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Regulation of Th1/Th17 cytokines and IDO gene expression by inhibition of calpain in PBMCs from MS patients

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Abstract

Multiple Sclerosis (MS) pathology is marked by the massive infiltration of myelin-specific T cells into the central nervous system (CNS). During active disease, pro-inflammatory Th1/Th17 cells predominate over immunoregulatory Th2/Treg cells. Here, we show that calpain inhibition downregulates Th1/Th17 inflammatory cytokines and mRNA in MS patient peripheral blood mononuclear cells (PBMCs) activated with CD3/28 or MBP. Interestingly, calpain inhibition elevated IDO gene expression in MS PBMCs, which was markedly decreased in calpain expressing cells. Functional assay showed that incubation of MS patient PBMCs with calpain inhibitor or recombinant IDO attenuates T cell proliferation. These results suggest that calpain inhibition may attenuate MS pathology and augment the efficacy of standard immunomodulatory agents used to treat this disease.

Keywords

Calpain; Multiple sclerosis; Th17; Peripheral blood mononuclear cells; Indoleamine 2, 3-dioxygenase

1. Introduction

Multiple sclerosis (MS) is an autoimmune, demyelinating disease of the central nervous system (CNS) with clinical symptoms including fatigue, paralysis, and visual dysfunction (Williams et al., 1994). There are 250,000–350,000 people with MS in the United States, and it is the leading cause of neurologic disability in early-to-middle adulthood (Anderson et al., 1992). Increased activity and expression of the Ca²⁺-activated protease, calpain, has been detected in CNS tissue, immune cells of MS patients, and in the corresponding animal model, experimental allergic encephalomyelitis (EAE) (Banik, 1992; Cuzner et al., 1975; Guyton et al., 2005; Hassen et al., 2006; Shields et al., 2000; Shields and Banik, 1998). The

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calpains are a family of at least 15 cysteine proteases that are activated at neutral conditions in the cells by Ca^{2+} (Wu et al., 2007). Both ubiquitous and tissue-specific calpains have been identified which play roles in many molecular processes including cell proliferation and differentiation, T cell activation (Cuzzocrea et al., 2000; Schaecher et al., 2002), immune cell migration (Butler et al., 2009), signal transduction (Kanungo et al., 2009), necrosis (Gill and Perez-Polo, 2009), and apoptosis (Colak et al., 2009; Guyton et al., 2005).

The activation of myelin-specific T cells is thought to be a major event in MS disease development and progression. Production of pro-inflammatory Th1 cytokines (e.g. TNF α , IL-2, and IFN γ) by CD4⁺ T cells is increased in MS patients during an exacerbation (Kivisakk et al., 2003; Lassmann and Ransohoff, 2004). In contrast, anti-inflammatory Th2 cytokines (e.g. IL-4, IL-10, and IL-13) are predominant during disease remission (Issazadeh et al., 1995; Kennedy et al., 1992). We have determined that calpain expression and activity are increased in activated peripheral blood mononuclear cells (PBMCs) during MS disease exacerbations, and inhibition of calpain in these cells attenuates secretion of IL-2 and IFN γ , thus promoting an anti-inflammatory cytokine bias (Imam et al., 2007). Calpain is involved in T cell activation through direct modulation of signaling proteins that lead to cytokine production (Hendry and John, 2004; Schaecher et al., 2004). Also, an indirect modulation of T cell activity by calpain, via cleavage of myelin protein into antigenic peptides that could activate myelin basic protein specific T cells, (Deshpande et al., 1995; Imam et al., 2007; Medveczky et al., 2006; Schaecher et al., 2001) has been observed. An important consequence of T cell activation is the induction of transcription factor nuclear factor kappa-B (NF κ B), which is regulated by its inhibitors, I κ B α and I κ B β (Baldwin, 1996). In turn, pro-inflammatory NF κ B further activates T lymphocytes via production of IL-2 and CD25 (Algarde et al., 1995; Gerondakis et al., 1998; Zuckerman et al., 1998). The cell-permeable calpain inhibitor, calpeptin, inhibits I κ B α degradation in a time and dose-dependent manner, through direct interaction with calpain-cleavage sites on the I κ B α protein (Schaecher et al., 2004). Since Th1/Th2 cytokines differentially regulate chemokine signaling, the effect of calpain activity on migration of immune cells toward inflammatory lesions has been investigated. In the acute Lewis rat model of EAE, calpain expression was correlated with T cell, macrophage, and neutrophil migration into the CNS (Shields and Banik, 1999); (Katsube et al., 2008; Lokuta et al., 2003). These studies implicate the use of calpain as a target for disease modification in MS.

