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Involvement of Calpain in the Process of Jurkat T Cell Chemotaxis

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Abstract

Massive T cell infiltration into the central nervous system is a hallmark of multiple sclerosis (MS) and its rodent model experimental autoimmune encephalomyelitis (EAE), resulting in the induction of many of the pathophysiological events that lead to neuroinflammation and neurodegeneration. Thus, blocking T cell migration into the central nervous system may reduce disease severity in MS and EAE. One potential target for reducing T cell migration is inhibition of the Ca²⁺-activated neutral protease calpain. Previous studies in other cell types have demonstrated that migration is reduced by incubation of cells with calpain inhibitors. Thus, we hypothesize that calpain inhibition will reduce migration of T cells in response to and toward the chemokine CCL2. To test this hypothesis, the intracellular free Ca²⁺ levels in Jurkat E6-1 T cells was first measured by the fura-2 assay to assess whether the intracellular ion environment would support calpain activation. The intracellular free Ca²⁺ levels were found to increase in response to CCL2. The cells were next treated with the calpain inhibitor calpeptin in a multiwelled Boyden chamber with CCL2 used as the chemoattractant. These studies demonstrate that inhibition of calpain with its inhibitor calpeptin produces a dose-dependent inhibition of chemotaxis. Calpain activity, as measured by live cell imaging, was also increased in response to CCL2, providing further evidence of its involvement in the process of chemotaxis and migration. These studies provide evidence for the involvement of calpain in the mechanisms of chemotaxis and warrants further exploration in MS patient and EAE animal samples.

Keywords

calpain; chemotaxis; EAE/MS; T cells

T cell involvement in the disease process of multiple sclerosis (MS) (Hickey, 1991; McFarland and Martin, 2007) and its animal model experimental autoimmune encephalomyelitis (EAE) are well established (Waksman and Adams, 1962; Swanborg, 2001). T cell infiltration into the central nervous system (CNS) is greatly increased in the plaques of MS patients (Hickey, 1991) as well as of EAE animals immediately before the onset of pathological symptoms and during clinical course of the disease (Waksman and Adams, 1962). Increased activity and expression of the Ca²⁺-activated protease calpain has been demonstrated in inflammatory cells (Shields et al., 1998, 1999) during the disease state in EAE animals. Moreover, inhibition of

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calpain has been demonstrated to decrease the T cell infiltration during acute EAE (Hassen et al., 2006). Calpain has signaling functions and is involved in a multitude of cellular processes including apoptosis (Zhivotovsky et al., 1997), T cell activation (Penna et al., 1999; Schaecher et al., 2004), cell cycle (Janossy et al., 2004), and cell migration (Huttenlocher et al., 1997; Franco and Huttenlocher, 2005).

T cell exclusion from the CNS is the therapeutic strategy used by the $\alpha 4 \beta 1$ -integrin blocking monoclonal antibody natalizumab (Ransohoff, 2007), which has been approved by the U.S. Food and Drug Administration (FDA). However, use of the FDA-approved drug natalizumab has not been without problems including several patients on this medication developing progressive multi-focal leukoencephalopathy (Koralnik, 2006) leading to the temporary withdrawal of the drug from the market. In addition, alternative targets for MS therapy are urgently warranted as a result of the low effectiveness of many of the current standard therapies available (Kleinschnitz et al., 2007). Targeted exclusion of T cells from the CNS would be of great benefit not only to MS but also to many other autoimmune diseases that have a T cell component in their pathology, including rheumatoid arthritis (Cope et al., 2007) and amyotrophic lateral sclerosis (Graves et al., 2004). Inflammatory cells entering the CNS in MS and EAE have been shown to increase calpain levels (Shields et al., 1998). If calpain was indeed involved with T cell migration then calpain inhibition would provide an alternative method for reducing pathological T cell entry into the CNS.

Calpain has been shown to be involved in the migration of numerous cell types including CHO cells (Huttenlocher et al., 1997), fibroblasts (Shiraha et al., 2002), myoblasts (Leloup et al., 2006), and neutrophils (Lokuta et al., 2003). The chemokine CCL2 (previously known as monocyte chemoattractant protein-1) and its predominant receptor CCR2, a G protein-coupled receptor, have been extensively associated with inflammatory pathologies (Jee et al., 2002; Mahad et al., 2006). CCL2 has been extensively shown as a specific chemoattractant for monocytes and T cells (Carr et al., 1994; Sanders et al., 2000). Even though the initiating stimulus for migration is well established, the downstream mechanism of T cell chemotaxis as of yet remains incomplete.

