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Calpastatin prevents NF-κB mediated hyperactivation of macrophages and attenuates colitis

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Abstract

Calpain enzymes proteolytically modulate cellular function and have been implicated in inflammatory diseases. In this study, we found that calpain levels did not differ between intestinal tissues from inflammatory bowel disease (IBD) patients and healthy controls, but IBD tissues showed increased levels of the endogenous calpain inhibitor, calpastatin (CAST). To investigate the role of CAST in the immune system during IBD, mice were x-ray irradiated and reconstituted with either CAST knockout (KO) or wild-type (WT) bone marrow and subjected to dextran sulfate sodium (DSS)-induced colitis. CAST KO recipients with induced colitis exhibited more severe weight loss, bloody diarrhea, and anemia compared to WT controls. Histological evaluation of colons from KO recipients with colitis revealed increased inflammatory pathology. Macrophages purified from the colons of KO recipients had higher IL-6, TNFα, and IFNγ mRNA levels compared to WT controls. Mechanistic investigations using siRNA and KO bone marrow to generate CAST deficient macrophages showed that CAST deficiency during activation with bacterial PAMPs including heat-killed *E. faecalis* or CpG DNA led to increased IκB cleavage, NF-κB nuclear localization, and IL-6 and TNFα secretion. Thus, CAST plays a central role in regulating macrophage activation and limiting pathology during inflammatory disorders like IBD.

Introduction

Inflammatory bowel diseases (IBD) are comprised of the relapsing, remitting inflammatory disorders Crohn's disease and ulcerative colitis. The specific tissues of the gut affected by these two disorders are different, but both types of IBD share symptoms indicative of

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[3]Abbreviations used: bone marrow-derived macrophages (BMDM), calcium (Ca^{2+}), calpastatin (CAST), dextran sulfate sodium (DSS), hemoglobin (Hb), human monocyte-derived macrophages (HMDM), inflammatory bowel disease (IBD), knockout (KO), wild-type (WT)

chronic intestinal inflammation (1). Recent progress has been made in understanding the onset or progression of IBD pathology, particularly how interactions between the mucosal immune system and the microbiota drive the initiation and progression of the diseases (2). However, more information is needed regarding cellular factors in immune cells that regulate disease susceptibility or pathology in IBD patients (3, 4). Identification of specific factors that function to limit hyperactivation of intestinal immune cells is a crucial step toward understanding and treating these and other chronic inflammatory disorders.

Emerging evidence suggests that some inflammatory conditions may be driven by the actions of calpains, which are cytosolic Ca^{2+}-activated cysteine proteases that cleave specific targets to modulate cellular functions (5-8). There are two major isoforms of this enzyme, μ-calpain (or calpain 1) and m-calpain (or calpain 2), which require μM and mM Ca^{2+} concentrations for activity, respectively (9). Calpain enzymes are comprised of an 80 kDa catalytic subunit and a 30 kDa regulatory subunit. Activation occurs after Ca^{2+}-binding induces conformational changes that lead to autocleavage of the N-terminal inhibitory domain of the 80 kDa subunit (10, 11). Following activation by Ca^{2+}, calpain cleaves a specific subset of cellular proteins, including cytoskeletal proteins, membrane receptors, calmodulin binding proteins, G proteins, protein kinase C and other signal transduction), and certain transcription factors (12).

Because the Ca^{2+}-induced activation of calpain is an irreversible reaction, an additional mechanism has evolved to modulate its activity involving calpastatin (CAST), which is an endogenous inhibitor that binds strongly to calpains. CAST contains four tandem repeats of a calpain inhibitory domain and each CAST molecule is capable of inhibiting more than one calpain molecule (13). We have recently reported that Toll-like receptor (TLR)-stimulation of macrophages leads to the upregulation of CAST, an effect that was not observed in other immune cells (14). This is important in the context of IBD given that the gut mucosa represents the most extensive interface between the body and the external environment and it contains the largest population of resident macrophages (15, 16). Inflamed tissues from IBD patients exhibit increased recruitment of blood-derived $CD14^+$ monocytes into the lesions and an upregulation of $CD40^+$ and $CD80^+$ activated macrophages, particularly adjacent to the epithelium (17-19).

A recent study in mice suggested that a pharmacological inhibitor of calpains may be useful in treating IBD (20), and the calpain/CAST system may represent an effective target for modulating inflammatory diseases (21). Understanding the role of the endogenous inhibitor, CAST, in regulating the inflammatory processes that drive IBD may facilitate a more effective use of pharmacological calpain inhibitors. Also, there is a need to better understand whether CAST deficiency or an impairment in its function may predispose individuals to increased susceptibility or severity of IBD. We used a mouse bone marrow transplant model to investigate the role of CAST in the immune system during colitis and found CAST deficiency in immune cells increased the development and severity of colitis. CAST was highly upregulated by intestinal macrophages during colitis, and in vitro experiments demonstrated a key role for this inhibitor of calpain activity in preventing hyperactivation of macrophages upon exposure to commensal bacteria or bacterial CpG DNA. These results present novel insight into how impaired CAST expression may lead to heightened susceptibility to IBD or increase disease severity from chronic inflammatory disorders in general.

Materials and Methods

Human Tissues and Cells

De-identified human samples were purchased from BioServe Biotechnologies (Beltsville, MD) and Bio-Options, Inc. (Brea, CA). Also, human biopsy tissues were generously provided by Dr. William Faubion (Mayo Clinic, Rochester, MN) and Dr. Zhenyou Jiang (Jinan University Medical School, Guangzhou, China). For Crohn's disease and ulcerative colitis tissues, diagnoses were confirmed for each patient sample and normal tissues from equivalent intestinal regions from healthy donors were used as controls. Blood for human monocyte-derived macrophages (HMDM) was obtained from healthy volunteers under a separately approved protocol. Monocytes were isolated using Histopaque-1077 (Sigma-Aldrich, St. Louis, MO) and cultured in X-Vivo-10 culture media (BioWhittaker; Walkersville, MD) containing 10% autologous serum. Media was replaced with fresh media on day 3 of culture and fully differentiated human macrophages used for experiments on day 6 of culture. All human samples were provided in a de-identified manner under IRB-approved human protocols (CHS#19345). Blood samples for the culture of human macrophages were obtained from healthy donors under a separately approved protocol (CHS#19442).

Bone marrow transplants and colitis model

The CAST knockout (KO) mice with more than ten generations of back-crossing onto a C57BL/6 background have been previously described (22). A colony generated from C57BL/6J mice (Jackson Laboratories) were used as wild-type (WT) controls. The CAST KO mice express a smaller CAST protein generated from the targeted allele generated by exon 6-skipping, which results in defective CAST activity and a lighter, more rapidly migrating band in western blots. Male 5-wk-old C57BL/6J mice were used as bone marrow transplant recipients. Bone marrow transplantation was performed as previously described (23, 24). Briefly, bone marrow cells (2×10^6 in PBS) from donor mice were administered to recipient mice by intravenous tail vein injection 24 h after an ablative dose of whole-body x-ray irradiation (10.5 Gy). Mice were allowed to recover for 4 weeks, bone marrow reconstitution of the immune system confirmed (Supplemental Fig. 1), and then were subjected to an established model of chronic, relapsing and remitting colitis (25). For this model, mice were given 1.0% dextran sulfate sodium (DSS; CAS#9011-18-1; MP Biomedicals, Santa Ana, CA) in drinking water alternated weekly with regular drinking water for 7 d, for total of 35 d and weight loss typically peaks around day 10. Control mice received normal drinking water throughout the 35 d period. Importantly, the effect of irradiation on intestinal macrophages was assessed by irradiating Ly5.2 mice (C57BL/6) followed by transplantation with Ly5.1 congenic bone marrow and transplantation with Ly5.2 bone marrow as a control. Flow cytometric analyses demonstrated that macrophages in both the colons and spleens of mice receiving Ly5.1 bone marrow were 90-95% derived from the donor marrow, with small contributions (5-10%) from recipient sources (data not shown). All animal protocols were approved by the University of Hawaii Institutional Animal Care and Use Committee.

