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Calpain inhibition impairs TNF- α -mediated neutrophil adhesion, arrest and oxidative burst

Andrew J. Wiemer^{1,2}, Mary A. Lokuta³, Jill C. Surfus¹, Sarah A. Wernimont¹, and Anna Huttenlocher^{*,1,4}

¹Department of Medical Microbiology and Immunology, University of Wisconsin, Madison, Wisconsin, USA

²Institute on Aging, University of Wisconsin, Madison, Wisconsin, USA

³Stratatech Corporation, Madison, WI

⁴Pediatrics, University of Wisconsin, Madison, Wisconsin, USA

Abstract

Proinflammatory cytokines, such as tumor necrosis factor alpha (TNF- α), are increased in many chronic inflammatory disorders, including rheumatoid arthritis, and contribute to recruitment of neutrophils into areas of inflammation. TNF- α induces a stop signal that promotes neutrophil firm adhesion and inhibits neutrophil polarization and chemotaxis. Calpain is a calcium-dependent protease that mediates cytoskeletal reorganization during cell migration. Here, we show that calpain inhibition impairs TNF- α -induced neutrophil firm adhesion to fibrinogen-coated surfaces and the formation of vinculin-containing focal complexes. Calpain inhibition induces random migration in TNF- α -stimulated cells and prevents the generation of reactive oxygen species, but does not alter TNF- α -mediated activation of p38 MAPK and ERK MAPK. These findings suggest that the TNF- α -induced neutrophil arrest requires the activity of calpain independent of p38 MAPK and ERK signaling seen after TNF- α stimulation. Together, our data suggest that therapeutic inhibition of calpain may be beneficial for limiting TNF- α -induced inflammatory responses.

Keywords

migration; stop signal; neutrophil; oxidative burst; tumor necrosis factor; motility

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*Corresponding author address: Anna Huttenlocher, M.D., Department of Medical Microbiology and Immunology, University of Wisconsin, 4205 Microbial Sciences Building, 1550 Linden Avenue, Madison, WI 53706, Phone: 608-265-4642, Fax: 608-262-8418, huttenlocher@wisc.edu.

Other author addresses: Andrew J. Wiemer, Sarah A. Wernimont, Jill C. Surfus, Department of Medical Microbiology and Immunology, University of Wisconsin, 4225 Microbial Sciences Building, 1550 Linden Avenue, Madison, WI 53706, Phone: 608-265-4669, Fax: 608-262-8418

Mary A. Lokuta, Stratatech Corporation, Suite 169, 505 S. Rosa Road, Madison, WI 53719, phone (608) 441-8358, fax (608) 441-2757, mlokuta@stratatechcorp.com

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INTRODUCTION

The inflammatory response is essential for proper wound healing (Gillitzer and Goebeler, 2001); however, in conditions including asthma, rheumatoid arthritis and inflammatory bowel disease excessive inflammation contributes to the development chronic inflammation and tissue damage (Aloisi and Pujol-Borrell, 2006). Neutrophils are early responders to inflammatory stimuli including cytokines, chemokines and extracellular matrix ligands (Mayadas and Cullere, 2005). The inflammatory response involves neutrophil adhesion to and migration out of the microvasculature into the affected tissue, and the subsequent retention of neutrophils within tissues (Granger and Kubes, 1994). Consequently, studying neutrophil migration is important for understanding the mechanisms that mediate the development of acute and chronic inflammation. Development of agents that either selectively inhibit or activate neutrophil migration represents an intriguing approach for treatment of inflammatory disorders (Mackay, 2008).

Tumor necrosis factor alpha (TNF- α) is a central mediator of the inflammatory response that is released by macrophages and other cells in response to a variety of proinflammatory stimuli (Locksley et al., 2001). Elevated levels of TNF- α are often associated with chronic inflammation, and there are therapeutic benefits to blocking TNF- α with agents such as etanercept or infliximab in patients with chronic inflammatory disorders (Aggarwal, 2003). TNF- α causes a cascade of events including cytokine and chemokine production, proliferation of immune cells, and increased cell adhesion, thus recruiting and retaining leukocytes in inflammatory sites. TNF- α activates several neutrophil functions including oxidative burst (Nathan, 1987) and degranulation (van der Poll et al., 1992). While TNF- α does not increase cytosolic free calcium levels (Richter et al., 1989), the ability of TNF- α to induce activation of neutrophils is dependent upon calcium release and can be blocked by calcium chelators (Richter et al., 1990) or calcium channel blockers. TNF- α also up-regulates the adhesion molecules CD11b and CD15 on the surface of neutrophils and induces neutrophil arrest (Lokuta and Huttenlocher, 2005; Mayadas and Cullere, 2005).