In this study, our aim was to gather evidence for the involvement of calpain in the process of T cell migration. This analysis used the Jurkat E6-1 T cell line to model for T cells as well as the normal human peripheral blood mononuclear cells (PBMCs) to model for primary cells. We observed an increase in activity of calpain in response to the CCL2 stimulation as well as the restriction of role of calpain in involvement in the directed migration of chemotaxis. This is in contrast to other immune cell types where calpain has been demonstrated to play a role in the negative regulation of chemokinesis (Lokuta et al., 2003). Thus, our current study provides an evidence for the involvement of calpain in T cell chemotaxis. Further examination of calpain involvement in the chemotaxis of native T cells and pathological T cells from MS patients and EAE animals is supported by the findings of this study.

MATERIALS AND METHODS

Cell Culture and Reagents

The T cell type used was the Jurkat E6-1 line (ATCC, Manassas, VA), which was grown in RPMI 1640 (MediaTech, Herndon, VA) media supplemented with L-glutamine, 100 U/mL penicillin and 1 μ g/mL streptomycin (Gibco, Grand Island, NY), and 10% fetal calf serum (FCS) (Hyclone, Logan, UT) at 37°C in a 5% CO₂ atmosphere (Thermo Fisher Scientific, Waltham, MA) to desired confluency for each of the assays performed. Fura-2 (Molecular Probes, Carlsbad, CA) was used as the Ca²⁺ indicator dye for the intracellular free Ca²⁺ assays. Calcein AM (Invitrogen, Carlsbad, CA) was used in the migration assay as the cellular tracking dye. Calpeptin (Calbiochem, San Diego, CA) was used as the pan-calpain inhibitor and CCL2

(Santa Cruz Biotechnology, Santa Cruz, CA) was the chemokine used as the chemoattractant in the cell migration assays. Cytochalasin D (Sigma, St. Louis, MO) was also used in the migration assay. CMAC, *t*-BOC-Leu-Met A6520 (Invitrogen) was used as the fluorescent calpain specific substrate in the calpain activity assay.

Isolation of PBMCs

Blood samples (20 mL) were taken from normal individuals and PBMCs were isolated as previously described (Imam et al., 2007). Briefly, whole blood was drawn from normal individuals and mixed with Hank's balanced salt solution (MediaTech, Herndon, VA) and layered on top of Ficoll-Paque PLUS (GE Healthcare, Piscataway, NJ) and centrifuged. The lymphocyte layer was removed and washed with phosphate-buffered saline (PBS) and then resuspended in RPMI 1640 without phenol red (MediaTech, Pittsburgh, PA) supplemented with 0.1% bovine serum albumin (BSA) (Fisher Scientific). The cells were then treated the same way as described below in the migration assay.