Assessment of colitis in mice

Weights of mice were monitored over the 35 d period to assess effectiveness of DSS. To evaluate blood in the feces of the mice, cecums were excised and homogenized in water to extract hemoglobin (Hb), and Hb quantified using ELISA (ICL, Inc., Portland, OR). The colons were removed, and homogenized into single cell suspensions using a GentleMacs (Miltenyi), of which 10^6 cells were removed and analyzed for different immune cell markers by flow cytometry as previously described (26).

Histology

Colon tissue embedded in optimal cutting temperature (O.C.T.) was cut into 5 μm sections using a Leica EG1160 microtome and adhered to glass slides. Upon thawing, tissue sections were covered in 100 μL PBS and treated with 1 μg anti-CD16/32 (BioLegend, San Diego, CA) to block Fcγ receptors for 30 min on ice. Anti-CD68 (BioLegend) was then added (1:200 final dilution) for 1 h, followed by three washes with PBS. Cy3-anti-mouse IgG (1:500 final dilution) was then added for 1 h, followed by three washes with PBS. The tissue was then fixed with 2% paraformaldehyde for 15 min, followed by 3 washes with PBS. Permeabilization was carried out by adding 0.03 % Triton X-100 for 5 min, the tissue washed with PBS, and non-specific sites blocked with 5% normal goat serum for 1 h. Anti-CAST (1:50 final dilution; Santa Cruz Biotechnology, Santa Cruz, CA) was added for 1 h at room temperature, followed by 3 washes with PBS. Alexa488-anti-rabbit IgG (Life Technologies-Molecular Probes) was then added (1:500 final dilution) for 1 h at room temperature, followed by three washes with PBS. The tissue was mounted in DAPI-containing media (Vector Labs, Burlingame, CA) covered with a glass coverslip and sealed. Sections were examined and images captured using a Zeiss inverted confocal microscope with LSM5 dual lasers and camera. For these experiments, isotype controls were used for each primary antibody to determine the specificity of staining for each antigen. In separate experiments, colon tissues were washed, cut open on Kim-wipes™ (Kimberly-Clark) and fixed in 10% buffered formalin. Standard H&E staining of paraffin-embedded tissue samples was conducted as previously described (27). Five H&E stained sections from each mouse were blindly scored as previously described (28). Images captured using a Zeiss Axioskop 2 Plus upright light microscope and camera.

Isolation of Macrophages from Mouse Colons and Realtime PCR

Colons were rinsed with PBS and incubated in DMEM + 5% FBS containing 1 mM DTT for 30 min at room temperature to remove mucus, then the tissue was digested in DMEM media with 5% FBS, 2 mg/mL collagenase IV (Sigma-Aldrich), and 30 μg/mL DNase I (Roche Applied Sciences, Indianapolis, IN) for 2 h at 37ºC on a shaking incubator. The digested tissue was then filtered through 70 μm cell separators, cells from 2 mice within each group were pooled and washed with DMEM with 5% FBS and counted using a Sceptor (Millipore-GE Healthcare, Billerica, MA). Cells were then incubated with magnetic beads in the ratio of 5×10^6 cells per 25 μL of Dynabeads (Life Technologies-Invitrogen) pre-coupled with 5 μg anti-CD68 (BioLegend). These cell and bead mixtures were incubated in eppendorf tubes at 4ºC for 30 min and tubes added to a magnetic stand (Life Technologies/Invitrogen) for 2 min. The non-bound cells were removed, then magnetically bound cells washed with complete media, and the process repeated two more times and tubes removed from magnetic stand and resuspended in PBS. Cells were enumerated using a Scepter 2.0 cell counter (Millipore). Purity was confirmed using flow cytometric detection of CD68 using PE-anti-C68 (BioLegend), PE-anti-F4/80 and PE-anti-CD11b (Ebioscience, San Diego, CA) using a FACScaliber flow cytometer (BD Biosciences, San Diego, CA). Total RNA extraction from purified CD68-positive cell pellets was performed using RNeasy (Qiagen), cDNA synthesized using a High Capacity cDNA Synthesis Kit (ABI), and real-time PCR used to evaluate levels of inflammatory mRNA using protocols previously described (29).

Flow cytometry

Single cell suspensions of colons and spleens were prepared using a GentleMacs cell dissociator (Miltenyi, Inc.). Cells (5×10^6) were stained with recommended dilutions of fluorochrome-conjugated antibodies in 100 μL FACS Buffer (PBS with 2% FBS) after pre-incubation with FcBlock (BD Pharmingen). Antibodies included PE-anti-CD45.1, PE-CD45.2, APC-conjugated anti-CD4, anti-CD8, anti-CD11b, anti-Gr-1 (all from Ebioscience, Inc.). For intracellular staining, cells were permeabilized using Ebioscience

Permeabilization Buffer and intracellular CAST detected with PE-anti-CAST or PE-conjugated isotype IgG as a control (Bioss, Inc.). Cells were evaluated on a FACScaliber flow cytometer (BD Biosciences). All flow data were analyzed using FlowJo software.

Mouse macrophages

Preparation of bone marrow-derived macrophages (BMDM) was performed as previously described (14). In brief, bone marrow was flushed from femurs and tibiae with HBSS using a syringe with a 25-gauge needle. Cells were released from clumps by drawing the suspension through a syringe with an 18-gauge needle and cell suspensions were then passed through a 70 μm pore cell strainer (BD Falcon, San Diego, CA) to remove tissue debris. The cells were plated in DMEM containing 10% FBS, 1% penicillin-streptomycin-L-glutamine (Life Techologies-GIBCO, Grand Island, NY), and 10% L929 conditioned media. On day 5 the cells were removed with Cellstripper™ solution (Mediatech, Inc., Manassas, VA), replated with fresh media in plates needed for each experiment, which were conducted on day 6 of culture.

