraphysiologic concentrations of TGF- β 1 failed to fully antagonize the effects of IL-5. In addition, exogenous TGF- β 1 had no effect on spontaneous eosinophil apoptosis occurring in the absence of exogenous pro-survival cytokines. These data suggest that IL-5 pro-survival signaling can only be partially antagonized by TGF- β 1 and that spontaneous eosinophil cell death is not dependent on TGF- β 1.

Recently we reported that pro-survival signaling prevented Bax cleavage and activation [7]. Calpeptin, a cell permeable calpain-1 inhibitor, blocked Bax cleavage as well as apoptosome activation and eosinophil apoptosis [7]. These data suggested Bax cleavage by calpain-1 was a critical control point in the induction of eosinophil apoptosis. Therefore PBEos were treated with IL-5 or TGF- β 1 alone or together with cell permeable inhibitors to calpain-1 and -2 or caspase 3. Inhibitors to caspase 3 or calpain significantly attenuated spontaneous eosinophil apoptosis (Figure 1A, 1C), suggesting eosinophil apoptosis

requires both calpain and caspase 3. The viability of cells treated with both TGF- β 1 and IL-5 and caspase 3 or calpain inhibitors was nearly identical to those cells treated with IL-5 alone. Cells treated with IL-5 plus Z-VAD and/or calpeptin showed slightly greater 3 day survival (75% vs. 82%, $n=3$ donors, $p>0.05$) than after IL-5 alone. These results suggest that the proapoptotic effects of TGF- β 1 involves the activation of calpain and caspase 3 while IL-5 suppresses both, consistent with prior data. Immunoblot analysis of total eosinophil lysates revealed only calpain-1 was expressed at detectable levels by resting or IL-5 stimulated cells (data not shown). Therefore, we conclude that calpain-1 was the target of calpeptin and this isoform is required for spontaneous or TGF- β mediated apoptosis.

Calpain-1 activation is increased during eosinophil apoptosis

Calpain-1 is expressed as an 80KD (p80) pro-enzyme that associates with an endogenous inhibitor, calpastatin [20,24]. Cleavage to a 76KD (p76) active form is triggered by increased intracellular calcium which reduces binding to calpastatin [20,25]. Given the ability of exogenous calpain inhibitor to attenuate eosinophil apoptosis [Figure 1A, 1C], we

