Targeting Gut T Cell Ca\(^{2+}\) Release-Activated Ca\(^{2+}\) Channels Inhibits T Cell Cytokine Production and T-Box Transcription Factor T-Bet in Inflammatory Bowel Disease


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Targeting Gut T Cell Ca\(^{2+}\) Release-Activated Ca\(^{2+}\) Channels Inhibits T Cell Cytokine Production and T-Box Transcription Factor T-Bet in Inflammatory Bowel Disease


Prolonged Ca\(^{2+}\) entry through Ca\(^{2+}\) release-activated Ca\(^{2+}\) (CRAC) channels is crucial in activating the Ca\(^{2+}\)-sensitive transcription factor NFAT, which is responsible for directing T cell cytokine and T-bet expression. To establish whether targeting CRAC might counteract intestinal inflammation, we evaluated the in vitro effect of a selective CRAC inhibitor on T cell cytokine production and T-bet expression by lamina propria mononuclear cells (LPMC) and biopsy specimens from inflammatory bowel disease (IBD) patients. The inhibitory activity of the CRAC blocker was investigated through patch-clamp experiments on rat basophilic leukemia cells and fluorometric imaging plate reader intracellular Ca\(^{2+}\). We found that the CRAC blocker concentration-dependently inhibited CRAC current in rat basophilic leukemia cells and that the specific contribution of CRAC to T cell activation is useful for limiting ongoing mucosal inflammation (3). There is also evidence that Th1 cytokines are key drivers of sustained mucosal inflammation in CD. Tissue from the gut of patients with CD contains abundant transcripts for IL-2 and IFN-\(\gamma\), and isolated mucosal T cells secrete large amounts of IFN-\(\gamma\) (4). The increased expression of Th1 cytokines, at least in CD, is associated with T-bet, an IFN-\(\gamma\)-inducible member of the T-box family of transcription factors (5). T-bet up-regulation correlates with IFN-\(\gamma\) production, and together with IL-12 appears essential for the development of Th1-mediated immunopathology in CD (6, 7).

Given the critical role of T cells in IBD, immunomodulatory therapies aimed at targeting T cell activation and cytokine production have been used in the management of patients with IBD (8). Among these therapies, cyclosporine and FK506 inhibit T cell activation by interfering with IL-2 production (9), whereas mAbs have been used to neutralize the T cell-derived cytokine IFN-\(\gamma\) (fotolizumab) (10), block T cell surface molecules, including the IL-2R \(\alpha\)-chain CD25 (daclizumab, basiliximab) (11, 12), and deliver negative signals to T cells via CD3 (visilizumab) (13) and CD4 (eM-T412) (14).

Recently, understanding of the molecular nature and regulation of the most important Ca\(^{2+}\) signaling pathway in T cells, the store-operated Ca\(^{2+}\) (SOC) entry through Ca\(^{2+}\) release-activated Ca\(^{2+}\) (CRAC) channels, has suggested that interference with Ca\(^{2+}\) signaling may also be a useful approach to control excessive T cell activation in vivo (15). A rise in intracellular free Ca\(^{2+}\) concentration is one of the early critical steps in the activation of T lymphocytes and it then has an essential role in determining the strength and the type of the T cell response (16, 17). One of the earliest biochemical steps in this process is the activation of phospholipase C-\(\gamma\), which generates inositol-1,4,5 trisphosphate, thus

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\(\text{Ca}^{2+}\) T cells are thought to be major players in the processes leading to inflammatory bowel disease (IBD)\(^3\) (1). Both Crohn’s disease (CD) and ulcerative colitis (UC) are characterized by an increased mucosal infiltration and activation of CD4\(^{+}\) T lymphocytes (2). Studies using models of experimental colitis have shown that blocking CD4\(^{+}\) T cell activation is useful for limiting ongoing mucosal inflammation (3). There is also evidence that Th1 cytokines are key drivers of sustained mucosal inflammation in CD. Tissue from the gut of patients with CD contains abundant transcripts for IL-2 and IFN-\(\gamma\), and isolated mucosal T cells secrete large amounts of IFN-\(\gamma\) (4). The increased expression of Th1 cytokines, at least in CD, is associated with T-bet, an IFN-\(\gamma\)-inducible member of the T-box family of transcription factors (5). T-bet up-regulation correlates with IFN-\(\gamma\) production, and together with IL-12 appears essential for the development of Th1-mediated immunopathology in CD (6, 7). Given the critical role of T cells in IBD, immunomodulatory therapies aimed at targeting T cell activation and cytokine production have been used in the management of patients with IBD (8). Among these therapies, cyclosporine and FK506 inhibit T cell activation by interfering with IL-2 production (9), whereas mAbs have been used to neutralize the T cell-derived cytokine IFN-\(\gamma\) (fotolizumab) (10), block T cell surface molecules, including the IL-2R \(\alpha\)-chain CD25 (daclizumab, basiliximab) (11, 12), and deliver negative signals to T cells via CD3 (visilizumab) (13) and CD4 (eM-T412) (14).