Stimulation of macrophages, western blots, and calpain activity assays

BMDM or HMDM were plated at a density of 2×10^6 cells per well in 6-well plates in 2 mL of DMEM with 5% FBS overnight at 37ºC and 5% CO_2. In some cases, siRNA for CAST or non-targeting control siRNA (Santa Cruz Biological) were transfected using a Neon electroporator (Invitrogen). The next day the cells were stimulated for 20 h with LPS (0111:B4; 100 ng/mL; Sigma), or BioLegend cytokines including TNFα (20 ng/mL), IFNγ (100 ng/mL), IL-17A (5 ng/mL), IL-10 (10 ng/mL), and IL-4 (10 ng/mL). Other reagents used for stimulation included CpG (5 ng/mL, TriLink Biotechnologies) and heat-killed *Entercoccus faecalis* (1 ug/mL; ATCC). Cell pellets were then harvested and lysed in 10 mM Tris pH 7.5, 1% Triton X-100, 5 mM EDTA, 1X proteinase inhibitors (Calbiochem-GE Healthcare), and 5 mM NaCl using a probe sonicator. In some cases, nuclear lysates were separated from cytosolic lysates using the NF-κB Activation Assay Kit (FIVEphoton Biochemicals, San Diego, CA). Protein concentration in the lysates was measured by Bradford assay reagent (Bio-Rad) and 15 μg total protein was combined with reduced Laemeli buffer, boiled at 95ºC for 10 min, cooled on ice, and loaded into wells of 10 - 14.5% polyacrylamide gels (Bio-Rad). After gel electrophoresis, protein was transferred to PVDF membranes, which were blocked for 1 h with low fluorescence blocking agent (Li-Cor) and incubated with primary antibodies including anti-CAST, anti-IκB (detects both IκBα and κBβ), anti-NF-κB (all from Cell Signaling, Danvers. MA), anti-μ-calpain and anti-m-calpain (Millipore), or anti-β-actin (Sigma). After washing with PBS, membranes were incubated with secondary antibodies from Li-Cor for 1 h, membranes washed with PBS, and signals detected with densitometry conducted using the Li-Cor Odyssey imaging system. Calpain activity was measured using a Calpain activity assay kit (BioVision, Inc., Milpitas, CA). This fluorometric assay is based on the detection of cleavage of calpain substrate Ac-LLY-AFC and each sample was measured for fluorescence in triplicate using a SpectraMax M3 fluorometer (Molecular Devices, Inc.).

Statistical analyses

Comparison of means was carried out using an unpaired Student's t test using GraphPad Prism version 4.0 (GraphPad, La Jolla, CA). Standard curves and regression analyses were also conducted using GraphPad Prism version 4.0. All comparisons were considered significant at P < 0.05.

Results

CAST is increased at chronic stages of IBD, while calpain levels remain constant

While emerging evidence suggests that calpain cleavage may be involved in regulating inflammation (30), little is known regarding how levels of calpains or their inhibitor, CAST, may be altered in inflamed versus healthy intestinal tissues. Based on the conventional notion that calpain activity promotes inflammation, we expected higher levels in inflamed intestinal tissues from human IBD patients. However, analyses of human tissues revealed that calpain activity was decreased in tissues from both ulcerative colitis and Crohn's disease patients compared to healthy controls (Fig. 1A). This decreased calpain activity coincided with increased CAST, but not with any changes in levels of μ- or m-calpain (Fig. 1B-E).

The relationship between IBD and the calpain/CAST system was further investigated using an established mouse model of colitis in which mice were given 1.0% DSS-treated water alternated weekly with normal drinking water for 35 days to induce a remitting, relapsing colitis (25). Induction of colitis was confirmed in mice receiving DSS-treated/regular water compared to control mice that only received regular water by evaluating weight loss, macroscopic and microscopic analyses of colon tissues (data not shown). Calpain activity was increased at early stages of acute colitis, but was decreased by day 10 and remained lower than healthy tissues at the chronic stage (day 35) (Fig. 2A). In this sense, the chronically inflamed intestinal tissues exhibited lower calpain activity compared to healthy controls similar to human IBD tissues. Also similar to human IBD, CAST was increased in chronic colitis tissues while calpain levels did not differ between colitis mice and healthy controls (Fig. 2B-C). Thus, decreased calpain activity during chronic colitis correlates with increases in the endogenous inhibitor, CAST, while no changes in μ- or m-calpain levels are found.

CAST deficiency in the whole animal or only in immune cells leads to more severe colitis

To investigate the role of CAST in the immune system during IBD, we performed DSS-induced colitis experiments using two different models. The first model involved wild-type (WT) and whole animal CAST knockout (KO) mice and the second model focused on CAST-deficiency only in the immune system. For the latter model, irradiated C57BL/6 mice were transplanted with bone marrow from WT (WT→WT) or CAST KO mice (KO→WT). After 4 wk of recovery, blood samples were evaluated to confirm equivalent immune system reconstitution in WT→WT and KO→WT mice as well as effective KO of CAST in immune cells (Supplemental Fig. 1), and the mice were then subjected to the DSS-induced model of chronic colitis.

With the first model, inducing colitis in the mice with CAST-deficiency in all tissues resulted in one death (at day 21) and the remaining KO mice exhibited more severe weight loss compared to WT mice with colitis (Fig. 3A). Similar results were obtained using the second model in which two deaths (days 21 and 24) occurred in the KO→WT mice subjected to colitis, and the surviving KO→WT mice with colitis had more severe weight loss compared to WT→WT mice with colitis. CAST deficiency in the immune system alone or in the whole animal were similar in producing more severe bloody diarrhea (Fig. 3B) and inflammatory pathology in colon tissue compared to controls (Fig. 3C-D). Histological evaluation revealed that CAST deficiency in the immune system alone or in the whole animal led to more abundant hallmarks of inflammation such as disrupted epithelial crypts and cellular infiltration and, in some cases, serosal ulceration (Supplemental Fig. 2). Both CAST deficiency in the immune system and whole animal resulted in increased calpain activity during colitis, whereas WT→WT and WT controls both showed lower calpain activity in colitis tissues consistent with results described above. Importantly, KO and

KO→WT mice given normal drinking water showed no signs of illness or pathology, suggesting CAST deficiency in the immune system is not sufficient for triggering the inflammatory pathology or symptoms of colitis. Overall, CAST deficiency only in the immune system was similar to CAST deficiency throughout all tissues in causing more severe colitis compared to controls.

CAST is required for limiting intestinal macrophage activation during colitis

To evaluate macrophage numbers in the colons of bone marrow chimeric mice, we used anti-CD68-coated magnetic beads to purify macrophages from the colons of colitis mice from the WT→WT and KO→WT groups and enumerated the numbers of macrophages per colon. The purity of the magnetically separated macrophages was confirmed with three surface markers including CD11b, CD68, and F4/80 (Fig. 4A). Both WT→WT and KO→WT mice with colitis had a greater than 3-fold increase in macrophage numbers in the colons compared to respective healthy controls (Fig. 4B). There were no differences in macrophages numbers between WT→WT and KO→WT mice with colitis, which suggested that CAST deficiency in the immune system did not affect the number of infiltrating macrophages. Despite similar numbers, there remained the possibility that CAST deficiency may influence the inflammatory state of these macrophages. To investigate this issue, we analyzed mRNA levels in purified macrophages from the colons of WT→WT and KO→WT mice with colitis for different inflammatory factors. While levels of mRNAs for several cytokines as well as iNOS did not differ between WT and KO macrophages (Fig. 4C), three inflammatory cytokine mRNAs including TNFα, IFNγ, and IL-6 were significantly increased during colitis in CAST KO macrophages compared to WT controls (Fig. 4D). This supports the notion that CAST does not affect the number of infiltrating macrophages, but plays a role in regulating the inflammatory state of macrophages during colitis in a manner that modulates expression of specific cytokines.

The effect of CAST deficiency on macrophages led us to re-examine the colons from WT mice in healthy control versus colitis groups for amounts of CAST in the macrophages compared to other cells in this tissue. A high frequency of CD68-positive macrophages expressing CAST was detected in the intestinal tissue from colitis mice, while detection of CAST-positive macrophages in these regions from colons of healthy mice was much less frequent (Fig. 5A). Confocal microscopy at higher power confirmed colocalization of CAST and CD68 in inflamed intestinal tissue from colitis mice (Fig. 5B). For a more quantitative assessment, CD68-positive macrophages were purified from colons of colitis and control mice and analyzed for CAST levels by western blot. The purified CD68-positive macrophages showed striking increases in CAST during colitis, while the CD68-negative cells did not show significant increases in CAST during colitis (Fig. 6A-B). Consistent with these western blot data, flow cytometric analyses of CAST levels in different immune cell types from colons showed that macrophages contained higher levels of CAST during colitis compared to healthy controls (Fig. 6C-D).