There are several well-characterized signaling pathways activated by TNF- α binding to its two predominant receptors, TNFR1 and TNFR2. Binding of TNF- α leads to increased NF- κ B activity and expression of its target genes, including increased expression of adhesion molecules E-selectin, VCAM, and ICAM-1 (Read et al., 1995). In addition, TNF- α stimulation activates signaling through MAPKs (mitogen activated protein kinases) including p38 and ERK (McLeish et al., 1998), and signaling through the small GTPase Cdc42 (Puls et al., 1999). Ultimately, TNF- α signaling leads to integrin activation and firm adhesion of neutrophils to a variety of surfaces (Williams and Solomkin, 1999), via mechanisms that are currently not well-characterized.

Calpains are intracellular, calcium-dependent proteases (Franco and Huttenlocher, 2005) that are constitutively active in resting neutrophils (Lokuta et al., 2003). Calpains contribute to polarization and formation of lamellipodia during chemotaxis (Nuzzi et al., 2007). In most cell types, calpain inhibition decreases migration (Huttenlocher et al., 1997), but in human neutrophils calpain inhibition enhances random neutrophil migration (Lokuta et al., 2003; Katsube et al., 2008). Through cleavage of their substrates, calpains regulate activity of the Rho family of small GTPases (Kulkarni et al., 1999), activation of integrins (Stewart et al., 1998), and organization of the cytoskeleton (Huttenlocher et al., 1997). Inhibition of calpains has been reported to induce activation of p38 MAPK and ERK MAPK under some circumstances (Katsube et al., 2008). Calpain activity reportedly is elevated in several human diseases including muscular dystrophy (Huang and Wang, 2001). Inhibition of calpain activity in an animal model of collagen-induced arthritis blocked neutrophil recruitment and prevented inflammation (Cuzzocrea et al., 2000).

The signaling pathways involved with neutrophil migration are complex (Niggli, 2003), and the regulation of neutrophil arrest by TNF- α is not well-characterized. In other processes and cell types, TNF- α -induced signaling requires calpain activity for its effects. For example, inhibitors of calpain reverse TNF- α -induced apoptosis in the U937 monocytic cell line (Vanags et al., 1996) and the Jurkat T lymphocytic cell line (Diaz and Bourguignon, 2000). In neutrophils, TNF- α signaling has been reported to cause apoptosis through separate caspase- or calpain-dependent pathways (Chen et al., 2006).

Here, we investigated how calpain inhibition alters TNF- α -mediated neutrophil adhesion and migration. In this report, we demonstrate that calpain inhibition alters several consequences of TNF- α signaling in neutrophils. Treatment with calpain inhibitors decreases TNF- α -induced neutrophil firm adhesion to fibrinogen, induces random migration in TNF- α -stimulated cells, and prevents TNF- α -induced generation of reactive oxygen species. Taken together, the data indicate that calpain inhibition can impair TNF- α -mediated neutrophil activation.

MATERIALS AND METHODS

Reagents

Recombinant human TNF- α was purchased from R&D Systems (Minneapolis, MN). Fibrinogen and f-Met-Leu-Phe (fMLP) were purchased from Sigma Chemical Co (St. Louis, MO). Calpain inhibitors ALLN, ALLM, Z-LLY-FMK, PD150606, and calpastatin peptide were purchased from EMD Biosciences (San Diego, CA). The calpain inhibitors used in this study were cell permeable peptide aldehydes ALLN (50 μ g/mL, 130 μ M) and ALLM (50 μ g/mL, 125 μ M) (concentrations which inhibit ~80% of calpain activity in neutrophils (Lokuta et al., 2003)), the fluoromethyl ketone Z-LLY-FMK (45 μ M), and the small molecule PD150606 (1 μ M).