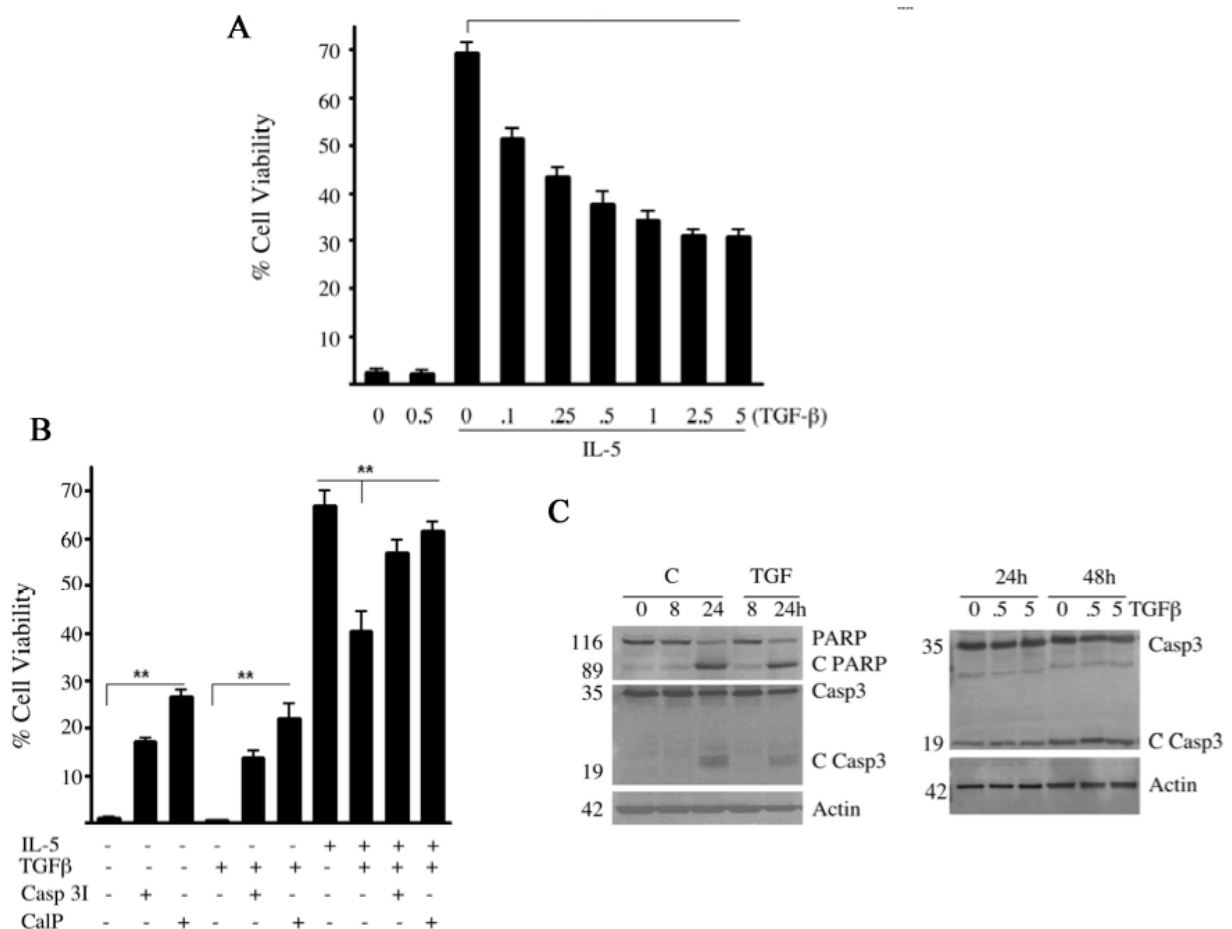


Figure 1: Calpain and caspase inhibitors enhance the survival of eosinophils. (A) PBEos were left untreated (0), treated with TGF- β 1 (0.5 ng/ml), IL-5 (3pM) or IL-5 (3pM) plus TGF- β 1 (as shown) and stained with annexin-V/PI and analyzed by FACS after 72 h. Data presented as a percentage of viable cells at time 0. (B) PBEos were left untreated (-) or treated with TGF- β 1 (0.5ng/ml), IL-5 (3pM), calpeptin (CalP, 20 μ M) or caspase 3 inhibitor (CaspI, 25 μ M), assessed by FACS as in (A) after 72 h of incubation and presented as a percentage of viability at time 0. (C) PBEos were untreated (C) or treated with TGF- β 1, lysed and analyzed by immunoblot for full-length or cleaved PARP (C PARP) and full-length or cleaved caspase 3 (C Casp3). For panels (A) and (B), **, $P<0.01$ (one-way ANOVA). Data are representative of at least three experiments with 3 different donors (error bars, SEM of triplicate donors).

evaluated the kinetics of calpain cleavage after the addition of IL-5, TGF- β 1, neither or both cytokines by immunoblot analysis. As shown in Figure 2A, freshly isolated PBEos contained very little active p76 calpain. In the absence of exogenous cytokines, increasing amounts of p76 appeared over the next 24h. Consistent with its failure to accelerate spontaneous apoptosis, TGF- β 1 alone had no discernable effect on the conversion of p80 to p76, which was indistinguishable from untreated controls even after 48 h (not shown). IL-5, however, significantly reduced calpain cleavage which was antagonized by TGF- β 1. These results suggest that TGF- β 1 may antagonize IL-5 by inducing calpain cleavage.

Based on these results, we evaluated calpain activity in cells using a fluorescently labeled, model peptide. Extracts were prepared immediately after cell isolation as well as 6 h thereafter. Over this time, there was a significant increase of calpain-1 activity in lysates from untreated eosinophils that was almost entirely suppressed by IL-5 [E2]. Consistent with these results, IL-5 treatment prevented the cleavage of α -spectrin, an endogenous calpain substrate [26] that was increased to untreated levels by TGF- β 1 plus IL-5 (Figure 2B). Therefore, calpain-1 activation is a cytokine dependent process that occurs early in the pro-death pathway and likely drives apoptosis in IL-5 deprived or TGF- β 1 treated eosinophils.