Specific types of inflammatory stimulation of mouse and human macrophages increase CAST levels

To determine if the higher levels of CAST found in the macrophages from colitis mice shown above were a direct result of macrophage activation, we performed experiments using both mouse and human primary macrophages. Results from time-course experiments showed that LPS- and TNFα-stimulation increased CAST levels in both BMDM and HMDM (Fig. 7A-B). The increases in CAST coincided with decreases in calpain activity over a 24 h period in the BMDM and HMDM stimulated with either LPS or TNFα, with significantly lower calpain activity apparent in BMDM at earlier timepoints compared to HMDM (Fig. 7C). The CAST KO BMDM did not show substantial decreases in calpain

activity, which supports the notion that CAST is the major mechanism by which calpain activity is regulated during activation. We next explored whether the increased CAST levels observed upon macrophage activation were specific to certain types of stimulation. In particular, pro-inflammatory stimuli including IFNγ, TNFα, and LPS but not stimulation with IL-4, IL-10, or IL-17A led to increased CAST (Fig. 7D). Levels of m-calpain were not affected by any of these stimuli.

Impaired CAST response during macrophage activation results in increased activation of the NF-κB pathway

Our in vivo data above showed that CAST deficiency in intestinal macrophages led to increases in mRNA levels for TNFα, IL-6, and IFNγ, which are induced by the transcription factor NF-κB. NF-κB is sequestered in the cytosol and translocation to the nucleus by prevented IκB, which has been shown in other cell-types to be a target of calpain cleavage (31). Thus, we used BMDM to investigate the possibility that CAST deficiency in macrophages leads to increased IκB cleavage and hyperactivation of the NF-κB pathway. Two independent methods were used to generate CAST deficiency in macrophages under conditions of TNFα priming: siRNA was used to knockdown CAST in WT BMDM with nontargeting siRNA serving as a negative control, or in separate experiments WT and CAST KO BMDM were used. After 24 h of TNFα priming, the BMDM were then stimulated with bacterial PAMPs including heat-killed *E. faecalis* or CpG DNA for different times. Western blot analyses of total IκB demonstrated that CAST deficiency induced by either knockdown or KO of CAST led to decreased levels of IκB with either bacterial PAMP (Fig. 8A). Consistent with these results, levels of nuclear NF-κB induced by the PAMPs were increased under conditions of CAST deficiency (Fig. 8B). These experiments were replicated in three independent experiments and densitometry performed showing that CAST deficiency resulted in significantly lower IκB levels and higher nuclear NF-κB (Supplemental Fig. 3).

To determine how CAST deficiency affected events downstream from NF-κB translocation to the nucleus, we evaluated cytokine production in WT and CAST KO BMDM primed with TNFα followed by CpG DNA stimulation. Cytokines that were secreted at detectable levels included TNFα, MCP-1, IL-6, and IL10 (IFNγ and IL-12 were not detected), and CAST KO BMDM secreted higher levels of TNFα and IL-6 (Fig. 9). In contrast, MCP-1 was not induced by CpG and there were no differences between WT and KO BMDM in IL-10 secretion. These data suggest that CAST serves to limit secretion of particular inflammatory cytokines targeted by NF-κB in macrophages (e.g. TNFα and IL-6, but not MCP-1), while the anti-inflammatory cytokine induced by CpG, IL-10, is not affected by CAST deficiency.

Discussion

The calpain/CAST system regulates cellular function through the proteolytic modulation of structural and signaling proteins. Importantly, Ca^{2+} induces activation of calpain in an irreversible manner, and thus the endogenous calpain inhibitor CAST is crucial in the regulation of calpain activity. Our recent publication suggested a unique role for CAST in regulating macrophage activation (14), and the results presented in the current study extend those findings to suggest a dynamic, regulatory role for CAST in macrophages during IBD. Calpains have been suggested to promote inflammatory conditions for different disease states (20, 32, 33), and we predicted IBD tissues would exhibit higher levels of calpain activity. However, our data showed that calpain activity was initially increased during acute inflammation and then decreased in chronically inflamed intestinal tissue compared to healthy controls. Despite decreases in calpain activity in the colon during chronic IBD, levels of both μ- and m-calpain remained unchanged. In contrast, the endogenous inhibitor of calpains, CAST, was increased during chronic IBD and this appears to contribute to the

decreased calpain activity observed in chronically inflamed intestinal tissues. Thus, the regulation of calpain activity during inflammatory conditions arising during colitis largely relies on the protein dynamics of CAST, mainly upregulated in macrophages. This highlights the crucial role this endogenous inhibitor plays in limiting the level of inflammation during IBD and perhaps other macrophage-driven inflammatory diseases.

A key finding in this study is that CAST deficiency in the immune system alone is similar to CAST deficiency in the whole animal in causing more severe colitis compared to controls. This suggests that CAST is particularly important in the immune system for regulating the level of inflammatory response during colitis. There were some minor differences in the whole animal and immune system CAST KO models. In particular, the bone marrow chimeric mice with induced colitis exhibited more persistent weight loss compared to the mice included in the whole animal knockout model. This may be due to effects on the gut resulting from the x-ray irradiation such as chronic epithelial cell shedding, differences in appetites, or some other factor included in the bone marrow reconstitution process. Regardless, the similar patterns of weight loss combined with similar levels of colitis pathology suggest that CAST deficiency in the immune system is sufficient to reveal an important regulatory role for CAST in limiting the severity of this disease.

One previous study showed that the type-I calpain inhibitor, which inhibits both μ- and m-calpain isoforms, significantly reduced the severity of DNBS-induced acute colitis in rats (20). Daily treatment of the rats with the calpain inhibitor reduced colon injury and clinical symptoms such as haemorrhagic diarrhoea and weight loss, and also reduced inflammation as indicated by lower levels of mucosal myeloperoxidase activity and colonic expression of ICAM-1 and P-selectin. This suggests that calpain inhibitors may be useful in the treatment of inflammatory bowel disease (21), although the lack of information regarding mechanisms by which the calpain/CAST system modulates inflammatory processes is a major impediment to the development of drugs targeting these proteins. Our data from the mouse model of colitis demonstrated a much higher level of CAST in intestinal macrophages compared to other cell types, with CAST levels mainly upregulated in macrophages in the colon during colitis. Importantly, western blot analysis showed a higher degree of increased CAST (83% increased) compared to results from intracellular flow cytometric analyses of CASTß (38%), which highlights the how these two very different approaches may give different results. This may be due to differences in recognition of CAST in the fixed/permeablized cells involved in the flow cytometric assay or other factors. Regardless, these results suggest that macrophages may serve as a more specific target of pharmacological modulation of the calpain/CAST system.

We found that the total number of macrophages in colons from mice with induced colitis was increased compared to healthy controls, which is consistent with other studies (17-19). However, enumeration of other immune cell types was not performed and despite our data suggesting that CAST was largely expressed in macrophages from colitis tissues, other immune cell types are likely to be involved in the inflammatory processes underlying colitis. In fact, emerging evidence suggest that calpains most certainly play important roles in other immune cell types. Levels of calpain mRNA and, in some cases, protein have been shown to increase during activation of T and B cells (5). This same study suggested increased calpain mRNA and protein results from IFNγ-induced activation of the human monocytic cell lines. Migrating T cell adhesion via LFA-1 is also regulated by calpain (7). Thus, different components of inflammatory responses contributing to the pathology of IBD likely depend on CAST and these mechanisms are currently under investigation.