The contribution of IL-17 producing T helper cells (Th17) to autoimmune pathogenesis is a current focus of MS research. A recent study concluded that IL-6 selectively promotes the proliferation of Th17 cells by activating the T cell gp130–STAT3 pathway (Nishihara et al., 2007). Since IL-6 exerts only a minimal effect on Treg development, a drug that effectively blockades the IL-6–gp130–STAT3 pathway in CD4⁺ T cells could inhibit harmful Th17-mediated effects. The development of Th17 cells may depend on the presence of IL-23 during antigen stimulation (Harrington et al., 2005; Park et al., 2005), where IL-23 has a key role in Th17-mediated inflammation *in vivo* (Chen et al., 2006). Moreover, recent data suggest that human Th17 differentiation is under control of TGF β -enhanced responsiveness to IL-23 (Mangan et al., 2006). In MS, increased numbers of PBMCs have been shown to express high levels of IL-17 mRNA, particularly during exacerbations (Matusevicius et al., 1999).

Conversely, dendritic cells (DCs) of MS patients and EAE mice express abnormally low levels of cytoplasmic indoleamine 2,3-dioxygenase (IDO) (Thackray et al., 2008). IDO is a heme-containing enzyme that catalyzes the aerobic metabolism of L-tryptophan to N-formylkynurenine, which is the first and rate-limiting step in the kynurenine pathway. Initiation of the IDO-kynurenine pathway facilitates immune inhibitory function, and transcription of IDO can be induced in DCs by CTLA4-B7 interaction, (Fallarino et al.,

2003) NF κ B activation (Puccetti and Grohmann, 2007), and upregulation of type I and type II interferons (Kahler and Mellor, 2009). IDO-mediated tryptophan depletion in the local microenvironment leads to starvation and stress of Th1 cells, impaired function of bystander Th1 cells, and apoptosis (Fallarino et al., 2006; MacKenzie et al., 2007; Munn and Mellor, 2007; Stone and Darlington, 2002). Thus, a high level of IDO expression may precede a favorable shift in Th1/Th2-mediated immune responses in chronic inflammatory diseases such as MS. We have previously shown that increased calpain activity is correlated with Th1/Th2 cytokine dysregulation in MS patient PBMCs during relapse and remission. In the current study, we examined the effect of calpain inhibition upon expression of Th1/Th17-associated cytokines and their mRNA and protein levels in MS patient PBMCs. We found that calpain inhibition downregulated several inflammatory cytokines (IL-17, IL-23, TNF α , G-CSF, and IL-12) in MS PBMCs while it upregulated IDO expression and limited T cell proliferation, indicating that inhibition of calpain may ameliorate immune pathology in MS.

2. Materials and methods

2.1. Study subjects

Subjects were enrolled according to a protocol approved by the Medical University of South Carolina Institutional Review Board. Informed consent was obtained from all participants. Patients were considered eligible if they were diagnosed with relapsing-remitting MS, determined by the McDonald criteria, (McDonald et al., 2001) and if they were presently taking interferon therapy or not on any treatment. Fourteen RRMS patients were recruited, (10 female, 4 male) all during study visits in which they were not presenting with a disease attack. The mean(SD) patient age was 46(14) years, 69% were Caucasian and 31% were African-American. Control blood was collected from age and sex matched donors with no history of autoimmune disease.