Extracellular Ca^{2+} is essential for calpain-1 activation

Calpain-1 is activated after the regulatory, C-terminal EF-hands

bind calcium, likely facilitating a conformational change and autolysis [20]. To address if calcium flux was required for calpain activation, cells were untreated or activated with TGF- β 1, IL-5 or both in the presence or absence of EGTA or BAPTA/AM. After 6 hrs, BAPTA/AM as well as EGTA alone, or in combination with TGF- β 1, reduced or eliminated calpain-1 autolysis, (Figure 2C), suggesting calcium flux from the extracellular to intracellular space was likely necessary for calpain-1 cleavage.

Calpastatin cleavage is induced by TGF- β 1

As mentioned above, calpain activation is also regulated by calpastatin, a highly specific endogenous inhibitor [20,27]. Given the above data, we hypothesized that changes in the intracellular levels of calpastatin likely preceded calpain activation. Therefore, freshly isolated PBEos were left untreated or treated with IL-5 alone or together with TGF- β 1 prior to immunoblot analysis of calpastatin. In agreement with other cell types [19], multiple calpastatin isoforms are present in eosinophils (Figure 3A), with freshly isolated cells predominantly expressing the 110 kDa form. In the absence of IL-5, calpastatin was rapidly degraded which occurred contemporaneously with calpain autolysis (Figure 3A). Immunoprecipitation/immunoblot of calpain (Figure 3B) revealed that TGF- β 1 blocked the interaction between procalpain-1 (80 kDa) and full-length calpastatin. Thus, prosurvival cytokines such as IL-5 prevent apoptosis, in part by stabilizing the calpastatin-calpain interaction which prevents calpain activation.