Our data show that TNFα priming of macrophages increases CAST, and without this increased CAST a subsequent exposure to PAMPs like *E. fecaelis* and CpG results in

hyperactivation. While our data reveals a critical role for CAST in limiting NF-κB activation in macrophage-driven inflammation, the notion of NF-κB as a critical regulator of IBD is not novel. NF-κB activation has been shown to be markedly induced in IBD patients, particularly in macrophages and epithelial cells isolated from inflamed intestinal tissues from these patients (34, 35). Our data in macrophages place CAST upstream of NF-κB activation through its inhibitory actions on calpain-cleavage of IκB, suggesting CAST may serve as a master regulator of hyperactivation of these cells. The multiple cellular functions regulated by the calpain/CAST system pose significant challenges to determining how treatment with calpain inhibitors may interrupt the development of IBD. Targeting macrophages with calpain inhibitors to reduce NF-κB pathway activity may more specifically attenuate inflammation in IBD patients. CAST appears to be critical in determining the extent to which IκB is cleaved by calpain and serves to limit the activation state of macrophages during IBD. Interestingly, despite both iNOS and MCP-1 also being transcriptional targets of NF-κB regulation, our data suggest these are not influenced by CAST deficiency. Also, several other cellular targets of calpain cleavage are likely affected by the dynamic regulation of CAST in macrophages. Calpains cleave a large subset of cellular proteins, including cytoskeletal proteins, membrane receptors, calmodulin binding proteins, G proteins, protein kinase C, other enzymes involved in signal transduction, and many transcription factors (12). Some of the signaling molecules that have been shown to be regulated by calpain cleavage in other cell types include Stat3, Stat5 and Stat6 in mast cells as well as SHP-1 and -2 in T cells (6, 36, 37). Cell motility and adhesion are important immune cell functions that are also regulated by the actions of calpain (7, 38). Thus, in addition to IκB, other calpain targets are likely involved in macrophage activation and the role CAST plays in the activation and differentiation process is currently under investigation.

Another possible interpretation of the findings in the present study is that CAST deficiency may increase susceptibility to severe IBD in humans. This raises the question of whether single nucleotide polymorphisms (SNPs) in the CAST gene or regions that regulate mRNA expression for CAST may increase susceptibility for onset or severity of human IBD. In fact, 48 CAST SNPs have been identified, 20 within the CAST gene itself (39). None of these SNPs have to date been associated with either Crohn's disease or ulcerative colitis through genome-wide association studies. This may reflect a role for CAST in regulating the severity of IBD, not necessarily triggering the onset of disease. The lack of data associating CAST SNPs with IBD is not surprising, given the complex genetic architecture of these diseases in humans (4). One disease that has been linked to CAST SNPs is Parkinson's Disease, which is a very different inflammatory disease (39, 40). In fact, the study of the calpain/CAST system in disease pathogenesis has to a large extent focused on its role in apoptosis related to ischemia/reperfusion injury, cancer, muscular dystrophy, or neurological disease (41). However, the novel findings presented in this study as well as recently published data from our laboratory (14) suggest an additional and critical role for CAST in regulating macrophage activation and colitis. We observed a dynamic role for CAST during macrophage activation that was not found in other immune cells such as T cells or B cells (14). These findings suggest that CAST levels in macrophages are particularly important during in vivo inflammation. Indeed, the present study showed that macrophages purified from inflamed colons exhibited very high levels of CAST compared to the non-macrophage population including other immune cells. The combination of the increased numbers of macrophages in the inflamed intestines together with the increased CAST in these cells highlight the particularly important role of CAST in regulating macrophage-driven inflammation. Based on these findings, we propose that, in addition to its widely studied roles in apoptosis-related pathologies, the calpain/CAST system plays an important but underappreciated role in chronic inflammatory diseases, with CAST acting as a crucial factor in limiting macrophage activation and attenuating inflammation. Studies are ongoing

to determine the exact role that CAST plays in attenuating activation of macrophages and the development of IBD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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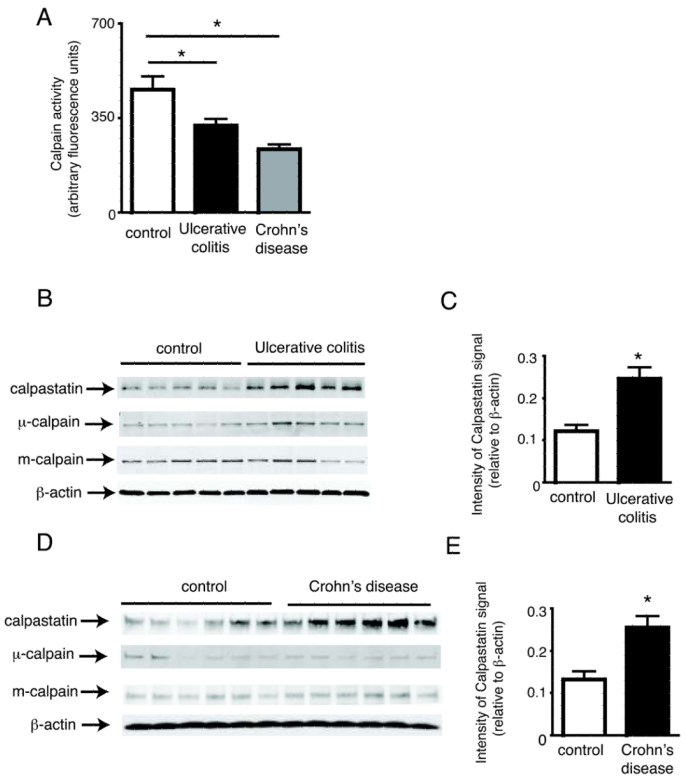

Figure 1.

Calpain activity is decreased and CAST is increased in human intestinal tissue during IBD . **(A)** Intestinal biopsy tissues from healthy controls (N = 7), ulcerative patients (N = 5), or Crohn's disease patients (N = 6) were analyzed for calpain activity, with relative fluorescent units per mg total protein measured using a fluorometer. Data represent mean ≠ S.E. and one-way ANOVA with Tukey post-test was used to compare means, *P < 0.05. **(B)** Western blot analysis of CAST and calpain levels in intestinal tissue from ulcerative colitis patients and five of the healthy individuals (15 μg/well). β-actin was used as a loading control. **(C)** Densitometry was performed to determine the intensity of western blot signals for CAST relative to β-actin for each lane. Data represent mean ≠ S.E. and means were compared using a student's t test, *P < 0.05. **(D-E)** Western blot and densitometry were used to analyze CAST and calpain levels in six healthy intestinal tissue and six tissues from Crohn's disease patients similar as described above for ulcerative colitis. Each experiment was repeated in two independent experiments.