2.2. Isolation and stimulation of PBMCs

Blood samples (20 mL) from MS patients and control individuals were collected. PBMCs were isolated from these blood samples and washed twice in Hanks Balanced Salt Solution (HBSS) as described (Imam et al., 2007). Briefly, anticoagulant-treated whole blood was mixed with equal volumes of HBSS and layered on top of Ficoll-Paque PlusTM, and centrifuged. The upper layer of plasma was carefully drawn off, leaving the lymphocyte layer undisturbed at the interface. This layer was transferred to a centrifuge tube and suspended in 6 ml of HBSS. After centrifugation, the supernatant was removed. The pellet was re-suspended in 6 ml of HBSS and centrifuged once more. PMBCs in the pellet were counted and diluted in RPMI 1640 medium containing 1% penicillin/streptomycin and 10% Fetal Bovine Serum to a concentration of 3×10^6 cells/ml. An equal volume of cells in medium (2×10^6 per well) was distributed into 3 wells of a 6-well plate. To one well, 100 μ M of calpeptin dissolved in DMSO (Sigma) was added. To the same well, 10 μ g/mL of anti-CD3 and 5 μ g/mL of anti-CD28 (Santa Cruz) was immediately added to activate T lymphocytes. To a second well, only 10 μ g/mL of anti-CD3 and 5 μ g/mL anti-CD28 were added. The third well, containing unstimulated and un-treated cells, served as a control. To determine cytokine expression in response to stimulation of myelin basic protein (MBP)-specific T cells, MS patient PBMCs (2×10^6 per well) were distributed into 6-well plates and stimulated with 40 μ g/mL purified whole MBP in the presence or absence of 100 μ M calpeptin. Plates activated with CD3/28 were incubated in a CO₂ incubator at 37°C for 24 hours, and MBP-activated plates were incubated for 3 days. The cells were collected, washed with sterile PBS, and the cell pellet and supernatant were stored at -80°C.

2.3. Multi-analyte ELISArray

The Human Th1/Th2/Th17 Cytokines Multi-Analyte ELISArray™ protocol was followed according to the manufacturer's protocol (SA Biosciences). Briefly, 50 µl of assay buffer was pipetted into each well of the 8-well ELISA strips. Serial dilutions of the antigen standards and experimental samples (50 µl each) were added to the appropriate wells of the ELISA strips then incubated for 2 hours at room temperature. After washing, 100 µl of detection antibody was added for 1 hour followed by washing and a 30-minute incubation with avidin-conjugated horseradish peroxidase. After appropriate washing, 100 µl of substrate solution was added to each well for 15 minutes at room temperature in the dark, and 100 µl of stop solution was added. Absorbance was read at 450 nm in an EL800 ELISA reader (Bio-Tek) within 30 minutes of stopping the reaction.