Determination of Intracellular Free Ca^{2+} Concentration

The intracellular free Ca^{2+} concentration, $[\text{Ca}^{2+}]$, was measured in Jurkat cells with the ratiometric fluorescent Ca^{2+} indicator fura-2 according to the method, as we described previously (Das et al., 2005), with slight modification for cell type. Restated is the modified method for intracellular free $[\text{Ca}^{2+}]$ determination (Grynkiewicz et al., 1985). Briefly, cells were grown to $5\text{--}6 \times 10^5$ cells/mL in supplemented RPMI 1640, pelleted by centrifugation, washed once with PBS and resuspended in 1 to 2 mL Locke's buffer (154 mM NaCl, 5.6 mM KCl, 3.4 mM NaHCO_3 , 1.2 mM MgCl_2 , 5 mM HEPES, 1 mM CaCl_2 and 5.6 mM glucose, adjusted to pH 7.4). The cells were then counted on a hemocytometer and Locke's buffer was added to adjust the concentration to $0.5\text{--}1 \times 10^6$ cells/mL for dye loading. Fura-2 AM was dissolved in dimethylsulfoxide (DMSO) and diluted in the cell culture medium to a final loading concentration of 5 μM . The cells were loaded with the dye at 37°C for 30 min with shaking and protected from light. The cells were then washed 2 times with ice cold Locke's buffer without Ca^{2+} and diluted with ice-cooled (4°C) Locke's buffer to a final concentration of 0.5×10^6 cells/mL and stored on ice until the assay was performed but no longer than 1 hr. Assays were performed on 1×10^5 cells in 180 μL in a 96-well plate format with a Gemini XPS fluorescent plate reader (Molecular Devices, Sunnyvale, CA) and fluorescence was read with dual excitation and a fixed emission at excitation 340 nm/380 nm and emission 510 nm. The plate was loaded with the appropriate number of cells and then incubated at 37°C for 10 min to stabilize the temperature then a basal 2 min kinetic reading was taken. The experimental test compound was then added to the cells in 20 μL and 5 min kinetic reading was taken. The fluorescence at the maximum free $[\text{Ca}^{2+}]$ was then determined by adding 25 μL of 250 μM digitonin (Fisher Scientific). The fluorescence at the minimum free $[\text{Ca}^{2+}]$ was then determined by the addition 25 μL of 50 mM EGTA (Sigma). The intracellular free $[\text{Ca}^{2+}]$ was calculated from these fluorescence values by using the following equation: $[\text{Ca}^{2+}] = K_d (R - R_{\min}) / (R_{\max} - R) \times (F_{\max}^{380} / F_{\min}^{380})$. The cell specific value of K_d was determined experimentally to be 127 nM for Jurkat T cells with the Calcium Calibration Buffer Concentrate kit (Invitrogen). The value R is the ratio of fluorescence emission from the two excitation wavelengths after exposure to the test compound. The values $R_{\max}$ and $R_{\min}$ are the ratio of fluorescence emissions after exposure to digitonin and EGTA, respectively. The value $F_{\max}^{380}$ is the resultant fluorescence emission that is measured by excitation at 380 nm when the free $[\text{Ca}^{2+}]$ is zero and is obtained during the EGTA exposure. The value $F_{\min}^{380}$ is the resultant fluorescence emission that is measured by excitation at 380 nm for saturating levels of free $[\text{Ca}^{2+}]$ and is obtained during the digitonin exposure.

Migration Assays

The migration assays were performed with the 96-well format Boyden chamber (Neuroprobe, Gaithersburg, MD) following the previously described method (Frevert et al., 1998) with modification for the cell types (Jurkat T cells and PBMCs) and chemoattractant optimization. Specifically, the cells were grown to confluency as described above to a final concentration of 1×10^6 cells/mL. Cells were pelleted by centrifugation and resuspended in RPMI 1640 without phenol red (MediaTech) supplemented with 0.1% BSA (Fisher Scientific). Calcein AM was dissolved in DMSO and added to the cells at a final concentration of 2 μ M and incubated at 37°C for 45 min with shaking. The cells were then washed 2 times with 37°C RPMI 1640 without phenol red and resuspended at a concentration of 4×10^6 cells/mL in preparation for loading the chamber. The chemokine CCL2 was diluted to the proper concentration in RPMI 1640 without phenol red and 30 μ L solution was added to a 96-well plate (Neuroprobe). A polycarbonate filter with 5 μ m pores (Neuroprobe) was then attached to the plate. The migration chamber was then assembled and cells in 50 μ L were added to the top well. The top well volume was then filled with 150 μ L of media or media containing an appropriate test compound. The whole chamber was incubated for 3 hr protected from light in 37°C humidified incubator with 5% CO₂ atmosphere. The chamber was then disassembled and the filter was gently scraped with a cell scraper (Fisher Scientific) and washed with PBS to remove any attached cells. The plate, with the filter still attached, was then centrifuged at 400g for 10 min (Eppendorf, Westbury, NY). The filter was then carefully detached and read on the fluorescent plate reader at excitation 485 nm and emission 535 nm.

Calpain Activity Assay

The intracellular calpain activity was assessed following the published method (Shao et al., 2006) with modification for cell type and instrumentation. Briefly, Jurkat T cells were grown to desired confluency as described before and then washed into RPMI 1640 without phenol red and 0.1% BSA. The cells were placed in 35-mm diameter culture plates (Corning, Lowell, MA) with incorporated cover slip for live cell microscopic imaging and kept at 37°C until labeling. The plates were then placed into a temperature and atmospherically controlled stage of a Zeiss LSM 510 NLO laser scanning confocal/multiphoton microscope (Zeiss, Thornwood, NY) equipped with a Coherent Chameleon tunable femtosecond Ti-Sapphire laser (Coherent, Santa Clara, CA) with META spectral detection (Zeiss). The instrument was focused before labeling was performed. The amino acid conjugated coumarin based fluorescent dye CMAC, *t*-BOC-Leu-Met was then injected into the dish at a final concentration of 50 μ M. Time lapse microscopic images were then captured every 15 sec for 20 min. Various treatments including addition of chemoattractant and calpain inhibitor were made and then further time lapse captures were performed. Average spot density from cells in the field was then calculated and plotted against time to determine increasing fluorescence levels over time (dF/dt).