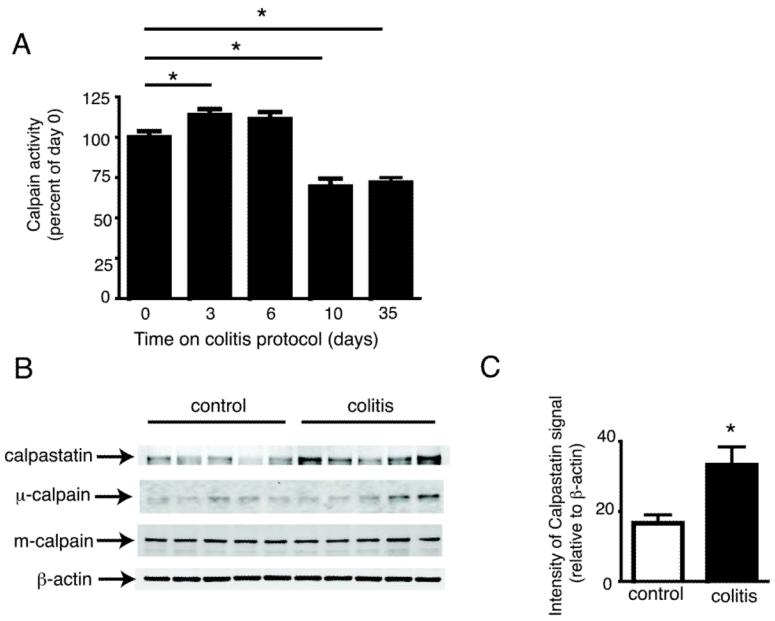

Figure 2.

Chronic colitis results in decreased calpain activity and increased CAST in colons of mice. **(A)** A mouse model of colitis involving DSS-treated water was performed as described in the Methods section. At different days of the colitis model, colon tissues (N = 5 each) were analyzed for calpain activity and relative fluorescent units per mg total protein was measured using a fluorometer. Data represent mean ± S.E. and a student's t-test was used to compare means, *P < 0.05. **(B)** Western blot was used to analyze CAST and calpain levels in lysates prepared from colons of mice subjected to DSS-induced chronic colitis (day 35) and healthy controls (15 μg/well). β-actin was used as a loading control. **(C)** Densitometry was performed to determine the intensity of western blot signals for CAST relative to β-actin for each lane. Data represent mean ± S.E. and means were compared using a student's t test, *P < 0.05. Each experiment was repeated in two independent experiments.

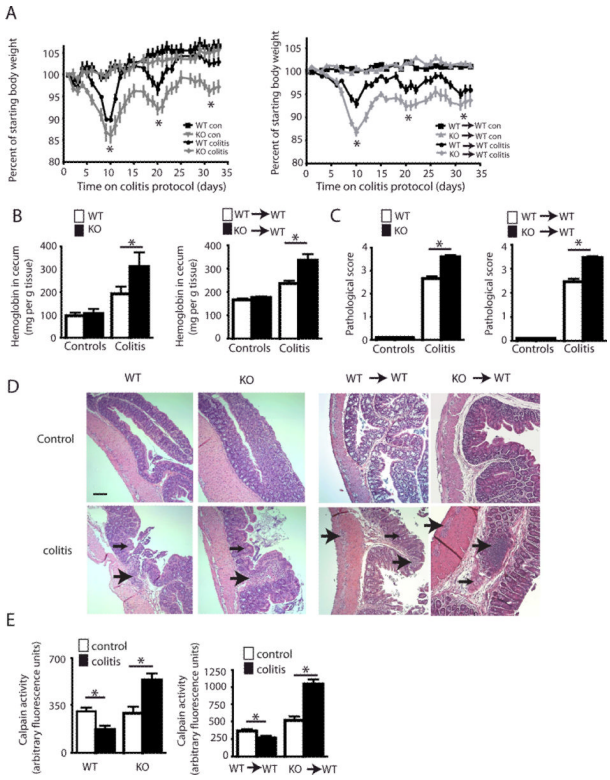

Figure 3.

CAST deficiency in the whole animal and in the immune system alone similarly lead to more severe colitis. **(A)** The graph on the left shows weights of mice with CAST-deficiency in the whole animal compared to WT controls using a model of DSS-induced chronic colitis involving DSS-treated water alternated with untreated water (N = 10 WT and KO) or untreated water alone (N = 8 WT and 9 KO). The graph on the right shows WT mice that were x-ray irradiated and reconstituted with WT bone marrow (WT→WT) or CAST KO bone marrow (KO→WT), then subjected to DSS-treated water or untreated water. For both graphs, weights of mice were measured over a 35 d period with decreased weights indicating colitis. **(B)** For colitis models involving CAST-deficiency in the whole animal (left) or in the immune system alone (right), mice were sacrificed on day 35 and ELISA measurement of hemoglobin in cecums was used for determining level of blood in feces. **(C-D)** For colitis models involving CAST-deficiency in the whole animal (left) or in the immune system alone (right), tissue sections from colons of mice were analyzed by H&E staining for features of inflammation and pathology. Scores for levels of inflammatory pathology were derived as described in the Methods section. The KO and KO→WT mice with induced colitis showed a higher degree of cellular infiltration into the submucosa and muscularis propria (large arrows) and highly disrupted crypt architecture (small arrows) compared to WT and WT→WT controls, respectively. Spacebar = 100 μm. **(E)** For colitis models involving CAST-deficiency in the whole animal (left) or in the immune system alone (right), calpain activity was measured at day 35. In WT and WT→WT mice, colitis resulted in decreased calpain activity, while in KO and KO→WT mice colitis resulted in increased calpain activity. In (A-C, and E), data pooled from two independent experiments with a minimum of 5 mice/group and are represented by mean + S.E. Means were compared using a student's t test, *P < 0.05. In (A), means at each timepoint were compared between WT and KO colitis mice.

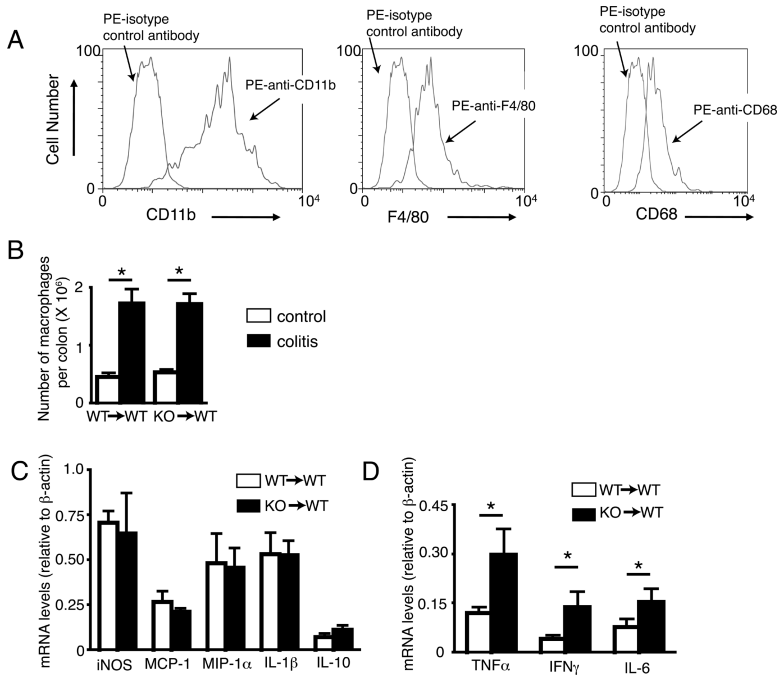

Figure 4.