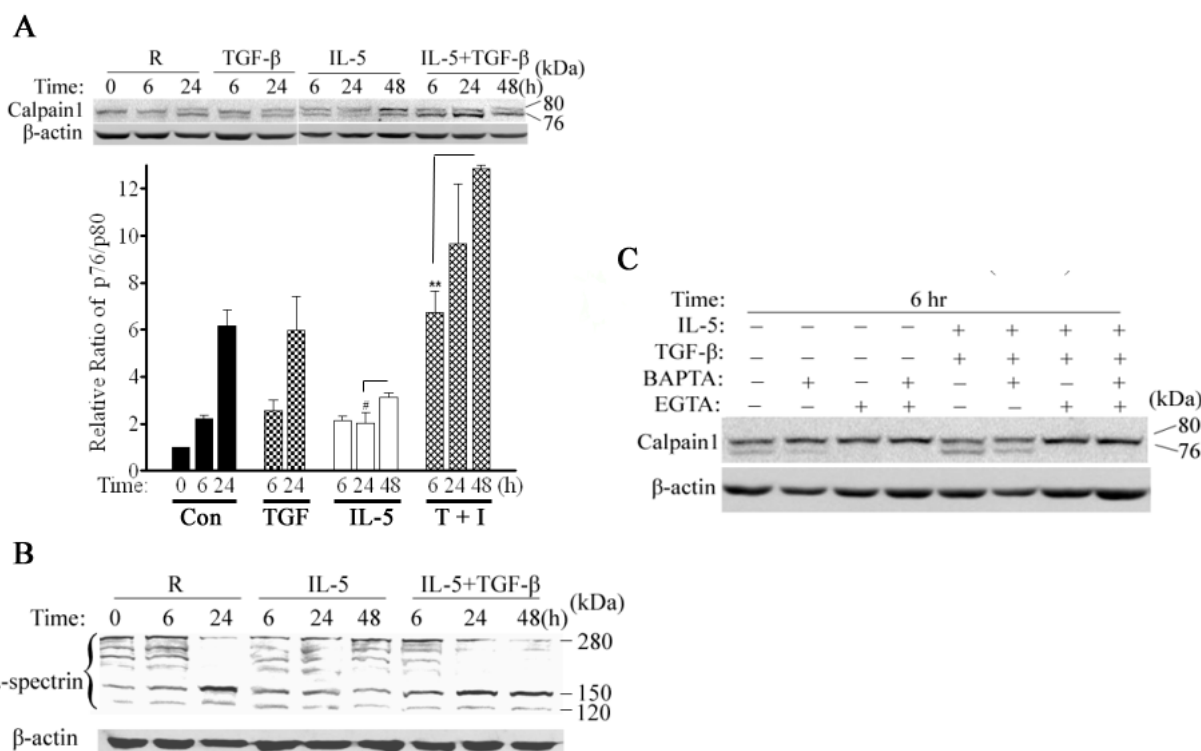


Figure 2: Calpain-1 is activated by calcium during eosinophil apoptosis. (A) Calpain-1 immunoblot analysis in PBEos lysed immediately (0), left untreated (R), or treated as shown. The p80 and p76 calpain signals were quantified with Image J software 1.34S (NIH) and ratio calculated and normalized to that observed for control untreated PBEos. (B) Immunoblot analysis of α -spectrin in eosinophils treated as shown. (C) PBEos were treated as shown, lysed and calpain-1 autolysis was determined by immunoblot. ** $p < 0.01$; # $p < 0.05$ (one-way ANOVA). Data representative of at least 3 independent experiments from different donors and expressed as the mean $\pm$ SEM.

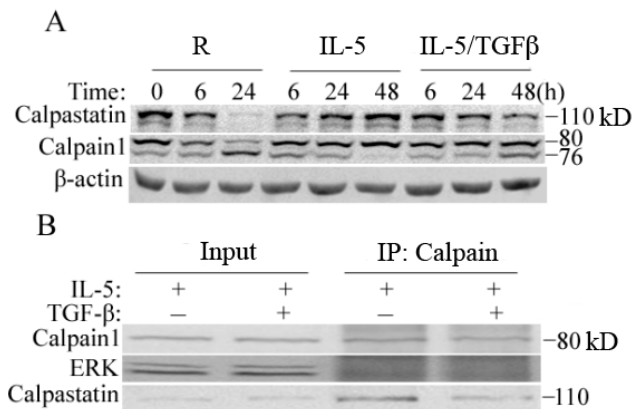


Figure 3: Calpastatin degradation is induced by TGF- β , blocked by IL-5 and precedes calpain cleavage and activation. (A) Immunoblot analysis for calpastatin and calpain-1 in PBEos treated as shown. (B) Immunoprecipitation (IP) and immunoblot analysis of PBEos treated as shown for 24h. IP's analyzed as shown and representative of at least 3 independent experiments from different donors.