Recently, understanding of the molecular nature and regulation of the most important Ca\(^{2+}\) signaling pathway in T cells, the store-operated Ca\(^{2+}\) (SOC) entry through Ca\(^{2+}\) release-activated Ca\(^{2+}\) (CRAC) channels, has suggested that interference with Ca\(^{2+}\) signaling may also be a useful approach to control excessive T cell activation in vivo (15). A rise in intracellular free Ca\(^{2+}\) concentration is one of the early critical steps in the activation of T lymphocytes and it then has an essential role in determining the strength and the type of the T cell response (16, 17). One of the earliest biochemical steps in this process is the activation of phospholipase C-\(\gamma\), which generates inositol-1,4,5 trisphosphate, thus
release Ca$^{2+}$ stored within the endoplasmic reticulum into the cytoplasm. The resulting decrease in the level of free Ca$^{2+}$ in the endoplasmic reticulum activates a more sustained Ca$^{2+}$ influx through CRAC channels in the plasma membrane (16). The three highly homologous Orai-1, Orai-2, and Orai-3 plasma membrane proteins, which form the CRAC channel pore subunit, have a crucial role in mediating CRAC channel current activity in T cells (15). Prolonged Ca$^{2+}$ entry through CRAC is crucial in activating the Ca$^{2+}$-sensitive transcription factor NFAT, which is responsible for directing T cell proliferation and the long-term expression of cytokine genes (18). The key role of CRAC in T cell responses is strongly supported by the lethal severe combined immunodeficiency syndrome in patients lacking CRAC (19).

Dysregulated Ca$^{2+}$ responses have been associated with several autoimmune and inflammatory diseases, including systemic lupus erythematosus, rheumatoid arthritis, psoriasis, and IBD (20). Consequently, CRAC channels have been proposed as potential targets in the therapeutic management of autoimmune and inflammatory disorders. Recent in vitro studies showed that pyrazole derivatives potently inhibit Ca$^{2+}$ influx through CRAC, thus counteracting T cell activation and NFAT-driven IL-2 production by activated T cells (21, 22).

On this basis, to establish whether targeting CRAC would offer T cell selective immune modulation in IBD, we used a range of in vitro pharmacological assays to define a selective CRAC inhibitor compound and evaluated its effect on T cell cytokine production and T-bet expression by gut-derived lamina propria mononuclear cells (LPMC) and biopsy specimens from inflamed areas of patients with IBD.