Neutrophil Isolation

Human peripheral blood neutrophils were purified from blood using Polymorphprep from Nycomed (Zurich, Switzerland) according to manufacturer protocol and as described previously (Lokuta et al., 2007). All donors were healthy and informed consent was obtained at the time of the blood draw. The human subject protocol was approved by the University of Wisconsin Center for Health Sciences Human Subjects Committee.

Immunofluorescent Staining

Neutrophils were plated on glass coverslips coated with 10 μ g/mL fibrinogen and incubated for 30 minutes at 37°C and 7.5% CO₂ in the presence of TNF- α and/or calpain inhibitor. Cells were fixed with 6.6% paraformaldehyde, 0.05% glutaraldehyde, and 0.25 mg/mL saponin in PBS for 15 minutes. This reaction was quenched with 0.15 M glycine in PBS for 15 minutes. Samples were blocked with PBS containing 10% heat-inactivated FBS and 0.25 mg/mL saponin at 4°C for 24–48 hr. Anti-vinculin antibody (V-9131; Sigma) was used at 1:400 in blocking buffer. Anti-actin antibody (AC-15; Sigma) was used at 1:500, DAPI (Sigma) was used at 1:10,000, and FITC-anti-tubulin antibody (Sigma) was used at 1:200. Sheep-anti-mouse-FITC from Valeant (Costa Mesa, CA) was used at 1:250 or goat-anti-mouse-rhodamine Red X was used at 1:250 (Invitrogen (Carlsbad, CA)). Coverslips were imaged using a 100X oil-immersion lens on a Nikon Eclipse TE300 inverted fluorescent microscope. Images were acquired with a Hamamatsu cooled CCD video camera and captured into Metamorph v7.1 from MDS (Toronto, Canada).

Adhesion Assay

Neutrophils were incubated in EGM-2MV containing 1.93 μM of the cell-permeable fluorescent indicator, calcein-AM (Invitrogen) for 15 minutes at 37°C and 7.5% CO_2 . Cells were then washed with PBS without $\text{Ca}^{2+}/\text{Mg}^{2+}$. Neutrophils were brought to 1×10^6 cells/mL in DPBS without $\text{Ca}^{2+}/\text{Mg}^{2+}$ for the standard curve or brought to 1×10^6 cells/mL in EGM-2MV and treated accordingly. Neutrophils were allowed to adhere for 30 minutes on 2.5 $\mu\text{g}/\text{mL}$ fibrinogen- or ICAM-1-coated, black, medium-binding 96-well plates from Greiner (Monroe, NC). Unbound cells were removed by washing and firm shaking. Fluorescence excitation/emission at 485/535 nm was determined using a TECAN SPECTRAFluor Plus fluorometer. Samples were run in quadruplicate, a standard curve was included on each plate, and linear regression was performed with Magellan v2.50 software to determine the number of neutrophils adhered in each well.

Oxidative Burst Assays

Extracellular oxidative burst was assayed using homovanillic acid. Briefly, 100 μL of 400 μM homovanillic acid with 4 U/mL horseradish peroxidase in HBSS and 75 μL of EGM-2MV containing treatment were equilibrated to 7.5% CO_2 and 37°C in black 96-well plates. Then, 1×10^5 neutrophils were added in 20 μL and incubated for 30 minutes at 7.5% CO_2 and 37°C. For the last 10 minutes of the incubation 5 μL of media or fMLP to a final concentration of 100 nM was added. The plate was then spun, 100 μL of the supernatant transferred to a clean well, and fluorescence excitation/emission at 355/405 determined using a Perkin Elmer Victor³V 1420 Multilabel Counter.

Time-lapse Microscopy

Neutrophils were plated for 30 minutes at 37°C and 7.5% CO_2 onto non-tissue-culture-treated dishes in EGM-2MV from Cambrex (East Rutherford, NJ) containing TNF- α and/or inhibitor as noted. Dishes were then placed in The Box closed system from Life Imaging Services (Basel, Switzerland), and maintained at 37°C and imaged on an Olympus (Center Valley, PA) IX-70 inverted microscope using a 20X phase objective. Images were collected using a Coolsnap fx cooled CCD camera from Photometrics (Tucson, AZ) and captured into MetaView v6.2 (MDS) every 15 seconds for 15 minutes.