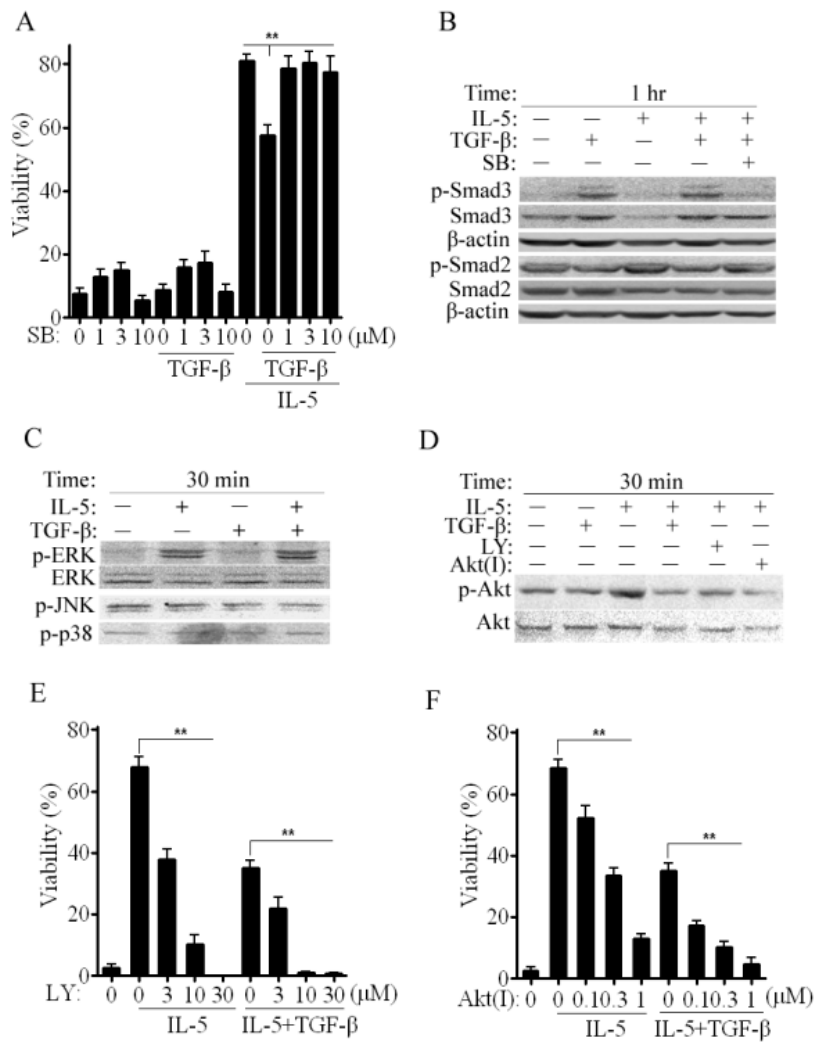


Figure 4: TGF- β 1 inhibits Akt phosphorylation induced by IL-5 signaling. (A) 72 hr viability of PBEos left untreated (0), treated as shown with or without SB431542 (SB). (B, C and D) Immunoblots of PBEos treated with SB, LY294002 (LY) or Akt Inhibitor IV (AktI). (E) and (F) Viability of PBEos treated as shown and presented as a percentage of viability at time 0. **, $p < 0.03$ (one-way ANOVA). All blots are representative of at least 3 independent experiments with different donors and data are expressed as the mean $\pm$ SEM.

TGF- β 1 inhibits Akt phosphorylation induced by IL-5 signaling

Our data shows that TGF- β 1 functions as a negative modulator of the prosurvival action of IL-5 in eosinophils. In most cell types, TGF- β 1 triggers Smad2/3 phosphorylation [11]. Therefore, we analyzed the effect of the TGF- β 1 receptor kinase I inhibitor, SB431242 on Smad 2/3 phosphorylation and the survival of untreated cells or those treated with TGF- β 1, IL-5 or both cytokines. As shown in Figure 4B, Smad3 was heavily phosphorylated after TGF- β 1 or TGF- β 1 plus IL-5. Consistent with a Type I receptor mediated event, phosphorylation was blocked by SB431242. Smad2 was constitutively phosphorylated and unaffected by cell treatment with cytokines or Type I Receptor blockade. Therefore, in eosinophils, Smad3 but not Smad2, is responsive to TGF- β 1 through Type 1 Receptor signaling. We next assessed if Smad3 signaling mediated TGF- β 1 effects on eosinophil survival. As shown in Figure 4A, low concentrations of SB431242 alone had modest, positive effects on eosinophil survival. Notably, Type I Receptor blockade fully restored IL-5 mediated survival despite the presence of TGF- β 1. These results indicate that Type I Receptor-Smad3 signaling likely initiates the proapoptotic effects of TGF- β 1.

IL-5 rapidly induces ERK phosphorylation and is essential for promoting eosinophil survival [21]. Therefore we evaluated the effects of TGF- β 1 on ERK as well as p38 and JNK. After 30 minutes incubation, IL-5 induced robust ERK phosphorylation which was unaffected by TGF- β 1 (Figure 4C). Neither JNK nor p38 were phosphorylated under these conditions. The PI-3-K/Akt pathway has been reported to be involved in eosinophil survival and allergic inflammation [6]. Thus, Akt phosphorylation was assessed under identical conditions. Like ERK, Akt was phosphorylated in response to IL-5 (Figure 4D, p-Akt:Akt ratio = 2.3 fold $\pm$ 0.3 unstimulated control, n=3 experiments). TGF- β 1 alone had no effect but prevented IL-5 induced phosphorylation as did the PI-3-K inhibitor LY294002 or Akt Inhibitor IV. These data suggest that TGF- β 1 may antagonize IL-5 signaling by preventing Akt activation via PI-3-K.

We tested this hypothesis by exposing untreated or cytokine treated eosinophils to PI-3-K or Akt inhibitors and measuring survival. As shown [Figure 4E,4F], both PI-3-K or Akt inhibitors individually block the prosurvival effects of IL-5 and are additive with TGF- β 1. In aggregate, these results suggest that TGF- β 1 antagonizes IL-5 prosurvival signaling through a pathway involving activated Smad3 which prevents Akt phosphorylation. This is consistent with data from other cell types [28,29]. However, immunoprecipitations with anti-Akt failed to pull-down Smad3 or calpastatin/calpain (data not shown). These data suggest that the interactions, if they exist, are transient.