The inflammatory state of intestinal macrophages is increased with CAST deficiency during colitis. Colons from WT→WT or KO→WT in control or colitis groups were processed into single cell suspensions and anti-CD68-coated magnetic beads were used to purifiy $CD68^+$ macrophages. **(A)** Purity of homogenous populations was confirmed using flow cytometric evaluation of three macrophage markers including CD11b, F4/80, and CD68. Purity was confirmed for each experimental group from two independent experiments and representative data show staining for isotype control antibody and each macrophage marker for purified macrophages. **(B)** Macrophages purified as described above were enumerated for WT→WT and KO→WT (N = 5 mice/group). Total RNA was extracted from purified $CD68^+$ macrophages, cDNA was synthesized, and real-time PCR used to measure relative mRNA levels of different inflammation markers. **(C)** In colitis tissues, no differences were found between WT→WT and KO→WT for several inflammatory markers. **(D)** Three inflammatory markers found to be higher in macrophages from KO→WT compared to WT→WT tissues included TNFα, IFNγ, and IL-6. Samples for (B-D) were derived from cell pellets described in (A), thus the numbers of experiments and mice were the same and data represent mean ≠ S.E. with means compared using a student's t test, *P < 0.05.

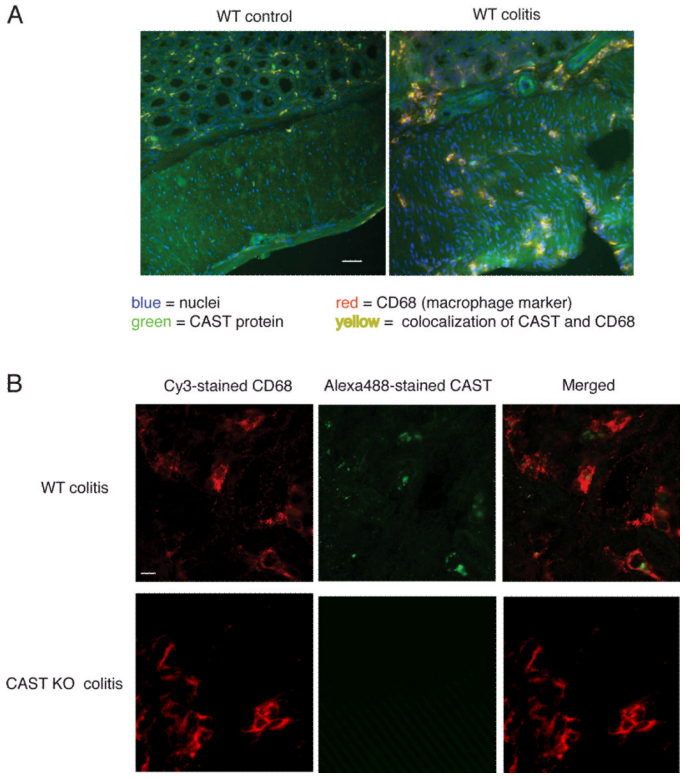

Figure 5.

CAST is increased in macrophages in mouse colitis tissue. **(A)** Colons from WT mice subjected to DSS-treated water or control mice were stained by immunofluorescence for CD68 (Cy3, red) and CAST (Alexa Fluor® 488, green), and colocalization (yellow) determined to identify $CAST^{+}CD68^{+}$ macrophages. Infiltrating $CAST^{+}CD68^{+}$ macrophages were abundant in the submucosa and muscularis propria of IBD colons. Images were captured using a 20X objective and scalebar = 50 μm. **(B)** Confocal microscopy of colitis colon tissue confirmed $CD68^{+}$ (Cy3, red) macrophages with CAST (Alexa Fluor® 488, green). To demonstrate specificity of CAST staining, colitis tissue from CAST KO mice was stained and no Alexa Fluor® 488 signal was detected. Isotype control antibodies were used to determine appropriate concentration of primary antibodies (Data not shown). Images were captured using a 63X objective, scalebar = 10 μm.

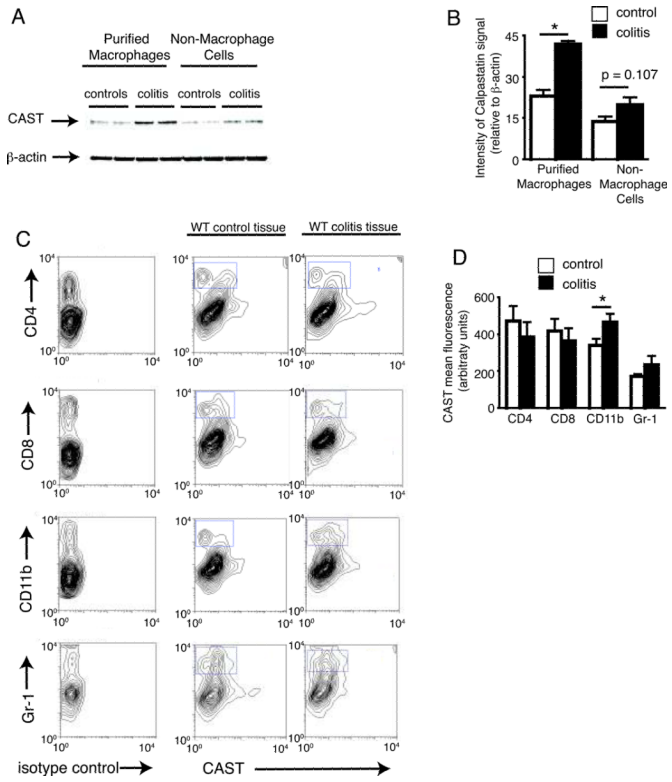

Figure 6.

CAST is increased in macrophages during colitis. **(A)** Macrophages were purified from colons of WT mice subjected to DSS-induced colitis or healthy controls and, because cell numbers in isolated cell pellets were low, cells from individual mice within groups were pooled. Western blot was used to analyze CAST levels in control (two pools, N = 4 and 3 mice each pool) and colitis (two pools, N = 2 and 3 mice each pool) mice. CAST levels were particularly increased in the purified macrophages and β-actin was used as a loading control. **(B)** Two independent IBD experiments were performed and western blots analyzed by densitometry to determine the intensity of signals for CAST relative to β-actin for each lane. Controls included 4 pools (N = 3, 3, 4, and 4 mice each pool) and IBD mice (N = 2, 2, 3, and 3 mice each pool). Data represent mean ± S.E. and means were compared using a student's t test, *P < 0.05. **(C)** Colons from WT controls (N = 8) or WT mice with colitis (N = 9) were processed into single cell suspensions for each mouse and flow cytometry used to detect intracellular levels of CAST in four different leukocytes including $CD4^+$, $CD8^+$, $CD11b^+$, and $Gr\text{-}1^+$ cells. **(D)** Mean fluorescence for CAST in each cell type shows that CAST increased in $CD11b^+$ macrophages from colitis mice compared to controls. Data represent mean ± S.E. from two independent experiments and means were compared using a student's t test, *P < 0.05.

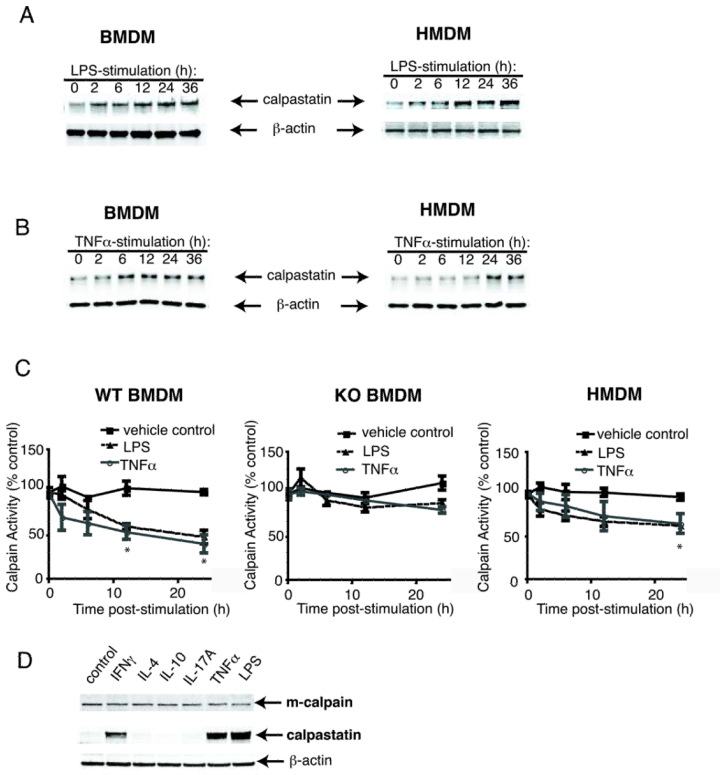

Figure 7.