**Materials and Methods**

**Patients and tissues**

Endoscopic biopsy or surgical specimens were taken from macroscopically and microscopically inflamed mucosa of 28 patients affected by active CD (mean age 33.4 years, range 22–66) and 23 patients affected by UC (mean age 34.9 years, range 20–63). Diagnosis of CD was ascertained according to the usual clinical criteria (23), and the site and extent of the disease were confirmed by endoscopy, histology, and enteroclysis in all patients. Disease activity was assessed by CDAI’s Disease Activity Index. Patients with scores below 150 were classified as being in remission, whereas those with scores over 450 had severe disease (23). In 18 patients the primary site of involvement was ileocolonic, and colonic in the remaining 10 patients. Seven patients were untreated at the time of biopsy, being at the first presentation; 14 were treated with mesalazine, steroids, or antibiotics; and seven were treated with only mesalazine at the time of biopsy, and had suspended the steroid treatment at least 3 mo earlier. None of the patients had ever been treated with cyclosporine, methotrexate, or infliximab. In patients with UC, disease activity was assessed according to the Clinical Activity Index of Rachmilewitz (24). Clinical remission was defined as a score below 4. Nine patients with UC had pancolitis, the remaining 14 had left-sided colitis. Six patients were untreated at the time of biopsy, being at the first presentation; 10 were treated with mesalazine and topical steroids; three were treated with only mesalazine at the time of biopsy, and had suspended the steroid treatment at least 3 mo earlier; and four were treated with mesalazine and azathioprine. None of the patients had been ever treated with cyclosporine, methotrexate, or infliximab. Mucosal samples were also collected from the colon of 11 subjects who had functional diarrhea at the end of their diagnostic work-up (mean age 38.1 years, range 29–68), and from macroscopically and microscopically unaffected colonic areas of 13 patients undergoing colectomy for colon cancer (mean age 48.8 years, range 38–70). Some of the mucosal samples were used to isolate LPMC, some others for organ culture experiments. Each patient who took part in the study was recruited after appropriate local Ethics Committee approval, and informed consent was obtained in all cases.

**Organ culture**

Biopsy specimens were placed on iron grids in the central well of an organ culture dish and the dishes placed in a tight chamber with 95% O$_2$ and 5% CO$_2$ at 37°C (25). Increasing concentrations (0.1, 1, and 10 μM) of the selective CRAC inhibitor Synta 66 (3-fluoro- pyridine-4-carboxylic acid (‘2,5-dimethoxy-biphenyl-4-yl)-amide, GS1K1349571A, patent ref. no. WO2005009954 and US2004/02379, Synta Pharmaceuticals), or 10 μM FK506 purchased from Sigma-Aldrich, were added to RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% HL-1 (Cambrex Bioscience), 100 U/ml penicillin, and 100 μg/ml streptomycin. As drugs were dissolved in DMSO (Sigma-Aldrich), 0.1% DMSO was used as negative control. After 24-h culture, supernatants were snap frozen and stored at −70°C.

**Cell isolation**

LPMC were isolated as previously described (25). Briefly, the epithelial layer was removed with 1 mM EDTA (Sigma-Aldrich). After stirring for 1 h at 37°C, the supernatant was removed and the remaining tissue was treated with type 1A collagenase (1 mg/ml; Sigma-Aldrich) for 2 h with shaking at 37°C. The crude cell suspension was allowed to stand for 5 min to permit the sedimentation of debris. Cells from the supernatant were washed twice, resuspended in 1 ml RPMI 1640 medium (Sigma-Aldrich) containing 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin and kept on ice until used. Cells were not used if viability did not exceed 90%. PBMC were isolated from heparinized peripheral blood of seven healthy volunteers (mean age 36.5 years, range 24–55) by Lymphoprep gradient centrifugation (Nycomed). Purified CD3$^+$ cells were obtained by positive MACS separation using a CD3$^+$ cell isolation kit (Miltenyi Biotech) after removing monocytes by adherence. The resulting cell preparation contained more than 95% CD3$^+$ T cells as assessed by flow cytometry.

**Cell culture**

Freshly isolated PBMC, LPMC, or CD3$^+$ LPMC (2 × 10^5 cells/well) were stimulated in anti-CD3-coated 96-well plates (BD Biosciences) with anti-CD28 Ab (0.5 μg/ml; ebioscience), and incubated for 48 h with medium containing a range of concentrations (0.01–10 μM) of the CRAC inhibitor or 10 μM FK506. Rat basophilic leukemia (RBL) cells (RBL-2H3), grown in MEM supplemented with 10% FBS, MEM nonessential amino acids, 2 mM L-glutamine at 37°C in a humidified atmosphere containing 5% CO$_2$, were used in electrophysiologic experiments. Cells were detached with Accutase (PAA Laboratories) and plated on poly-l-lysine-coated glass coverslips (BD Biosciences) at a density of ~20,000 cells/ml. Cells were tested 2–3 days after plating.