Statistical Analysis

All data are shown as means $\pm$ standard error of mean (SEM) of separate experiments ($n \geq 3$). Data from various experiments was analyzed by SPSS software (SPSS, Chicago, IL). Statistical significance was determined by either the unpaired Student's *t*-test or one-way analysis of variance with Games-Howell post hoc test at a 95% confidence interval when multiple group comparisons were required. Statistical significance was obtained at $P \leq 0.05$.

RESULTS

Changes in Intracellular Free [Ca²⁺] in Response to Chemokine and Calpain Inhibitor

To investigate if an environment that was favorable for calpain activation was present in the Jurkat T cells during exposure to chemoattractant, we examined the intracellular free [Ca²⁺]

in the cells. It has been reported that certain calpain inhibitors can block the increase of intracellular free $[Ca^{2+}]$ levels (Liu et al., 2002). Therefore, we also examined the effect of calpain inhibitor. The Jurkat T cells were first exposed to various extracellular $[Ca^{2+}]$ to determine the effects on intracellular free $[Ca^{2+}]$ levels (Fig. 1A). Intracellular free $[Ca^{2+}]$ was increased corresponding to the concentration dependent-increase in the extracellular $[Ca^{2+}]$ levels. In order to confirm that our method for measuring intracellular free $[Ca^{2+}]$ would measure Ca^{2+} spikes, indicative of extracellular Ca^{2+} signaling, 1 μ M of ionomycin was added to the assay with and without 1 mM extracellular $[Ca^{2+}]$ (Fig. 1B). This positive control demonstrated a large Ca^{2+} spike in contrast to the low-level steady-state increase. The exposures to the chemokine CCL2 concentrations of 1 ng/mL to 100 ng/mL caused an increase in intracellular $[Ca^{2+}]$ (Fig. 1C). When we exposed the cells to a subconcentration (0.01 ng/mL) of CCL2, we no longer observed a change in intracellular free $[Ca^{2+}]$ (data not shown). An increase in the intracellular free $[Ca^{2+}]$ in the Jurkat T cells was also observed with exposures to various concentrations (100 nM to 100 μ M) of the calpain inhibitor calpeptin (Fig. 1D). The observed trend was an increase in intracellular free $[Ca^{2+}]$ corresponding to increase in concentration of calpeptin. Increase in intracellular free $[Ca^{2+}]$ was also observed in response to various concentrations (10 nM to 1 μ M) of the calpain inhibitor P150606 (Fig. 1E), which binds to the Ca^{2+} binding site of calpain to prevent calpain activation.

CCL2 Induced Largely Chemotactic Migration of Jurkat T Cells

Cellular migration can be divided into increased random motion and increased directed motion. Chemokinesis is the increase in random motion caused by a cytokine, while chemotaxis is directed motion toward a chemokine. A standard method (Frevert et al., 1998; Wilkinson, 1998; Colvin et al., 2004) of measuring cellular migration is performed with the use of a Boyden chamber, which is composed of two compartments separated by a membrane with holes smaller than the resting diameter of the cell. In order for cells to move to the lower compartment and be detected by the assay, they must actively change their shape and migrate through a pore in the membrane. Jurkat T cells placed into a Boyden chamber will eventually equilibrate over time as random migration of the cells carry them through the filter membrane (Fig. 2A), which over the 3 hr of the experiment appears as migration for the unstimulated group. The effects of various types of chemokine stimulation can be assessed by where in the chamber compartments the compounds are placed relative to the cells. CCL2 stimulation both in a chemokinetic fashion, chemokine above and below the filter, and in a chemotactic manner, chemokine below the filter, produced significant migration ($P < 0.05$) when compared with the unstimulated group. The major difference that was observed in the chemotaxis stimulated group ($P < 0.05$) was significantly different than both the unstimulated (58% different) and chemokinetically stimulated (44% different) groups. Increased chemotaxis was observed as a concentration-dependent response (Fig. 3A) toward higher concentrations of CCL2 until the cells reached maximal chemotaxis at 50 ng/mL CCL2. Concentrations of CCL2 above 50 ng/mL did not increase chemotaxis.