2.4. Semi-quantitative RT-PCR and Real-Time PCR

Total RNA was extracted from PBMCs using the RNEasy Mini Kit (Qiagen) according to the manufacturer's protocol. The reverse transcription reaction was performed using the iScript cDNA Synthesis Kit (Bio-Rad). Briefly, the following components were combined to form a 20 µL reaction volume: nuclease-free water, 5x iScript Select reaction mix, oligo (dT)₂₀ primer, total RNA (2 µg), and Reverse Transcriptase (RT). The reaction tubes were incubated for 90 minutes at 42°C, then incubated for 5 minutes at 85°C (to inactivate RT). PCR was executed in a programmed thermal cycler (Biometra). β-actin mRNA was used as a control for each experiment using the primer sequence 5'-GACAGGATGCAGAAGGAGATTACT-3' (sense) and 5'-TGATCCACATCTGCTGGAAGGT-3' (anti-sense). Cytokine and IDO primers were used to test for IL-6 5'-GTGTGAAAGCAGCAAAGAGGC-3' (sense) and 5'-CTGGAGGTACTCTAGGTATAC-3' (anti-sense); IL-8 5'-TTGGCAGCCTTCCTGATT-3' (sense) and 5'-AACTTCTCCACAACCTCTG-3' (anti-sense); IL-12p40 5'-GGACCAGAGCAGTGAGGTCTT-3' (sense) and 5'-CTCCTTGTTGTCCCCTCTGA-3' (anti-sense); IL-17 5'-TTAGGCCACATGGTGGACAATCGG-3' (sense) and 5'-ATGACTCCTGGGAAGACCTCATTG-3' (anti-sense); IL-23p19 5'-TGTGGAGATGGCTGTGAC-3' (sense) and 5'-TTGAAGCGGAGAAGGAGA-3' (anti-sense); and IDO 5'-ACTCCATTGACATCATCTGTGG-3' (sense) and 5'-CTCACCAGCAGAATCCAGGAG-3' (anti-sense). Thermal cycling parameters were 95°C for 10 minutes followed by 40 cycles of amplifications at 96°C for 3 seconds, 55°C for 3 seconds, and 68°C for 5 seconds, followed by a final elongation step of 72°C for 10 minutes. PCR products were visualized using Geldoc software following electrophoresis in 1.5% agarose gel with ethidium bromide staining. Quantitative PCR analyses were performed using the QuantiTect SYBR green PCR kit (Qiagen) according to the manufacturer's protocol. We used β-actin with the following primer sequence 5'-GACAGGATGCAGAAGGAGATTACT-3' (sense) and 5'-TGATCCACATCTGCTGGAAGGT-3' (anti-sense) as endogenous control. The primer sequences for IDO were 5'-GATGAAGAAGTGGGCTTTGC-3' (sense) and 5'-CTCTGTGACTTGTGGTCTGT-3' (anti-sense); IL-17A 5'-AGGAATCAATCCACGAA-3' (sense) and 5'-GGAGATTCCAAGGTCGAGGTG-3' (anti-sense); and IL-12p40 5'-CTCCCTGACATTCTGCGTTC-3' (sense) and 5'-CATTTTTGCGGCAGATGAC-3' (anti-sense).

2.5. Calpain inhibition and T cell responses

MS patient PMBCs (5×10^6 cells per ml) were treated with 10 µg/mL of anti-CD3 and 5 µg/mL of anti-CD28 (Santa Cruz), in the presence or absence of calpeptin for 24 hours, and supernatant was collected and stored at -80°C. Healthy donor PMBCs were cultured in duplicate wells of a 96-well plate and treated with anti-CD3/28 (Santa Cruz) in the presence or absence of calpeptin or previously collected MS patient supernatant for 24 hours in a CO₂

incubator at 37°C. For the myelin basic protein (MBP)-specific proliferation studies, MS patient PBMCs (2.5×10^5 per well) were aliquotted into duplicate wells of a 96-well plate and stimulated for 3 days with 40 µg/mL purified whole MBP in the presence or absence of 100 µM calpeptin or 0.5 µg/mL recombinant IDO (R&D Systems). Following incubation, an MTS cell proliferation assay (Promega) was performed following the manufacturer's protocol. Data are represented as % T cell proliferation compared with activated untreated healthy PBMCs $\pm$ standard errors from duplicate wells.

2.6. Statistical analysis

The protein expression of cytokines measured by ELISArray was quantified and presented as the mean optical density (O.D.) $\pm$ the standard error of the mean. Differences in cytokine expression between experimental groups (unactivated, activated, activated with calpeptin) were analyzed for statistical significance ($p \leq 0.05$) using the Kruskal-Wallis rank sums test. Statistical significance for the T cell proliferation assay ($p \leq 0.05$, $p \leq 0.01$) was calculated using the Student's t-test. Real-time PCR data were analyzed using the delta-delta CT (ddCT) method (Fleige et al., 2006).