**Patch-clamp electrophysiology**

Whole cell patch-clamp recordings were performed at room temperature (20–24°C). Patch pipettes were pulled from borosilicate glass (0.69 mm ID; Harvard Apparatus) on a P-87 horizontal puller (Sutter Instrument), with a resistance of 2–3 MΩ when filled with an intracellular solution (in millimoles) comprising: 145 cesium glutamate, 8 NaCl, 10 HEPES, 10 EGTA, 1 MgCl$_2$, 2 Mg ATP, 0.05 inositol-2,4,5-trisphosphate adjusted (pH 7.4) with cesium hydroxide. A fragment of glass coverslip with adherent cells was placed in a recording chamber on an inverted microscope (Carl Zeiss) and continuously perfused with an external solution (in mM) comprising: 145 NaCl, 2.8 KCl, 2 MgCl$_2$, 2 CaCl$_2$, 10 HEPES, 10 glucose, 100 HEPES, adjusted (pH 7.4) with sodium hydroxide. For recording the CRAC current (I$_{CRAC}$), a 50 ms duration voltage ramp (−100 to +100 mV) was applied every 5 s to cells at a holding potential of 0 mV. The amplitude of I$_{CRAC}$ was measured at ~80 mV throughout the experiment. A control extracellular solution containing nominally “zero Ca$^{2+}”$ was applied for ~30 s to determine a zero-I$_{CRAC}$ baseline before and after any pharmacological manipulation. Compounds were used as fast switches and liquid filaments across excision systems driven by a solenoid valve (SF-77B; Warner Instruments). Whole cell patch-clamp recordings were acquired using an Axopatch 200B (Molecular Devices), filtered using a Bessel filter at 2 kHz and digitized at 50 μs. Leak current was subtracted offline by subtracting the average of currents recorded in the control nominal calcium solution before pharmacological manipulation. Data were captured and analyzed using pClamp 9.0 (Molecular Devices) and Origin software packages (OriginLab). All pooled data are given as mean ± SEM.

**Fluorometric imaging plate reader (FLIPR) intracellular Ca$^{2+}$ assay**

Jurkat T cells were loaded with assay buffer containing the Ca$^{2+}$-sensitive fluorescent dye fluo-4-AM (2 μM) for 2 h, and plated in the presence or absence of the CRAC inhibitor in Ca$^{2+}$-free Tyrode’s solution with 1 μM FK506 purchased from Sigma-Aldrich, which empties intracellular Ca$^{2+}$ stores. The plates were then placed into a FLIPR (Molecular Devices) to monitor cell fluorescence, and Ca$^{2+}$ was added at the pEC50 concentration (26).
Western blotting

Western blotting was performed according to a modified method previously described (27). In brief, tissue samples or cells were lysed in ice-cold lysis buffer (10 mM EDTA, 50 mM (pH 7.4) Tris-HCl, 150 mM NaCl, 1% Triton X-100, 2 mM PMSF, 2 mM sodium orthovanadate, 10 mg/ml leupeptin, and 2 mg/ml aprotinin) and the amount of protein was determined by the Bio-Rad Protein assay. A total of 50–75 µg of protein was loaded in each lane and run on 10% SDS-PAGE under reducing conditions. After electrophoresis, protein was transferred to nitrocellulose (Bio-Rad). The kit (Amersham Biosciences). Blots were stripped and analyzed for concentration-response analysis, based on n = 5 samples per concentration tested, yielded an IC_{50} of 1.4 µM.

Cytokine assay

IFN-γ, IL-2, IL-8, and IL-17 were analyzed in the cell and organ culture supernatants by the ECL multiplex ELISA system Sector 6000 imager (Meso Scale Discovery), according to the manufacturers’ instructions.

Flow cytometry

Apoptosis was quantified by flow cytometry using allophycocyanin-Annexin V (BD Biosciences) and propidium iodide in the gate of CD3+ cells. First, labeling of LPMC was performed on ice for 30 min with saturating concentration of FITC-conjugated anti-CD3 Ab (BD Biosciences). Then, cells were stained with 5 µl of allophycocyanin-Annexin V diluted 1/10 in buffer, and 2.5 µl of propidium iodide. After incubation for 15 min, the cells were analyzed by flow cytometry with standard FACScan equipment (BD Biosciences). Cells permeabilized with 0.1% saponin were used as the control for propidium iodide-positive cells.