Akt antagonizes calpain activation

The data above implicate Akt as well as calpain in signaling and apoptotic decisions by eosinophils treated with IL-5 and TGF- β 1. In order to better characterize the relationship between these regulators, we evaluated survival and calpain activation in the presence of IL-5, calpain and Akt inhibitors. Thus eosinophils were left untreated, treated with IL-5 alone or IL-5 in combination with the calpain inhibitor calpeptin or a caspase 3 inhibitor. As shown in Figure 5A, both calpeptin and caspase 3 inhibitor significantly increased the survival of untreated eosinophils. As seen above (Figure 4), IL-5 mediated survival was antagonized by Akt inhibition. However, this effect was significantly attenuated by either calpain or caspase 3 inhibitors. These data suggest that Akt activation is necessary for suppression of calpain cleavage. In

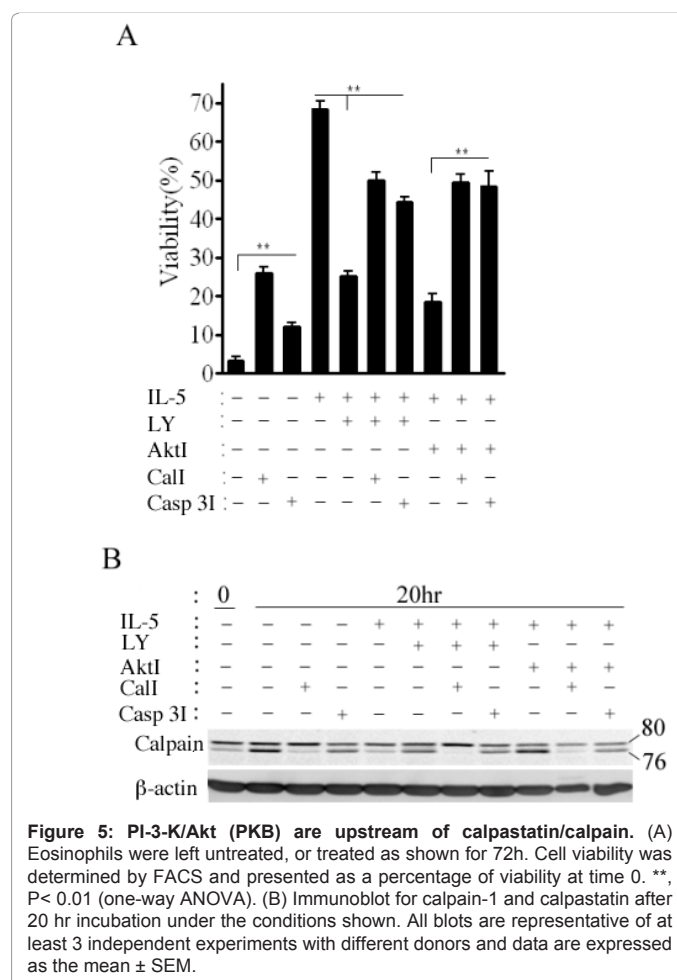


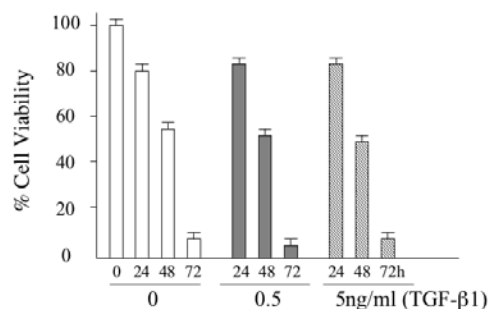
Figure 5: PI-3-K/Akt (PKB) are upstream of calpastatin/calpain. (A) Eosinophils were left untreated, or treated as shown for 72h. Cell viability was determined by FACS and presented as a percentage of viability at time 0. **, $P < 0.01$ (one-way ANOVA). (B) Immunoblot for calpain-1 and calpastatin after 20 hr incubation under the conditions shown. All blots are representative of at least 3 independent experiments with different donors and data are expressed as the mean $\pm$ SEM.