3. Results

3.1. Calpain inhibition attenuates inflammatory cytokines released from PBMCs of MS patients in remission

We have previously shown that calpain expression and activity is differentially regulated in PBMCs and that calpain inhibition downregulates production of IL-2 and IFN γ in activated PBMCs from relapse and remission patients suffering from MS (Imam et al., 2007). Based on these findings, we examined whether calpain inhibition alters the protein expression of inflammatory cytokines in activated MS patient PBMCs using an ELISArray. Activation of CD4⁺ T cells with CD3/28 antibody significantly augmented the expression of IL-12 ($p=0.010$), IL-17 ($p=0.004$), TNF α ($p=0.004$), and G-CSF ($p=0.037$), but not IL-6 or TGF β 1 (Fig. 1). An increase in TNF α was expected as biologically active TNF α promotes juxtacrine cell death and reactive proliferation of glia. Human G-CSF stimulates the proliferation and differentiation of hematopoietic progenitor cells committed to become granulocytes, in particular, neutrophils. Treatment with calpain inhibitor significantly decreased the expression of IL-17 ($p=0.004$), G-CSF ($p=0.004$), and TNF α ($p=0.010$). Furthermore, the release of IL-12, which is required for Th1 cell development, was significantly reduced by calpeptin ($p=0.006$).

3.2. The effect of calpain inhibition upon IL-17, IL-23, and IL-12p40 mRNA of activated MS PBMCs

Stimulation of PBMCs with anti-CD3/28 may enhance survival of both Th1 and Th2 populations, augmenting cytokine production and overcoming T cell anergy (Alegre et al., 2001; Shibuya et al., 2000). Here, expression of IL-17 mRNA was increased upon stimulation of MS PBMCs with CD3/28 or MBP, although IL-17 mRNA was not increased in activated control PBMCs, (Fig. 2A) thus reinforcing the contribution of IL-17 to MS pathology. We have previously shown that calpain expression and activity is upregulated in anti-CD3/28 activated healthy and MS patient PBMCs (Imam et al., 2007). Calpain inhibition by treatment with calpeptin (100 µM) prior to anti-CD3/28 or MBP stimulation downregulated IL-17 gene expression in MS patient cells as compared with the activation only group (Fig. 2A). Data obtained showed that the anti-CD3/28 activation of PBMCs increased IL-23 levels in the MS remission group of PBMCs and in control cells, (Fig. 2B) although activation with MBP did not produce an observable change in IL-23 transcription. Calpeptin-treated PBMCs stimulated with CD3/28 exhibited a decrease in the levels of IL-23 mRNA as compared to activated non-treated cells, regardless of disease state (Fig.

2B). The IL-12p40 subunit associates with the p19 subunit of IL-23 within the same cell to form biologically active IL-23 (Oppmann et al., 2000), and with IL-12p35 to form active IL-12 (Sospedra and Martin, 2005). Activation of PBMCs with anti-CD3/28 increased IL-12p40 mRNA expression both in control subject and in remission patient cells, and MBP stimulation of MS cells produced the same effect. Compared with activated cells, calpain inhibition reduced IL-12p40 mRNA expression in healthy subjects and in remission patients (Fig. 2C). In accord with the protein expression and RT-PCR results, Real-time PCR analysis confirmed that IL-17 and IL-12 gene expression was increased in activated cells by 5.8-fold and 1.2-fold respectively (Supplemental Table 1). Moreover, activated cells treated with calpeptin exhibited a 1.3-fold decrease in IL-17 expression and a 2.1-fold decrease in IL-12 gene expression.

3.3. The effect of calpain inhibition on IL-6 and IL-8 mRNA levels in activated MS PBMCs

Studies suggest that elevated IL-6 levels are directly injurious to oligodendrocytes that help produce the protective myelin sheath coating around nerve cells. Thus, the inflammatory cascades in MS could be lowered by downregulating the elevation of IL-6. Here, IL-6 gene expression was elevated upon activation of MS patient PBMCs (Fig. 3A). IL-6 mRNA expression was also elevated upon activation of PBMCs from healthy subjects. Calpain inhibition by calpeptin reduced IL-6 mRNA levels to an extent in patient PBMCs and in healthy PBMCs. IL-17 causes neutrophil recruitment primarily through the release of IL-8, a CXC chemokine for neutrophils, and induces neutrophil activation (Laan et al., 1999; Witowski et al., 2004). Although we observed an increase in IL-17 mRNA expression anti-CD3/28 stimulation produced virtually no effect on IL-8 mRNA expression of patient PBMCs, nor was it increased in activated healthy PBMCs (Fig. 3B). Furthermore, calpain inhibition did not produce a decrease in IL-8 gene expression in healthy cells and had no effect on IL-8 expression in MS remission cells. These data suggest that calpain is not involved in IL-8 chemokine gene expression.