Proliferation assay

Freshly isolated LPMC were labeled by incubation with 5 µM CFSE (Invitrogen) in PBS containing 1% FBS for 10 min at 37°C. Subsequently, cells (2 × 10^5 cells/well, in triplicate) were stimulated in anti-CD3-coated 96-well plates (BD Biosciences) with anti-CD28 Ab (0.5 µg/ml; eBioscience), and incubated for 72 h with medium containing a range of concentrations (0.01–10 µM) of the CRAC inhibitor. After culture, cells were harvested, stained with anti-CD3-allophycocyanin Ab (BD Biosciences), and analyzed by flow cytometry using CFSE dilution in gated CD3+ cells with standard FACScan equipment (BD Biosciences).

Statistical analysis

Data were analyzed in the GraphPad Prism statistical PC program (GraphPad) using the student’s t test and the Mann-Whitney U test. A level of p < 0.05 was considered statistically significant.

Results

Characterization of a selective CRAC inhibitor

Our initial experiments sought to define whether the claimed CRAC inhibitor compound Synta 66 (GSK1349571A) exerted a robust inhibitory activity on I_{CRAC} and possessed a suitable selectivity profile vs a range of additional receptor targets commensurate with its use as a tool compound to probe I_{CRAC} biology. We used whole cell patch-clamp electrophysiology on RBL cells and FLIPR intracellular calcium assays on thapsigargin-stimulated Jurkat T cells to characterize the primary pharmacological activity of the CRAC inhibitor. As shown in Fig. 1, A–C, I_{CRAC} was readily identified in the RBL cells and substitution of the extracellular solution with an equivalent bearing nominally zero Ca^{2+} concentration enabled the CRAC component of the current to be readily dissected (28, 29). The small magnitude of this Ca^{2+}-selective inhibition

FIGURE 1. The CRAC inhibitor compound inhibits I_{CRAC} recorded from RBL cells in a concentration-dependent manner. A, The voltage protocol applied for the study of I_{CRAC} consisted of a 50 ms voltage ramp from −100 mV to +100 mV applied every 5 s. B, Time course of an exemplar experiment showing the characteristic increase in inward current measured at −80 mV following intracellular perfusion of RBL cells with inositol-2,4,5-triphosphate and EGTA. Application of the CRAC inhibitor (1 µM) resulted in a clear block of this current. The magnitude of blockade was calculated as a difference between the maximum inward current before compound application and the average current obtained in control extracellular solution containing nominally zero calcium. C, The pooled concentration-response curve for the CRAC inhibitor generated an IC_{50} of 1.4 µM, n = 3 or 4 samples per concentration tested. D, The CRAC inhibitor decreased the intracellular Ca^{2+} response in the FLIPR after addition of extracellular Ca^{2+} (see Materials and Methods). A concentration-response analysis, based on n = 5 samples per concentration tested, yielded an IC_{50} of 1.4 µM.
current (mean current density (pA/pF) 0.76 ± 0.05, range 0.41–1.13 pA/pF, n = 19 samples) was in line with previous studies on this conductance (28, 29). Using this approach we were able to accurately quantify the activity of the CRAC blocker, which decreased $I_{\text{CRAC}}$ in a concentration-dependent manner with an $IC_{50} = 1.4$ μM. This inhibitory action on $I_{\text{CRAC}}$ also translated to a clear inhibition of an intracellular Ca$^{2+}$ response in the FLIPR after addition of extracellular Ca$^{2+}$ to thapsigargin-stimulated Jurkat T cells (Fig. 1D) with an equivalent $IC_{50}$ of ~1 μM. Next we investigated the selectivity profile of the CRAC inhibitor in a functional hippocampal slice assay (see supplemental Fig. 1A) and a panel of radioligand binding assays commercially available via CEREPEP (see supplemental Fig. 2). The CRAC inhibitor exhibited no significant activity at a concentration of 10 μM in these assays consistent with a lack of activity at a range of enzyme, receptor, and ion channel targets including metabotropic glutamate, muscarinic acetylcholine and GABA$\text{A}$ receptors and ligand-gated ion channels such as GABA$\text{A}$, AMPA, and NMDA receptors and voltage-gated channels such as tetrodotoxin-sensitive Na$^{+}$ channels, and N- and P/Q-type voltage-gated Ca$^{2+}$ channels (see supplemental material for further details).