Western Blot analysis

Neutrophils were stimulated with 250 ng/mL TNF- α for 30 minutes at 37°C and lysed in 50 mM HEPES pH 7.4, 1% Triton X-100, 1 mM EDTA, and 1 mM EGTA using a method modified from Suzuki et al. (Suzuki et al., 1999). Lysing buffer also contained freshly added phosphatase inhibitor cocktail (1:50 dilution, P-5726; Sigma), protease inhibitor cocktail (1:50 dilution, P-8340; Sigma), 2 mM phenylmethylsulfonylfluoride (PMSF), 100 μM sodium orthovanadate, 2 $\mu\text{g}/\text{mL}$ aprotinin, 2 $\mu\text{g}/\text{mL}$ leupeptin A, 900 μM benzamidine, 1 mM phenantroline, and 1 $\mu\text{g}/\text{mL}$ pepstatin A. Proteins were resolved by SDS-PAGE on 6–20% acrylamide gradient gels, transferred to nitrocellulose using standard methods, and blotted with anti-p38 MAPK or anti-phospho-p38 MAPK antibodies (Biosource). Detection was performed using Alexa-Fluor®680 goat-anti-mouse IgG (Molecular Probes) and IRDyeTM800CW goat-anti-rabbit IgG (Rockland) antibodies. Quantification was determined using an Odyssey Infrared-Imaging System.

Statistical Analysis

Statistical analyses were performed using Graph Pad (Prism). Statistical significance was calculated using one-way or two-way analysis of variance (ANOVA) where indicated to assess for significant differences in treatment and/or treatment day. Post-hoc analysis was performed using Tukey's HSD. Data were normalized relative to the mean and expressed as fold increase

relative to control. All columns in bar graphs represent the mean of the indicated number of replicates. Error bars on graphs represent standard error of the mean (SEM). An α level of 0.05 was set as the level of significance.

RESULTS

Calpain inhibition decreases TNF- α induced neutrophil adhesion

Stimulation with TNF- α induces firm neutrophil adhesion (Lokuta and Huttenlocher, 2005). To determine whether calpain activity is required for TNF- α -mediated adhesion, we treated human peripheral blood neutrophils with TNF- α alone (250 ng/mL) or in combination with a panel of calpain inhibitors and examined neutrophil adhesion to fibrinogen-coated or intercellular adhesion molecule 1 (ICAM-1)-coated coverslips (Figure 1). Cells were allowed to adhere to fibrinogen-coated coverslips for 30 minutes in the presence of TNF- α and/or indicated inhibitors. Cell morphology was initially assessed via light microscopy (Figure 1A and 1B). As expected, vehicle control treated neutrophils retained a rounded morphology and appeared only weakly adherent to the fibrinogen (Figure 1A). Control neutrophils exhibited stronger adhesion to ICAM-1 and displayed polarized morphology (Figure 1B). Following TNF- α treatment neutrophils developed a non-polarized and spread morphology on fibrinogen which was associated with increased adhesion (Lokuta and Huttenlocher, 2005). Treatment with calpain inhibitors alone changed the cell morphology of the adherent subpopulation from rounded to polarized (Lokuta et al., 2003). When added to TNF- α -treated cells, calpain inhibitors decreased overall adhesion relative to TNF- α alone and induced polarization.

Cell adhesion assays were performed to quantify the effect of calpain inhibition on TNF- α -mediated neutrophil adhesion (Figure 1C and 1D). Cells were fluorescently labeled with calcein-AM, allowed to adhere to fibrinogen- or ICAM-1-coated 96-well plates for 30 minutes in the presence or absence of calpain inhibitors, and adhesion was quantified by fluorescence detection. As expected, TNF- α increased adhesion of neutrophils to fibrinogen relative to vehicle controls almost 10-fold. Calpain inhibitors alone had no statistically significant effect on adhesion. However, ALLN, ALLM, and PD150606 significantly reduced the TNF- α -mediated increase in adhesion to fibrinogen. Z-LLY-FMK also appeared to reduce adhesion to fibrinogen, although the results were not statistically significant. Untreated neutrophils exhibited much stronger adhesion to ICAM-1 than to fibrinogen and consequently the TNF- α -mediated increase was not as pronounced on this substrate. In addition, although calpain inhibition also appeared to impair neutrophil adhesion to ICAM-1, these effects were more subtle, likely a result of the increased baseline adhesion, and were not statistically significant. Therefore, further studies were performed using fibrinogen coated surfaces.