3.4. Calpain inhibition upregulates IDO mRNA levels in activated MS PBMCs

Compared with unactivated MS PBMCs, treatment with anti-CD3/28 or MBP produced a variable change in the level of IDO mRNA (Fig. 4A–B). However, in every set of patient PBMCs tested, cells pre-treated with calpain inhibitor expressed a much higher level of IDO mRNA vs. the unstimulated or stimulated untreated group. Real-time analysis confirmed that IDO gene expression was decreased by 11-fold in activated cells and calpeptin-treated cells exhibited a 7.8-fold increase in IDO gene expression vs. activated (Supplemental Table 1). Interestingly, in healthy PBMCs, no upregulation of IDO was observed with calpeptin treatment (Fig. 4A). Furthermore, while IDO mRNA levels in unactivated and activated healthy PBMCs were much higher than those of MS patient cells, calpeptin-treated patient cells expressed IDO levels that did not differ from levels in healthy PBMCs.

3.5. Calpeptin treated MS patient supernatant suppresses T cell proliferation

To examine if increased IDO production in calpeptin-treated patient samples has an inhibitory role in T cell proliferation, we treated activated healthy patient PBMCs with calpeptin or supernatant from activated MS patient PBMCs co-incubated with calpeptin. Following incubation, an MTS cell proliferation assay was performed to quantify T cell proliferation. While anti-CD3/28 did activate healthy PBMCs, treatment with calpeptin significantly reduced ($p < 0.05$) T cell activation (Fig. 5A). Furthermore, when cells were incubated with conditioned medium from MS patient cells previously treated with calpeptin, T cell proliferation was significantly reduced ($p < 0.05$) to levels similar to calpeptin treatment alone (Fig. 5A). Taken together with the effects of calpeptin on IDO mRNA expression, these data suggest that calpeptin treatment may produce inhibitory factors, such

as IDO, that could suppress T cell activation, alleviating disease severity. Next, whole MBP was used to stimulate patient PBMCs in order to further mimic *in vivo* conditions of autoimmune T cell proliferation (Figure 5B–C). In response to incubation with calpeptin or recombinant IDO, the proliferation of T cells was significantly attenuated.

4. Discussion

The purpose of this study was to determine whether patterns of Th1 and Th17 cytokine expression in PBMCs of MS remission patients are altered by administering calpain inhibitor prior to activation. We previously found that higher levels of calpain are expressed in both unactivated and activated PBMCs from MS patients during relapse or remission, compared to controls, and no significant increase was noted in relapse as compared to remission patients. These data imply a disruption in normal calpain expression and activity in MS patients. We have demonstrated calpain-mediated activation and chemotaxis of T cells, and together with data that calpain inhibition can suppress production of IL-2 and IFN γ from CD4⁺ T cells, we propose a crucial role of calpain in propagating demyelinating disease (Butler et al., 2009; Imam et al., 2007; Schaecher et al., 2002; Shields and Banik, 1998; Shields and Banik, 1999).

In this study, compared with activated cells, calpeptin significantly decreased IL-12 release from patient PBMCs stimulated with CD3/28 or MBP, and downregulated transcription of IL-23 upon treatment with CD3/28. IL-12 activity augments CNS inflammation and glial activation via production of IFN γ and NO, and the IL-12p40 subunit interacts with IL-23p19 to form biologically active IL-23 (Brahmachari and Pahan, 2008). IL-23 is expressed by monocyte-derived dendritic cells, and CD4⁺ T cells activated with IL-23 produce IL-17 (Aggarwal et al., 2003; Kolls and Linden, 2004; Langrish et al., 2004). Studies have shown that EAE severity is greatly reduced on treatment with a monoclonal antibody to IL-17 and IL-23 (Langrish et al., 2005). Similarly, EAE IL-17 knockout mice exhibited a heavily attenuated form of the disease (Komiyama et al., 2006). In our study, it was observed that anti-CD3/28 or MBP activation of MS patient PBMCs significantly increased IL-17 protein and mRNA expression and calpain inhibition reduced both in remission patient cells. This agrees with reports that IL-17 mRNA levels are augmented in CD4⁺/CD8⁺ T cells from blood derived from both MS patients and mice with EAE (Lock et al., 2002; Matusevicius et al., 1999; Zhang et al., 2003). The attenuation of IL-17 transcription and translation may have resulted from lower levels of IL-23 and IL-12 activity, or a more direct interference of calpeptin with expression of IL-17, via known calpain interactions with STAT3 (Oda et al., 2002) or other transcription factors.