**Effect of CRAC inhibitor on T cell cytokine production**

Firstly, we explored by immunoblotting the expression of Orai proteins, which form CRAC channels (30), on T cells isolated from peripheral blood and gut mucosa. As shown in Fig. 2, we did find a higher expression of Orai-1 and Orai-2 proteins on IBMD CD3$^+$ LPMC in comparison to control CD3$^+$ LPMC and control CD3$^+$ PBMC. No difference was found in Orai-3 expression between control and IBM CD3$^+$ LPMC, whereas control CD3$^+$ PBMC showed lower Orai-3 protein expression in comparison to control and IBM CD3$^+$ LPMC.

Then we investigated the effect of CRAC blockade on T cell-derived cytokines, by measuring IFN-γ, IL-2, IL-8, and IL-17 concentration in the supernatants of anti-CD3/CD28-stimulated LPMC from normal mucosa and from inflamed areas of patients with IBM after incubation with the CRAC inhibitor. IFN-γ, IL-2, IL-17, but not IL-8 production by both control LPMC (Fig. 3A) and IBM LPMC (Fig. 3B) were inhibited in a concentration-dependent manner by the CRAC inhibitor. Of note, the maximal effect of the CRAC inhibitor on IBM LPMC production of IFN-γ, IL-2, and IL-17 was equivalent to that exerted by the FK506 (Table I). No appreciable difference in the efficacy of the compound between CD and UC samples was observed. Cytokine production by control LPMC, both in unstimulated and anti-CD3/CD28-stimulated conditions, was lower in comparison to that by IBM LPMC (Table I). We also investigated the effect of different concentrations of the CRAC inhibitor on IFN-γ, IL-2, and IL-17 production by purified CD3$^+$ LPMC from eight patients with IBM (four patients with CD and four patients with UC), and we found a concentration-dependent down-regulatory effect of the compound that reached a statistical significance at the concentrations of 10, 1, and 0.1 μM (Table II).

We then explored the influence of the CRAC inhibitor on T cell cytokine production in organ cultures of IBM biopsy specimens (Fig. 4). A significant dose-dependent decrease of IFN-γ, IL-2, and IL-17 concentration was observed in the supernatants of biopsy specimens from IBM inflamed areas incubated with the CRAC inhibitor in comparison to biopsy samples incubated with medium only: IFN-γ (mean, 214.2 ± 39.4 pg/ml with medium vs 98.1 ± 21.5 pg/ml at 1 μM ($p < 0.001$) and 18.7 ± 4.3 pg/ml at 10 μM ($p < 0.001$) of the CRAC inhibitor); IL-2 (mean, 84.4 ± 30.6 pg/ml with medium vs 38.7 ± 10.2 pg/ml at 1 μM ($p < 0.001$) and 13.3 ± 2.6 pg/ml at 10 μM ($p < 0.001$) of the CRAC inhibitor); and IL-17 (mean, 150.1 ± 41.2 pg/ml with medium vs 73.9 ± 18.5 pg/ml at 1 μM ($p < 0.001$) and 9.8 ± 4.3 pg/ml at 10 μM ($p < 0.001$) of the CRAC inhibitor). However, no significant inhibitory effect was exerted by the CRAC inhibitor on IL-8 production (mean, 2942 ± 576 pg/ml with medium vs 2831 ± 594 pg/ml at 1 μM and 2496 ± 481 pg/ml at 10 μM of the CRAC inhibitor). FK506 induced a significant decrease of all the cytokines examined: IFN-γ (mean, 49.2 ± 16.9 pg/ml, $p < 0.001$); IL-2 (mean, 32.1 ± 9.7 pg/ml, $p < 0.001$); IL-17 (mean, 19.7 ± 5.5 pg/ml, $p < 0.001$); and IL-8 (mean, 1074 ± 236 pg/ml, $p < 0.001$).