Calpain Inhibition Is Restricted to Reducing Chemotaxis in Jurkat Cells Stimulated With CCL2

Previous work on other immune cell types have reported that calpain is a negative regulator of cell migration and that calpain inhibition leads to increased chemokinesis (Lokuta et al., 2003). In order to determine if calpain plays a similar negative regulator role for chemokinesis in Jurkat T cells, we performed migration assays with a calpeptin gradient without chemokine stimulation (Fig. 2B). In this unstimulated environment (i.e., in absence of CCL2), cellular migration was not significantly different than control (no calpeptin) for any of the calpeptin concentrations tested. Jurkat T cells migration assays were also performed with chemokine stimulation in a chemokinetic environment (Fig. 2C). In this chemokinetically stimulated environment, CCL2 (50 ng/mL) above and below the membrane, cellular migration was also

not significantly different than control (no calpeptin) for any of the calpeptin concentrations tested. In contrast, calpain inhibition did produce a significant ($P < 0.05$) concentration-dependent reduction in chemotaxis toward CCL2 (50 ng/mL) (Fig. 2D). Chemotaxis was reduced by 31.1%, 39.5%, and 68.1% with calpeptin concentration at 1 μ M, 10 μ M, and 100 μ M, respectively.

Calpain Inhibition Also Reduces Chemotaxis in Human PBMCs Stimulated With CCL2

Migration assays were also performed with normal human PBMCs in response to chemotactic stimulation by CCL2 in the presence of the calpain inhibitor calpeptin (Fig. 3). Calpain inhibition produced a significant ($P < 0.05$) concentration-dependent reduction in chemotaxis toward CCL2 (100 ng/mL) (Fig. 3). Chemotaxis was reduced by 19.4%, 42.1%, and 41.7% with calpeptin concentration at 1 μ M, 10 μ M and 100 μ M, respectively. Chemotaxis also trended toward reduced migration with the two lower concentrations (10 nM and 100 nM) of calpeptin.

Calpain Is Part of a Chemotaxis Signaling Cascade

The number of cells that undergo chemotaxis toward a signal is dependent upon the concentration of that signal. The optimal concentration of CCL2 to induce maximal chemotaxis was determined by performing the migration assay with a chemoattractant gradient (Fig. 4A). The highest level of chemotaxis was observed with 50 ng/mL CCL2 even though the chemotactic effect appeared to plateau at CCL2 concentrations greater than 50 ng/mL. Calpain inhibition with calpeptin at the experimentally derived ID_{50} for Jurkat T cell chemotaxis toward CCL2 (50 μ M) produced a significant inhibition of chemotaxis ($P < 0.05$) for all the CCL2 concentrations tested when compared with chemotaxis in absence calpain inhibition (Fig. 4A). At the lower concentrations (1 ng/mL–50 ng/mL) of CCL2, there was significant inhibition ($P < 0.05$) of chemotaxis with calpeptin when compared with control levels. This significant inhibition of chemotaxis disappeared at the higher concentrations of CCL2 (100 ng/mL and 500 ng/mL). The polymerization of actin is a nonsignaling mechanism that is required for migration to occur (Vicente-Manzanares et al., 2005). Inhibition of this process of polymerization of actin with 25 μ M cytochalasin D produced a significant inhibition ($P < 0.05$) of chemotaxis with CCL2 concentrations of 10 ng/mL through 500 ng/mL (Fig. 4B). The percentages of chemotaxis in the cytochalasin D groups, compared with control, were not significantly different with any of the concentration of CCL2 tested. Both 25 μ M cytochalasin D and 50 μ M calpeptin were also tested together in order to determine if the migratory inhibition effect was additive (Fig. 4C). Inhibition of this process with 25 μ M cytochalasin D and 50 μ M calpeptin produced a significant inhibition ($P < 0.05$) of chemotaxis with CCL2 concentrations of 10 ng/mL through 500 ng/mL when compared with the media only group. Significant difference between the cytochalasin D group (Fig. 4B) and the combined treatment group (Fig. 4C) was not observed, though it trended toward slightly greater chemotactic inhibition.

Direct Observation of Calpain Activity in Response to CCL2 Exposure

The effect of calpain on chemotaxis has been indirectly inferred in the previous experiments from effects observed with a biochemical inhibitor. In order to directly assess whether calpain activity was increased in response to CCL2 stimulation, we used live cell imaging of a fluorescent dye coupled to a calpain substrate. The specificity of CMAC, *t*-BOC-Leu-Met as a calpain substrate and thereby indicator of calpain activity has been previously shown in hepatocytes (Shao et al., 2006). Jurkat E6-1 T cells used in this study displayed a basal level of calpain activity (Fig. 5) so the protocol was modified accordingly for this cell type. As a result of this basal calpain activity, simple fluorescence compared with non-fluorescence would not indicate altered activity. Sample micrographs from three groups of time lapse series (Fig.