Unstimulated neutrophils loosely adhere to fibrinogen and do not polarize. We (Lokuta and Huttenlocher, 2005) and others (Fuortes et al., 1999) have shown that TNF- α induces non-polarized neutrophil spreading. Conversely, calpain inhibition in the absence of TNF- α stimulation elicits polarized neutrophil spreading (Lokuta et al., 2003). We therefore asked whether calpain inhibition alters TNF- α -induced cytoskeletal polarization. Cells were treated with TNF- α in the presence or absence of calpain inhibitors (Figure 2) and assessed for actin architecture using fluorescence microscopy. Vehicle control cells were loosely attached with rounded morphology and peripherally localized actin. As expected, neutrophils treated with TNF- α exhibited non-polarized cell spreading and peripherally localized actin, while cells treated with calpain inhibitors alone exhibited polarized cell spreading with actin polarized to the leading edge. Calpain inhibition caused polarized neutrophil morphologies with actin reorganization to the leading edge even in the presence of TNF- α .

Calpain inhibition alters cytoskeletal organization of adherent neutrophils

Previous work has shown that treatment with TNF- α induces the formation of non-polarized vinculin-containing focal complexes, and increases distribution of NADPH oxidase to focal complexes (Yan et al., 1995). In order to determine whether calpain inhibition disrupts focal complex formation, cells were treated with TNF- α in the presence or absence of calpain inhibitors and the formation of vinculin-containing focal complexes was examined (Figure 3A). As expected, treatment with TNF- α alone elicited the formation of non-polarized vinculin-containing focal complexes. In the presence of ALLN alone, the cells took on a polarized morphology with few vinculin-containing focal complexes. When neutrophils were treated with both TNF- α and ALLN, cells exhibited an elongated and polarized morphology with few vinculin-containing focal complexes oriented to the cell rear. Similar results were observed with other calpain inhibitors including ALLM (data not shown). These data suggest that calpain inhibition impairs focal complex formation induced by TNF- α .

In order to further characterize cell polarization we examined the effects of TNF- α and calpain inhibitors on microtubule organization. Neutrophils were treated with TNF- α alone or in combination with calpain inhibitor and tubulin orientation was assessed by fluorescence microscopy (Figure 3B). In TNF- α treated cells, formation of the microtubule organizing center (MTOC) is evident, and the microtubules are not polarized but extend in all directions. However, in neutrophils treated with the calpain inhibitor ALLN and TNF- α , the MTOC localizes toward the rear of the cell with a distribution of microtubules which is typical of polarized neutrophils.

Calpain inhibition induces migration of TNF- α -arrested neutrophils

Several studies have indicated a role for TNF- α signaling in neutrophil migration (Spertini et al., 1991; Drost and MacNee, 2002). Our previous studies have demonstrated that TNF- α stimulation induces neutrophil arrest and impairs neutrophil random migration (Lokuta and Huttenlocher, 2005). To determine if calpain inhibition affects TNF- α -mediated neutrophil arrest, neutrophils were treated for 30 minutes with 50 ng/mL TNF- α in the presence or absence of calpain inhibitors and migration was assessed using time-lapse light microscopy (Figure 4). As expected, in the absence of TNF- α , neutrophils were only loosely adherent and speeds of migration were not determined (for a full description of how calpain inhibition affects migration of unstimulated neutrophils see reference (Lokuta et al., 2003)). In the presence of TNF- α , neutrophils migrated at a speed of $5.75 \pm 0.50 \mu\text{m}/\text{min}$. When neutrophils were treated with both TNF- α and a calpain inhibitor, the speed of migrating cells increased. The effects were most pronounced for ALLN and ALLM and were over two fold, to 17.0 ± 1.16 and $15.0 \pm 1.06 \mu\text{m}/\text{min}$, respectively. Both Z-LLY-FMK and PD150606 also significantly increased migration speeds although to a lesser extent than ALLN and ALLM. Together, the data indicate that calpain inhibition blocks TNF- α -mediated neutrophil arrest.