order to test this hypothesis, PBEos were treated for 20 h as above prior to lysis and calpain-1 immunoblot analysis. Despite the presence of IL-5, Akt inhibition was associated with procaspase cleavage (Figure 5B). Calpeptin, but not the caspase 3 inhibitor, blocked calpain-1 cleavage in untreated or IL-5 treated eosinophils. Concordant with these results, calpain activity was suppressed by IL-5 but increased by Akt inhibition (E3). These data together suggest that Akt is activated by IL-5 treatment and must remain active to prevent calpain autolysis and downstream apoptosis.

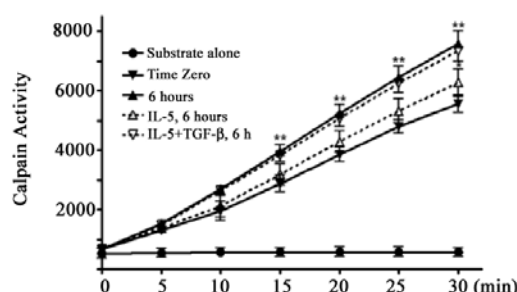
Discussion

In this study, we demonstrate that calpain-1 activation is a critical control point in apoptotic decisions by primary, peripheral blood human eosinophils. Blockade of calpain activation delayed spontaneous as well as TGF- β 1-facilitated eosinophil apoptosis. Calpain-1 activation was contemporaneous with loss of its endogenous inhibitor calpastatin both of which processes were antagonized by IL-5 signaling. TGF- β 1 antagonized IL-5 prosurvival signaling by inhibiting Akt phosphorylation which accelerated calpain activation. Finally we show that eosinophil apoptosis was initiated by increased intracellular free Ca^{2+} that is most likely mobilized from extracellular stores.

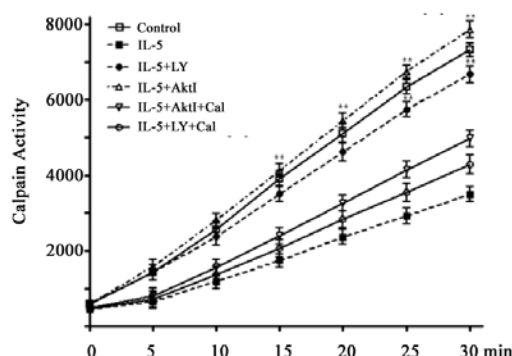
Calpains are highly conserved proteases found in nearly all mammalian cells whose deletion is embryonic lethal [30-32]. Calpain-calpastatin have been implicated in apoptotic decisions by other leukocytes including immature T and B cells [33-36] and neutrophils



E1: PBEos were stimulated with various concentration of TGF- β 1 for the indicated times followed by determination of cell viability by trypan blue exclusion. The results shown are representative of 3 independent experiments.



E2: Calpain activity was measured in cell extracts with fluorogenic α -spectrin substrate. Fresh eosinophils were immediately lysed (time zero), left untreated (6 hr) or treated with IL-5 alone or with IL-5 plus TGF- β 1 for 6 hr as shown. Substrate alone without cell lysate is the negative control.



E3: Calpain activity was measured in cell extracts with fluorogenic α -spectrin substrate after 6 hr incubation under the conditions shown.

[21]. While calpain has also been recently implicated in apoptotic decisions by G-CSF treated neutrophils [22], the pathways that lead to and control this decision-point remain poorly understood.

Upon binding calcium, calpains become proteolytically active and associate with intracellular membranes [15,37,38]. Here we show that both extra- and intracellular calcium chelation with EGTA or BAPTA, respectively, suppressed calpain-1 activation, although the effects of EGTA were greater. These results establish that extracellular calcium entry is likely required for calpain activation in eosinophils and is probably a very early event. Our data can not presently determine if

intracellular release also participates in this process. Based on these results, and the absence of calpain cleavage in the presence of IL-5, we conclude that prosurvival signaling likely suppresses calcium flux from extracellular sources. Calcium entry is critical for a variety of eosinophil functions including transmigration in response to cytokines and lysophosphatidylcholine induced β 2-integrin-mediated adhesion [39]. The relevant calcium channels have yet to be defined for any of these events. We were unable to block calpain-1 activation with L-type calcium channel inhibitors or antagonists of the NMDA receptor (data not shown) both of which are expressed by eosinophils.