5A) demonstrated the basal level of calpain activity with increased fluorescence over time as well as increased fluorescence over time in the CCL2 (50 ng/mL) group. The CCL2 (50 ng/mL) + calpeptin (100 μ M) series demonstrated the specificity of CMAC, *t*-BOC-Leu-Met as a calpain substrate. The masking examples (Fig. 5B) demonstrated the importance of choosing cells at random from the field of view because of the differential response in the culture between strong and weak responders. The same mask was used at each time point to track the same cells for the entire time of the experiment. The average spot density of the representative cells (10 for each field) was plotted against time for each of the exposures revealing the differential response (Fig. 5C). Both the basal and the CCL2 groups demonstrated calpain activity, while the CCL2 + calpeptin group did not show any activity. The results were quantitated by comparing the rate of increasing fluorescence (dF/dt) (Fig. 5D). The CCL2 stimulated group demonstrated a dF/dt that was significantly higher ($P < 0.05$) than the unstimulated basal group. The CCL2 + calpeptin group had a dF/dt significantly lower ($P < 0.05$) than both the basal and the CCL2 stimulated groups.

DISCUSSION

Migration of the adaptive immune system's cellular components into pathological areas in response to chemokine signaling is important for organisms to fight infection. The occurrence of autoimmune disease is evidence that this vital immune response does not always function in a way that is beneficial to the organism as a whole. The migration of T cells into the areas of inflammation is a critical step in their role in the pathology of the autoimmune conditions in which they participate. The infiltration of T cells into the CNS of MS patients and EAE animals has a fairly strong correlation with the disease pathology observed. The infiltrating inflammatory cells and T cells have been shown to increase calpain expression and activity (Shields et al., 1998). Even more importantly, treatment with calpain inhibitor has the ability to reduce the clinical score in EAE and also improve the morphology of the CNS tissue (Guyton et al., 2006; Hassen et al., 2006).

Calpain has been shown to have a role in the migration of various cell types but, as of yet, never in the adaptive immune cell type of T cells. Through our experiments we have provided the evidence for a direct involvement of calpain in chemotaxis toward CCL2 and its differing role in cellular migration from the innate immune cell type of neutrophils (Lokuta et al., 2003). We demonstrated that the intracellular environment in response to CCL2 is supportive for calpain activation (Fig. 1C). Other work (Bach et al., 2007) has also demonstrated an intracellular Ca^{2+} increase in response to the chemokine CXCL12 (SDF-1 α), which provides evidence for the involvement of calpain in the chemotactic response to various chemokines and is not limited to CCL2. Whenever intracellular Ca^{2+} levels change to another steady state, calpain activity should be investigated. Our use of calpeptin also appears to cause a mild increase in steady state intracellular free $[\text{Ca}^{2+}]$ (Fig. 1D). Previous work has demonstrated that certain calpain inhibitors have the ability to block further Ca^{2+} uptake (Liu et al., 2002). Calpeptin belongs to a class of protease inhibitors that binds to the active site of calpain and not to the Ca^{2+} binding site (Wang, 1990; Ray et al., 2003). This means that irrespective of the resulting intracellular Ca^{2+} level in response to calpeptin treatment, calpain activity will still be inhibited. The intracellular free Ca^{2+} levels were also measured in response to the calpain inhibitor PD150606, which inhibits calpain by binding to the Ca^{2+} binding site to prevent protease activation (Wang et al., 1996), and a similar mild increase of intracellular Ca^{2+} levels was also observed (Fig. 1E). This mild increase may be an inherent property of all calpain inhibitors because of homeostatic feedback control of basal calpain activity on intracellular free $[\text{Ca}^{2+}]$ but it is small enough to go undetected in most studies and is not large enough to alter the calpain inhibition.