**Effect of the CRAC inhibitor on T-bet expression**

To test whether CRAC blockade influenced T-bet expression, anti-CD3/CD28-stimulated LPMC or biopsy specimens from inflamed areas of patients with IBM were cultured in the presence or absence of different concentrations of the CRAC inhibitor and, after culture, T-bet was determined by immunoblotting on cell lysates or mucosal homogenates. A significant ($p < 0.0001$) increase of T-bet expression was observed when LPMC were stimulated with anti-CD3/CD28 Abs. This increase was significantly inhibited by the CRAC inhibitor in a dose-dependent manner ($p < 0.001$ at 10 μM; $p < 0.05$ at 1 μM). No effect was evident when cells were treated with the lowest concentration (0.1 μM) of the CRAC inhibitor (Fig. 5A). In organ culture biopsies, the highest concentration of the CRAC inhibitor significantly ($p < 0.001$) inhibited T-bet expression. The CRAC inhibitor at the concentration of 1 μM reduced, but not significantly, mucosal T-bet expression (Fig. 5B). FK506, used as positive control, significantly ($p < 0.001$) inhibited T-bet expression both in cells and tissues.

**Effect of the CRAC inhibitor on T cell proliferation**

To investigate the influence of the CRAC inhibitor on T cell proliferation, LPMC from inflamed areas of patients with IBM were labeled with CFSE and cultured for 72 h in presence or absence of anti-CD3/CD28, with or without increasing concentrations of the

**FIGURE 2.** Western blotting detection of the subunits of the CRAC channels Orai-1, Orai-2, and Orai-3 in purified CD3$^+$ PBMC from a healthy control (HC), in purified CD3$^+$ LPMC from the colonic mucosa of a healthy control, and from the inflamed colonic mucosa of a patient with CD. Blots of cell lysates were stripped and analyzed for β-actin as an internal loading control. Each sample is representative of experiments performed in three patients with CD, three patients with UC, and four control subjects.

($^4$) The online version of this article contains supplemental material.
CRAC inhibitor (0.01–10 μM), and the percentage of proliferating T cells was assessed by flow cytometry in gated CD3+ cells (see supplemental Fig. 3). T cell proliferation was significantly \((p < 0.001)\) increased by anti-CD3/CD28 stimulation (from mean 12.3 ± 3.7% to 82.01 ± 12.2%). When we added the CRAC inhibitor to stimulated LPMC, T cell proliferation significantly \((p < 0.001)\) decreased at the highest concentration of 10 μM (mean 15.6 ± 0.3%), whereas the other concentrations had no significant down-regulatory effect (mean 70.8 ± 15.5% at 1 μM; 78.3 ± 13.3% at 0.1 μM; 80.0 ± 9.5% at 0.01 μM).

**Effect of the CRAC inhibitor on T cell apoptosis**

To investigate the influence of the CRAC inhibitor on T cell apoptosis, LPMC isolated from IBD inflamed areas and stimulated with anti-CD3/CD28 Abs were cultured with increasing concentrations (0.1, 1, and 10 μM) of the CRAC inhibitor, and the percentage of apoptotic T cells was assessed after 48 h culture by flow cytometry with Annexin V and propidium iodide staining in gated CD3+ cells. Anti-CD3/CD28-stimulated LPMC cultured with 10 μg/ml infliximab or its isotype matched control (human IgG1) were used as positive control (Fig. 6A). Representative results show no difference in the percentage of apoptotic T cells (Annexin V-positive/propidium iodide-negative) cultured with medium only (mean, 33.5 ± 5.3%) or increasing concentrations of the CRAC inhibitor (mean, 33.1 ± 7.7% at 0.1 μM; 30.9 ± 6.4% at 1 μM, and 36.1 ± 7.1% at 10 μM). A significant \((p < 0.0005)\) higher percentage of apoptotic T cells was found in infliximab-treated cells (mean, 77.3 ± 11.6%) in comparison to IgG1-treated cells (mean, 30.2 ± 4.9%) (Fig. 6B).

### Discussion

In this study we have provided to our knowledge the first evidence that blocking CRAC channels with a suitably selective tool inhibitor attenuates T cell cytokine production and T-bet expression in LPMC and organ culture biopsy specimens taken from inflamed areas of patients with IBD. Importantly, these results confirm the crucial role of CRAC channels in modulating the T cell-based immune response, and suggest that CRAC blockade may offer a new therapeutic strategy in patients with IBD.