In remission patient PBMCs, TNF α secretion was significantly increased upon CD3/28 activation and was reduced with calpain inhibition. TNF α mediates cell destruction by direct cell-to-cell contacts and promotes the proliferation of astroglial cells and microglial cells, thus potentially contributing to astrogliosis and demyelination. Moreover, TNF α enhances the proliferation of T-cells induced by various stimuli in the absence of IL-2, and some subpopulations of T-cells only respond to IL-2 in the presence of TNF α . Calpeptin blocks secretion of both cytokines (Imam et al., 2007), and is thus an ideal inhibitor to prevent proliferation of CD4⁺ T cells. Human G-CSF was investigated as a biological marker for acute, neutrophil-predominant inflammation, as a positive correlation exists between inflammation and the degree of axonal transection in MS lesions (Bitsch et al., 2000; Ferguson et al., 1997; Trapp et al., 1998). Activation of CD4⁺ T cells with CD3/28 significantly increased G-CSF secretion and pre-treatment with calpeptin maintained G-CSF at control levels, and thus may allay exacerbation of MS symptoms.

Taken together with data that calpain inhibition attenuates pro-inflammatory cytokine expression, our finding of increased IDO transcription in calpeptin-treated MS patient PBMCs supports the theory that a high level of IDO expression may precede a favorable shift from Th1/Th17 to Th2-mediated immune responses in chronic inflammatory disease. Of interest, the increase in IDO gene expression upon calpeptin treatment appeared to be selective for MS patient cells, as this change was not detected in healthy control PBMCs (Figure 4). Moreover, our results indicate that IDO can directly suppress MBP-specific T cell proliferation. The role of IDO in promoting Th2 type cellular immune responses is supported by data from an EAE model induced by adoptive transfer of MBP-specific T cells, in which both IDO mRNA expression and the kynurenine-to-tryptophan ratio, which reflect IDO activity, are increased during the remission phase (Sakurai et al., 2002). We are currently investigating mechanisms by which calpain inhibition is upregulating IDO transcription, which may be via interactions with inducers such as NF κ B and the type I and II interferons. Due to our deduction that calpeptin is promoting an anti-proliferative cytokine milieu in patient PBMCs, we quantified proliferation of activated T cells in the presence or absence of supernatant from PBMCs that were previously incubated with calpeptin for 24 hours. Incubation of cells in conditioned medium exhibited a strong anti-proliferative effect that was comparable to direct treatment with calpeptin, supporting the hypothesis that calpeptin may produce indirect immunosuppressive effects on activated immune cells *in vitro*.

A limitation of the study is that some variation in cytokine mRNA expression was observed across the patient or control samples. This is likely due to the limited number of subjects enrolled in the study, and the fact that these patients likely represent a broad spectrum of disease severity. In future studies we will begin to use recent MRI reports in order to further stratify patients by disease severity for analysis.