Calpain inhibition functions independently of TNF-induced MAPK activation

Activation of p38 and ERK signaling occurs in neutrophils following TNF- α stimulation (Suzuki et al., 1999), and p38 activation is required for TNF- α -mediated neutrophil adhesion and arrest (Lokuta and Huttenlocher, 2005). Additionally, calpain inhibition has been reported by Katsube et al. to induce p38 and ERK phosphorylation in human neutrophils (Katsube et al., 2008). We investigated whether calpain inhibition activates phosphorylation of p38 and ERK in combination with TNF- α stimulation during the timeframe of our migration assays (30 minutes post-TNF- α addition). Neutrophils were treated with TNF- α in the presence or absence of calpain inhibitors and phosphorylation of p38 and ERK was assessed by Western Blot analysis (Figure 5). TNF- α induced an increase in levels of phosphorylated p38 and ERK at 15 and 30 minutes post-stimulation. Calpain inhibition alone did not induce an increase in p38 and ERK phosphorylation relative to vehicle control under the conditions of this assay.

Inhibition of calpain activity also did not significantly alter TNF- α -mediated p38 or ERK activation at these time points. These findings suggest that calpain inhibition likely affects neutrophil adhesion and migration in TNF- α -treated neutrophils independent of p38 and ERK MAPK signaling.

Calpain inhibitors prevent TNF- α mediated oxidative burst

One measure of neutrophil activation is the release of reactive oxygen species, which is promoted by TNF- α stimulation, enhanced in the presence of other activating agents such as fMLP and antagonized by inhibitors of calcium flux (Yuo et al., 1989). Therefore, we tested whether or not calpain inhibitors affect TNF- α -mediated release of reactive oxygen species (Figure 6). Neutrophils were stimulated with TNF- α for 30 minutes in the presence or absence of calpain inhibitors and generation of hydrogen peroxide in the media was quantified. Following TNF- α stimulation of fMLP-treated neutrophils, levels of hydrogen peroxide were increased approximately 2-fold relative to untreated control cells. Treatment with the calpain inhibitors alone had no statistically significant effect on the oxidative burst. Treatment with all four calpain inhibitors significantly reduced TNF- α -mediated hydrogen peroxide generation. These findings indicate that calpain activity is required for TNF- α -mediated oxidative burst.

DISCUSSION

TNF- α -targeted therapies are highly effective for the treatment of rheumatoid arthritis and other chronic inflammatory disorders. Here we provide evidence that calpain inhibition impairs TNF- α -mediated adhesion and arrest of primary human neutrophils. We also show that calpain inhibition induces cell polarization and random migration in TNF- α -stimulated cells and prevents the generation of oxidative burst, but does not alter TNF- α -mediated phosphorylation of p38 and ERK. Together, our findings suggest that therapeutic inhibition of calpain may be beneficial for limiting TNF- α -induced inflammatory responses and tissue retention.

It is well-documented that TNF- α induces integrin-mediated firm neutrophil adhesion to a variety of surfaces including fibrinogen and ICAM-1 (Nathan, 1987; Read et al., 1995; Lokuta and Huttenlocher, 2005). While neutrophil binding to ICAM-1 is likely mediated through MAC-1 (α M β 2) (Diamond and Springer, 1993), adhesion to fibrinogen may be mediated by both MAC-1 (α M β 2) and the vitronectin receptor (α v β 3) (Hendey et al., 1996). We demonstrate that treatment with a panel of calpain inhibitors decreases TNF- α -mediated neutrophil adhesion. This effect is most pronounced on fibrinogen, likely because on ICAM-1 neutrophils exhibited higher baseline adhesion in unstimulated controls. The use of a panel of calpain inhibitors is important since each inhibitor displays different specificities toward both calpains and other proteases, and primary neutrophils are generally not amenable to other modes of inhibition such as gene silencing because they are terminally differentiated and short-lived (Rock et al., 1994). The analysis of adhesion at early time points (30–45 minutes) is also critical because of the known involvement of TNF- α in regulating NF- κ B activity (Read et al., 1995). Previous studies have reported that ALLM and ALLN have different effects on adhesion mediated by NF- κ B due to their different inhibitory potencies with regard to the proteasome (Read et al., 1995). In our studies, ALLM and ALLN affected adhesion similarly in TNF- α -treated neutrophils, suggesting the effects on adhesion are due to calpain inhibition rather than non-specific effects on the proteasome.