Despite being nondividing and terminally differentiated, eosinophils have the capacity to significantly alter gene expression in response to prosurvival signaling. Microarray analysis revealed calpastatin mRNA levels increased after both IL-5 and GM-CSF while calpain-1 mRNA was largely unchanged after IL-5 but modestly elevated after GM-CSF [40]. Consistent with those results, calpastatin protein levels progressively increased over 48 h of IL-5 exposure while calpain levels (80KD) remained essentially constant. These results imply that in addition to suppressing calcium flux, IL-5 treatment also prevented calpain activation by increasing the steady state levels of calpastatin. Interestingly, TGF- β 1 antagonized these effects of IL-5, leading to calpain activation and the initiation of apoptosis when eosinophils were exposed to both cytokines. Calpain inhibitors reduced spontaneous and Fas receptor-mediated death of neutrophils [21], while calpastatin knock-down accelerated neutrophil apoptosis [41]. Therefore, the calpastatin-calpain system is clearly an important target of both pro-survival (IL-5) and pro-death (TGF- β 1) signaling and thus may be a therapeutic target for eosinophilic diseases such as asthma [42].

Through partially shared receptors, IL-5 and GM-CSF mediated signaling enhances eosinophil survival [7,8,39]. Ligation of the IL-5 receptor recruits several kinases, including JAK2, Lyn, and Raf-1, as well as the phosphatase SHP2 [43] leading to JAK-STAT, Ras-ERK [7,40,43] and PI-3-Ky-Akt signaling [6,44,45]. Here, using human eosinophils, we show that IL-5 induced serine phosphorylation of Akt and ERK while p38 and JNK were unaffected. PI-3-K and Akt antagonists attenuated IL-5-induced eosinophil survival, which was partially restored by calpain inhibitors. Therefore, PI-3-K/Akt is upstream of the calpain/calpastatin system. How Akt modulates calpain activation remains unclear. We were unable to identify a direct interaction between these 2 proteins by immunoprecipitation (data not shown).

TGF- β 1 is a multifunctional cytokine that controls the proliferation, differentiation, and function of many cells of the immune system. Most inflammatory cells including eosinophils secrete TGF- β [46] and are responsive to its signaling via canonical SMAD dependent pathways [10-13]. TGF- β 1 levels increase in the BAL of patients with allergic asthma and correlates well with airway and parenchymal remodeling [49]. TGF- β 1 has been shown to inhibit Jak-STAT signaling pathway induced by IL-5, although the exact mechanisms remain unknown. Here, we confirmed the proapoptotic effects of TGF- β 1/Smad signaling in eosinophils. Interestingly, the effects of TGF- β 1 were restricted to cells also treated with IL-5, suggesting TGF- β 1 prevented IL-5 dependent signaling. Based on its differential phosphorylation after combined stimulation, the likely target was Akt. This conclusion was strengthened by the observation that Akt inhibitors antagonized IL-5 prosurvival signaling in the absence of TGF- β 1 and are consistent with a recent report that Akt signaling maintains calpastatin levels in retinal

photoreceptors [47]. As TGF- β 1 induced p-Smad3 which was blocked by a Type I Receptor antagonist (SB431542), we also conclude that pro-death TGF- β 1 signaling occurred through canonical Smad pathways. This observation is consistent with the recent finding by Kanzaki et al. [11].

Conclusions

We have showed that calpain-1 plays an important and early role in spontaneous or TGF- β 1 mediated eosinophil apoptosis. Conversely, IL-5 antagonizes calpain activation and in doing so, prevents cell death. Eosinophils possess at least partially intact TGF- β 1/Smad signaling. Our data will be important in understanding the role of TGF- β 1 in the induction of eosinophil apoptosis, asthma and airway remodeling [48].

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Author Contributions

Q. X. designed and did most experiments and wrote the initial manuscript; Z.-J. S. and J. O. designed, performed experiments and helped with data interpretation; H. C. provided help in experimental design and critically read the manuscript and J. S. M. conceived the project, helped to design the experiments, interpreted data and wrote and edited the manuscript.

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