This study demonstrated that calpain inhibition reduces inflammatory cytokines including IL-12, IL-17, TNF α , and G-CSF in MS patient PBMCs. Investigation of the effect of calpeptin on cytokine gene expression illustrated that calpain inhibition differentially regulates IL-6, IL-8, IL-17, IL-23 and IL-12p40 cytokine gene expression in activated MS PBMCs. These results point to an ability of calpeptin to reduce the pro-inflammatory cytokine profile of MS patient PBMCs, a feature that could reduce disease severity and prolong patient remission time. Calpeptin treatment also upregulated IDO mRNA levels in activated MS PBMCs, an enzyme that can lead to starvation and stress of Th1 cells, impaired function of bystander Th1 cells, and immune cell apoptosis. Reductions in Th1 immune function through increased IDO production could further attenuate inflammatory cytokines, chemokines, and alleviate neutrophil migration, hallmarks of MS development and progression. Functional analysis confirmed the ability of calpeptin to suppress MBP-specific T cell proliferation. We also observed that treatment of MBP-stimulated PBMCs with exogenous IDO suppresses T cell proliferation. Whether the attenuation by calpeptin was a direct or indirect result of increased IDO production is currently being studied in our laboratory. Finally, calpeptin was administered to cells of patients already controlled on interferon beta therapy. Calpeptin may play a role in supplementing the immunomodulatory effect of interferon beta treatment, especially in patients who demonstrate decreased therapeutic responsiveness. (Boz et al., 2007; Runkel et al., 2001). Based on the efficacy of calpain inhibitors to prevent neuronal cell death and axon degeneration (Czeiter et al., 2009; Guyton et al., 2009), future studies will utilize combination therapy with calpeptin and interferon beta as an approach to not only attenuate inflammation but control or delay the neurodegenerative process. In conclusion, these data support the targeting of calpain in the treatment of MS, and provide new insight into the potential of calpain inhibition to alleviate the inflammatory responses associated with progression of the disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Calpain inhibition alters expression of Th17 and Th1 cytokines released from MS PBMCs. PBMCs from MS patients in remission (n=6) were unactivated, activated for 24 hours with anti-CD3/28, or activated after pre-treatment with 100μM calpeptin. Supernatants were immediately collected for analysis of cytokine levels using an ELISArray kit. Cytokine levels are presented as the mean O.D. value of each treatment group. *p<0.05 unactivated vs. activated; #p<0.05 activated vs. activated + calpeptin.

Fig. 2.

Effect of calpain inhibition on mRNA levels of Th17-associated cytokines. PBMCs from MS patients in remission (n=6) or controls (n=4) were unactivated, activated for 24 hours with anti-CD3/28, or activated after pre-treatment with 100μM calpeptin. Alternately, PBMCs from MS patients in remission (n=3) were incubated for 3 days with purified whole MBP (40μg/mL) in the presence or absence of 100μM calpeptin. Representative RT-PCR product using IL-17 (A), IL-23 (B), or IL-12p40 (C) primer with β-actin control.

Fig. 3.

Effect of calpain inhibition on mRNA levels of IL-6 (**A**) and IL-8 (**B**). PBMCs from MS patients in remission (n=6) or controls (n=4) were unactivated, activated for 24 hours with anti- CD3/28, or activated after pre-treatment with 100 μ M calpeptin. Representative RT-PCR product using IL-6 and IL-8 primers with β -actin control.

Fig. 4.

Effect of calpain inhibition on transcription of indoleamine-2,3 dioxygenase (IDO). (A) PBMCs from MS patients in remission (n=3) or healthy controls (n=3) were unactivated, activated for 24 hours with anti-CD3/28, or activated after pre-treatment with 100μM calpeptin. (B) PBMCs from MS patients in remission (n=2) were incubated for 3 days with purified whole MBP (40μg/mL) in the presence or absence of 100μM calpeptin. Representative RT-PCR product using IDO primer with β-actin control.

Fig. 5. (A) Healthy donor PMBCs were treated with anti-CD3/28 in the presence or absence of 100 μ M calpeptin, or with previously collected MS patient conditioned medium (n=2) treated with calpeptin. Following incubation for 24 hours in a CO₂ incubator (37°C), an MTS cell proliferation assay was performed following the manufacturer's protocol. (B–C) MS patient PBMCs (n=2) were stimulated in the presence of 40mg/mL purified whole MBP for 3 days with or without 100 μ M calpeptin (B) or 0.5mg/mL recombinant indoleamine 2,3-dioxygenase (C). Data are represented as % T cell proliferation compared with activated untreated cells $\pm$ standard errors from duplicate wells (*p $\leq$ 0.05, **p $\leq$ 0.01).