Our current work supports a previous study with CCL2 in that CCL2 functions in T cells primarily as a chemotactic cytokine and has limited chemokinetic properties (Carr et al., 1994). Calpain inhibition appears directly concentration-dependent with respect to chemotaxis reduction, showing that greater calpain inhibition corresponds to greater reduction in chemotaxis. Our current work differs from a previous report (Lokuta et al., 2003) in that we were not able to demonstrate a significant role for calpain in the process of chemokinesis but our results were primarily restricted to chemotaxis (Fig. 2). There are a number of possible reasons for these discrepancy including difference in cell types, sensitivity of assays, various migratory stimulations, and various calpain inhibitors used. Although the difference is noteworthy between previous studies, the disease relevance is more important for directed migration, which guides pathogenic T cells to the CNS in EAE and MS. The observation of this inhibitory effect of calpain inhibitor in chemotaxis of Jurkat T cells (Fig. 2D) as well as in normal human PBMCs (Fig. 3) adds increased evidence to relevance of calpain in the disease process. The exact role of calpain in chemokine induced chemotaxis as of yet remains unknown, but evidence points to its involvement in a signaling pathway. The ability of an increasing concentration of CCL2 to overcome calpeptin induced chemotactic inhibition (Fig. 4A) lends evidence to a role of calpain in a signaling pathway. When a known downstream effect, polymerization of actin, of migration is blocked with a pharmacological agent, cytochalasin D, the inhibition and recovery effect observed with the calpain inhibitor is not reproduced, further strengthening the evidence for the specific involvement of a calpain-mediated mechanism in T cell chemotaxis. Chemotaxis was also examined with a combined treatment of calpeptin and cytochalasin D to determine if the compounds produced an additive effect. The combined treatment (Fig. 4C) produced a profile similar to (Fig. 4B) and a non-significant trend toward slightly greater inhibition. This profile provides further evidence that the cytochalasin D acts downstream of calpain in the migration mechanism as the cytochalasin D profile alone (Fig. 4B) is reproduced.

The current investigation has been performed mostly on the Jurkat E6-1 T cells to produce results in a T cell-like cell before expansion of this study into cells directly obtained from MS patients and EAE animals. In order to add more strength to this work and indicate a possibility of transition to a more disease relevant model, we presented a similar role of calpain in chemotaxis in normal human PBMCs (Fig. 3). Although the current work does not provide direct evidence for therapeutic intervention of MS, it does support the expansion of this study into more disease relevant models. The current study also does not address the specific isoforms of calpain that may be involved with cell migration. The two major ubiquitous calpain isoforms, μ -calpain and m-calpain, are often expressed at different levels in various cell types and this may also be the case in the Jurkat T cells that we have studied. The current study identifies a basal level of calpain activity (Fig. 5) and also calpain expression (data not shown) in Jurkat T cells. We attempted to assess changes in calpain levels by Western blotting, which would allow identification of subtypes with specific antibodies. Unfortunately, the changes in calpain levels were not large enough to detect differences between treatment groups. The short time frames in which migration occurred would have resulted from changes in calpain activity rather than changes in protein expression. At present, the lack of inhibitors specific for the calpain subtypes is also a limitation for assessing variations in migration due to protease subtypes. Further studies are ongoing in our laboratory using more disease-relevant cell types and calpain RNAi to assess more specific changes and to narrow down the protease isotypes involved in migration.

Current treatments for MS are moderately effective at best (Kleinschultz et al., 2007) and most treatments eventually lose effectiveness in many patients. Therapies that selectively target a mechanism up-regulated or active during the disease state, such as increased calpain activity in T cells in MS patients (Imam et al., 2007), can provide a direct therapeutic approach and prove to be an effective treatment. Calpain activity contributes to many cellular processes

including apoptosis (Zhivotovsky et al., 1997), cell cycle regulation (Witkowski and Bryl, 2004), T cell activation (Schaecher et al., 2001), and T cell chemotaxis (as shown in this investigation).

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Fig. 1.

Changes in intracellular free $[Ca^{2+}]$ in response to various ionic stimulus, chemokine exposure, and protease inhibition. Changes in intracellular free $[Ca^{2+}]$ were measured with the ratio-metric intracellular fluorescent dye fura-2 loaded into Jurkat E6-1 T cells and read on a fluorescent plate reader after exposure to experimental compounds. **A:** Intracellular free $[Ca^{2+}]$ rises to a steady state in response to increased extracellular $[Ca^{2+}]$. **B:** Ionomycin, a calcium ionophore, effectively transports Ca^{2+} across the plasma membrane and creates an intracellular free $[Ca^{2+}]$ spike in the cell. **C:** The chemokine CCL2 causes an increase in intracellular free $[Ca^{2+}]$ at an optimum concentration of 10 ng/mL. **D:** The calpain inhibitor calpeptin causes an increase of intracellular free $[Ca^{2+}]$ in a concentration-dependent manner. **E:** The calpain inhibitor PD150606 causes an increase of intracellular free $[Ca^{2+}]$ in a

concentration-dependent manner. Each time point represents the mean $\pm$ SEM ($n = 6-8$ /time point).