CAST is increased in mouse and human macrophages with specific inflammatory stimuli. BMDM and HMDM were stimulated with **(A)** LPS (100 ng/mL) or **(B)** TNFα (20 ng/mL) for increasing time periods and levels of CAST analyzed by western blot (15 μg/well), with β-actin serving as a loading control. **(C)** Calpain activity was analyzed in WT or CAST KO BMDM and HMDM activated with LPS or TNFα (100 and 20 ng/mL, respectively), and results showed stimulation led to decreased calpain activity in WT BMDM and in HMDM, but not KO BMDM. Data represent mean *+* S.E. (N = 3), and means for both LPS and TNFα were compared to vehicle control at each timepoint using a student's t-test, *P < 0.05. **(D)** Increased CAST was found for BMDM stimulated 24 h with IFNγ, TNFα, or LPS, but not with IL-4, IL-10, or IL-17A. β-actin served as a loading control. Results are representative of two independent experiments conducted for **(A-C)**.

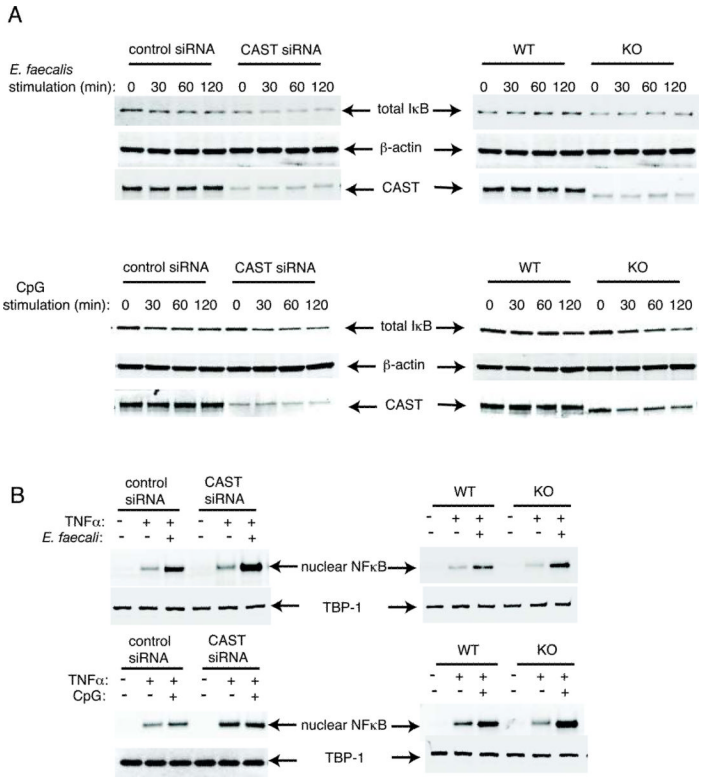

Figure 8.

CAST deficiency in macrophages leads to decreased IκB and increased NF-κB translocation to the nucleus. **(A)** CAST deficiency in BMDM was accomplished by two different methods: left panel, pretreating WT BMDM with CAST siRNA and or non-targeting siRNA as a negative control; right panel, WT vs. CAST KO BMDM. These macrophages were then primed with TNFα (20 ng/mL) for 24 h, followed by stimulation for different time periods with two different bacterial PAMPs, heat-killed *E. faecalis* (1 μg/mL) or CpG DNA (5 μg/mL). Levels of IκB were decreased to a greater extent under conditions of CAST deficiency. β-actin served as a loading control and CAST levels measured to confirm deficiency. As explained in the Methods section, CAST KO mice are characterized by low levels of a functionally impaired truncated CAST. **(B)** BMDM with CAST deficiency induced using either siRNA or KO as described above were stimulated with TNFα (20 ng/mL) for 24 h followed by either heat-killed *E. faecalis* or CpG DNA stimulation for 120 min. Nuclei were isolated and levels of NF-κB detected by western blot, with nuclear TATA-binding protein (TBP)-1 serving as a loading control. Results are representative of three independent experiments conducted for **(A-B)**.

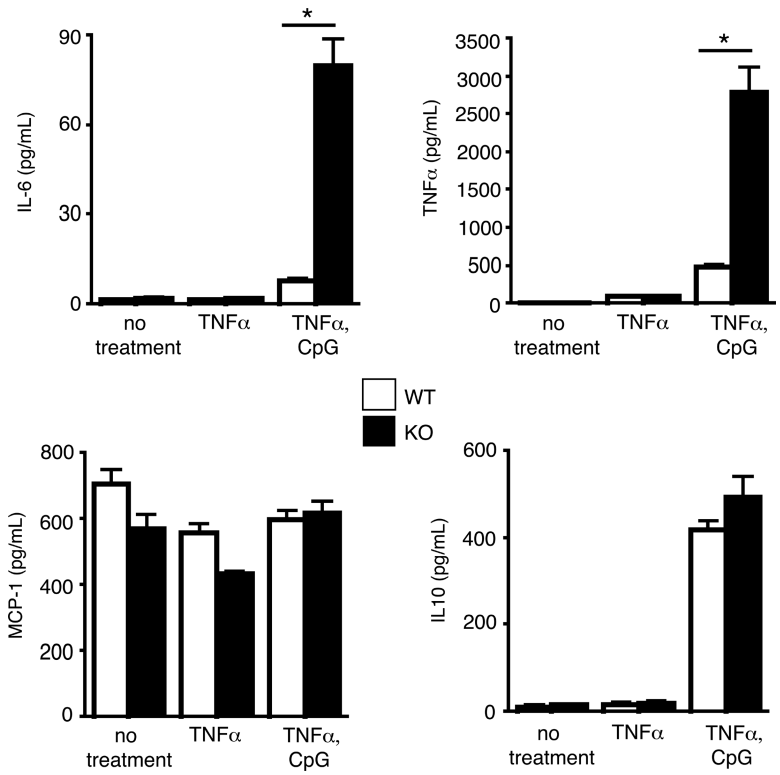

Figure 9.

Secretion of NF-κB induced cytokines is increased in CAST deficient macrophages. WT or CAST KO BMDM were primed with TNFα (20 ng/mL) for 24 h, media changed and cells stimulated for 24 h with CpG DNA (5 μg/mL). Supernatents were evaluated by Cytometric Bead Array for levels of six cytokines including IFNγ, IL-12, TNFα, MCP-1, IL-6, and IL-10. Only the latter four cytokines were detected, three of which were induced by CpG (TNFα, IL-6, and IL-10). TNFα and IL-6, but not IL-10, were secreted at higher levels by CAST KO BMDM compared to WT controls. Data represent mean ± S.E (N = 3) and represent results from two independent experiments.