It has been shown by us and others that TNF- α stimulation induces a flattened, non-polarized morphology characterized by the formation of vinculin-containing focal complexes (Fuortes et al., 1999; Lokuta and Huttenlocher, 2005). Here, we demonstrate that treatment of neutrophils with TNF- α in the presence of calpain inhibitors induces a polarized morphology with a concomitant relocalization of actin to the leading edge, tubulin orientation to the rear, and reduction of vinculin-containing focal contacts. Focal complexes form a platform for

NADPH oxidase-mediated generation of reactive oxygen species (Williams and Solomkin, 1999). We also find that calpain inhibition impairs TNF- α -mediated neutrophil oxidative burst. Our data are in agreement with work from others showing that both adhesion (Nathan, 1987) and calcium influx (Brechard and Tschirhart, 2008) are required for TNF- α induced oxidative burst. The disruption in the ability of TNF- α to induce stable focal complexes may well underlie the effect that calpain inhibition has on the generation of oxidative burst after TNF- α treatment.

We have demonstrated that calpain inhibition induces random migration in TNF- α -treated arrested neutrophils. While all four calpain inhibitors induced this effect, the magnitude was larger with ALLN and ALLM than for LLY-FMK and PD150606. This is not surprising because ALLN and ALLM are generally regarded as more potent inhibitors. These findings suggest that calpain activity is required for TNF- α -induced neutrophil arrest. We speculate based on these data and others in the literature that calpain may act to prevent protrusive behavior and as a result of the inhibition of calpain, arrested neutrophils exhibit decreased adhesion and enhanced chemokinesis. The effects of calpain inhibition are similar to the phenotype observed with inhibitors of microtubules such as colchicine, which increase random migration but decrease directional chemotaxis (Xu et al., 2005).

In primary human neutrophils TNF- α stimulation promotes phosphorylation of p38 and ERK, the former of which is required for TNF-mediated arrest (Lokuta and Huttenlocher, 2005). Calpain inhibition did not significantly change the amount of phosphorylated p38 or ERK induced by TNF- α . This suggests that the role that calpain plays in neutrophil adhesion regulation is independent of altered MAPK signaling. We hypothesize that calpain activity acts downstream of the TNF receptor and is required for neutrophil adhesion, arrest, and oxidative burst (Figure 7). However, it is unlikely that calpain is acting via a p38 MAPK or ERK pathway in order to carry out these functions. A more plausible scenario may involve calpain regulation of focal complex formation via cleavage of its known substrates such as talin.

There is now an accumulating body of evidence describing calpain-mediated effects of TNF- α signaling in neutrophils (Vanags et al., 1996; Diaz and Bourguignon, 2000; Chen et al., 2006). Under some circumstances TNF- α promotes survival of neutrophils, however TNF- α signaling also has been linked to neutrophil apoptosis (Chen et al., 2006). TNF- α -treatment alone induces neutrophil arrest, but was recently shown to enhance neutrophil migration in response to certain chemoattractants (Montecucco et al., 2008). While TNF- α signaling during migration, adhesion and apoptosis is complex, all of these important cellular processes appear to be dependent upon calpain activity and altered by calpain inhibitors. Our work strengthens this accumulating body of knowledge by showing that calpain inhibition impairs TNF- α -mediated adhesion, stop signal and oxidative burst.

It is unknown whether altering neutrophil adhesion and migration would be an effective means to treat inflammatory disorders. Neutrophil retention in inflamed tissues is enhanced by TNF- α and exacerbates various disease states. Calpain inhibition, by enhancing migration in TNF- α stimulated neutrophils and preventing their accumulation in inflamed sites, may be one approach to reduce inflammation. The data presented here could suggest a mechanism for the previous finding that calpain inhibition in models of arthritis prevents neutrophil accumulation in sites of inflammation (Cuzzocrea et al., 2000). Ultimately, data from more complex in vivo models would further clarify the precise physiological roles of calpains in mediating TNF- α signaling.