Fig. 2.

Effect of the calpain inhibitor calpeptin on CCL2-induced chemokinesis and chemotaxis in Jurkat E6-1 T cells. Cells were loaded with the fluorescent dye calcein AM to aid in cell tracking and then loaded into a 96-well Boyden Chamber with various treatments to assess migration status. **A:** CCL2 (50 ng/mL) was placed both above and below the chamber's filter membrane to study the chemokinetic response or was only placed below the filter to study the chemotactic response. Mean $\pm$ SEM ($n = 8$). * $P < 0.05$ vs. unstimulated; † $P < 0.05$ vs. chemokinesis. **B:** Calpain inhibition with any of the concentrations of calpeptin in the absence of CCL2 did not significantly alter the levels of migration. **C:** Inhibition of calpain with calpeptin did not significantly alter the migration of Jurkat T cells in response to chemokinetic stimulation (50

ng/mL CCL2 placed above and below the filter membrane). **D:** Calpeptin caused a concentration-dependent inhibition of migration of cells toward CCL2 (50 ng/mL). Mean $\pm$ SEM ($n = 8$). * $P < 0.05$ vs. vehicle (set as 100% chemotaxis).

Fig. 3. Effect of the calpain inhibitor calpeptin on CCL2-induced chemotaxis in human PBMCs. PBMCs were loaded with the fluorescent dye calcein AM to aid in cell tracking and then loaded into a 96-well Boyden chamber with various treatments to assess migration status. Calpeptin caused a concentration-dependent inhibition of migration of cells toward CCL2 (100 ng/mL). Mean $\pm$ SEM ($n = 3$). * $P < 0.05$ vs. control (set as 100% chemotaxis).

Fig. 4.

Effect of chemoattractant concentration on calpain inhibitor-induced reduction in chemotaxis: a response distinct from the inhibition by cytochalasin D. Chemoattractant was loaded in the lower wells of a 96-well Boyden chamber in increasing concentrations while Jurkat T cells were loaded in the upper chamber. **A:** The chemotaxis of Jurkat T cells in the increasing chemoattractant gradient was compared with chemotaxis in the same gradient when an IC_{50} concentration of calpeptin was added to the cells in the upper wells. Chemotaxis of Jurkat T cells was highest at an optimum CCL2 concentration of 50 ng/mL. Calpeptin-induced reduction in chemotaxis was observed only up to 50 ng/mL CCL2 and the effect was lost at higher CCL2 concentration. Mean $\pm$ SEM ($n = 8$). * $P < 0.05$ between groups for media in upper chamber

vs. calpain inhibitor; † $P < 0.05$ migration inhibition compared with control. **B:** The chemotaxis of Jurkat T cells in the increasing chemoattractant gradient was compared with chemotaxis in the same gradient when the actin polymerization inhibitor cytochalasin D was added to the cells in the upper wells. Cytochalasin D groups were not significantly different from control; however, it could significantly attenuate the CCL2-induced chemotaxis. Mean $\pm$ SEM ($n = 8$). * $P < 0.05$ between groups for media in upper chamber vs. cytochalasin D. **C:** The chemotaxis of Jurkat T cells in the increasing chemoattractant gradient was compared with chemotaxis in the same gradient when cytochalasin D and calpeptin was added to the upper wells. Combined treatment group displayed the same trend as cytochalasin D treatment alone. Mean $\pm$ SEM ($n = 4$). * $P < 0.05$ between groups for media in upper chamber vs. calpeptin and cytochalasin D.

Fig. 5.

Increased intracellular calpain activity in response to chemokine CCL2. Increased calpain activity was imaged in live Jurkat E6-1 T cells based on the increasing fluorescence from the calpain-specific substrate CMAC, *t*-BOC-Leu-Met after exposure to the chemokine CCL2 (50 ng/mL) or the calpain inhibitor calpeptin (100 μ M). **A:** Representative micrographs from a 15-min time lapse exposure measuring fluorescence of 50 μ M CMAC at 370 nm. **B:** Representative images of spot density selections of random cells, repeating for every time point. **C:** The average spot density of 10 individual cells over the time lapse series for each exposure group. The mean of 10 cells was also included on each of the graphs. **D:** The change in average fluorescence density for the mean of each experimental group. Mean $\pm$ SEM ($n = 10$). * $P < 0.05$ between the experimental groups and basal dF/dt; † $P < 0.05$ between the CCL2 group and CCL2 + calpeptin group. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]