Nonstandard abbreviations

TNF- α	Tumor Necrosis Factor alpha
MAPK	mitogen activated protein kinase

ICAM-1	intercellular adhesion molecule 1
FBG	Fibrinogen

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Figure 1. Calpain mediates TNF- α -induced cell adhesion

(A) Human peripheral blood neutrophils plated on fibrinogen (FBG) or intercellular adhesion molecule 1 (ICAM-1) (B) Neutrophils were treated for 30 minutes with TNF- α (250 ng/mL) in the presence or absence of calpain inhibitors ALLM (M) or ALLN (N) (50 μ g/mL), Z-LLY-FMK (L) (45 μ M), or PD150606 (P) (1 μ M). Results are representative images of at least three experiments. Neutrophils were assessed for adhesion to (C) FBG or (D) ICAM-1 coated wells (mean $\pm$ SEM, $n = 3$, $p < 0.05$ by one-way ANOVA, Tukey post hoc analysis indicates significant difference relative to control (*) or TNF(#)).

Figure 2. Calpain inhibition polarizes TNF- α -treated cells

Human peripheral blood neutrophils were treated for 30 minutes with TNF- α (50 ng/mL) in the presence of vehicle control or calpain inhibitors ALLN (50 μ g/mL) or ALLM (50 μ g/mL), Z-LLY-FMK (45 μ M), or PD150606 (1 μ M). Neutrophil actin arrangement was determined by rhodamine phalloidin staining. Results are representative images of at least three experiments.

Figure 3. Calpain inhibition causes altered organization of focal complexes and microtubules
Human peripheral blood neutrophils were treated for 30 minutes with TNF- α (50 ng/mL) in the presence or absence of calpain inhibitor ALLN (50 μ g/mL). (A) Formation of focal complexes was assessed by visualization of vinculin. Samples were co-stained with rhodamine phalloidin and DAPI. Results are representative images of at least three experiments. (B) Organization of microtubules was assessed by staining for tubulin. Samples were co-stained with DAPI. Results are representative images of at least three experiments.

Figure 4. Calpain inhibition reverses TNF- α -mediated migration stop signal

Human peripheral blood neutrophils were treated for 30 minutes with TNF- α (50 ng/mL) in the presence or absence of calpain inhibitor ALLM (50 μ g/mL). Cell movement was captured between 30–45 minutes post-treatment by time-lapse microscopy and cell speeds were quantified using Metamorph (mean $\pm$ SEM). A) ALLM increases displacement of TNF- α treated neutrophils (Data shown is representative of three separate experiments). B) Calpain inhibitors ALLM, ALLN, PD150505 and LLYFMK increase mean cell speeds of neutrophils treated with TNF- α . (Data from three separate experiments, * indicates $p < 0.01$).

Figure 5. Calpain inhibition does not affect TNF-mediated phosphorylation of p38 and ERK
Neutrophils were treated for 30 minutes with TNF- α (250 ng/mL) in the presence or absence of calpain inhibitor ALLM (50 μ g/mL). Levels of phosphorylated and total p38 were assessed by Western Blot analysis. A) Representative Western Blots and quantification of cells treated for 0, 15, or 30 minutes. (n=3, * indicates $p < 0.05$ relative to untreated controls).

Figure 6. Calpain inhibition prevents TNF- α -mediated oxidative burst

Human peripheral blood neutrophils were treated for 30 minutes with TNF- α (250 ng/mL) in the presence or absence of calpain inhibitors ALLN or ALLM (50 μ g/mL), Z-LLY-FMK (45 μ M), or PD150606 (1 μ M). H₂O₂ production was quantified relative to unstimulated controls (mean $\pm$ SEM, n = 4 or 5, p < 0.05 by one-way ANOVA, Tukey post hoc analysis indicates significant difference relative to control (*) or fMLP TNF(#)).

Figure 7. TNF- α and calpain regulate integrin activation in human neutrophils

Binding of TNF- α to its receptor initiates a signaling cascade that increases β -integrin mediated adhesion, promoting neutrophil arrest. Neutrophil arrest requires p38 activation (Lokuta and Huttenlocher, 2005). Calpain inhibition prevents calpain-mediated proteolysis of the focal contact substrates talin, paxillin, and β integrins, which may alter neutrophil adhesion.