Our initial work focused on the characterization of a CRAC inhibitor Synta 66 (GSK1349571A) using patch-clamp electrophysiology and FLIPR assays to directly assess its inhibitory action on \(I_{\text{CRAC}}\) and effects on intracellular Ca\(^{2+}\), respectively. We demonstrated that the CRAC inhibitor produced a robust and concentration-dependent inhibition of \(I_{\text{CRAC}}\) in RBL cells, and thapsigargin-induced Ca\(^{2+}\) influx through CRAC in Jurkat T cells with an IC\(_{50}\) of ~1 μM. We also extensively characterized its selectivity profile demonstrating a lack of additional pharmacological activity at a concentration of 10 μM vs a wide range of enzyme, receptor and ion channel targets sampled as part of a CEREPI panel of radioligand binding assays and a hippocampal slice assay (see supplemental Figs. 1 and 2). Additionally, we demonstrated by microarray a remarkably selective effect of the compound in down-regulating cytokine genes involved in T cell activation, including IFN-γ, IL-2, IL-3, IL-9, IL-17, IL-21, and IL-22 (data not shown). An additional evidence supporting the selectivity of our compound (Synta 66) for CRAC channels comes from the study by Ng et al. (31), who confirmed the selectivity of Synta 66 by siRNA technology.

### Table I Cytokine levels in the supernatants of total LPMC in IBD

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<th>Cytokine Level (pg/ml)</th>
<th>Controls</th>
<th>CD Patients</th>
<th>UC Patients</th>
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<td></td>
<td>%CD3/CD28</td>
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<td>IFN-γ</td>
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<td>oCD3/CD28</td>
<td>+ CRAC</td>
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<td>+ FK506</td>
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<td>oCD3/CD28</td>
<td>+ FK506</td>
<td>+ FK506</td>
</tr>
</tbody>
</table>

\(^a\) IFN-γ, IL-2, IL-8, and IL-17 levels in the supernatants of total LPMC isolated from 14 control subjects, 14 patients with CD, and 14 patients with UC, cultured in the absence or presence of anti-CD3/CD28 (oCD3/CD28) Abs, together with the selective CRAC inhibitor compound (10 μM) or FK506 (10 μM). Results are mean ± SD.

\(^b\) \(p < 0.0001\) vs medium.

\(^c\) \(p < 0.0001\) vs anti-CD3/CD28 only.
knockdown of Orai-1 in mast cells. Overall these data support the use of the CRAC inhibitor as a tool with which to further probe the role of CRAC in T cell signaling and IBD.

We firstly explored the expression of Orai proteins forming CRAC channels in CD3⁺ LPMC, and we found a higher expression of Orai-1 and Orai-2 in patients with IBD in comparison to controls, thus supporting the evidence of an increased activation state of CRAC channels in IBD inflamed mucosa. This in keeping with the data of Weigmann et al. (32) who demonstrated an increased NFAT expression in the mucosa of patients with IBD. Then, we took two approaches to determine whether blocking CRAC had any effect on mucosal T cell function in IBD. In the first approach, we cultured both total and CD3⁺ LPMC from patients with IBD using the CRAC inhibitor and then measured cytokines in the supernatants; in the second, we used explants of IBD mucosa in the same protocol. In both cases, blocking CRAC channels markedly decreased the levels of the T cell-derived cytokines IL-2, IFN-γ, and IL-17 in the supernatants, with a comparable efficacy to that of the immunosuppressant FK506. We interpret these results as indicating that Ca²⁺ entry through SOC channels in the mucosa and in LPMC actively modulates T cell activation and cytokine production, which fits in well with the higher level of Ca²⁺ influx observed in lamina propria T cells from active IBD patients (33, 34). These results are also consistent with the idea that increased Ca²⁺ signals promote stronger activation of NF-κB (16, 35), which is known to play a key role in intestinal inflammation (36). Activation of NF-κB induces the expression of a number of proinflammatory cytokines that act locally within the intestinal microenvironment and cause inflammation (37). All the cytokines we found to be inhibited by the CRAC inhibitor, namely IFN-γ, IL-2, and IL-17, are involved in causing gut inflammation (1, 38). Among these cytokines, the most recently identified is IL-17, a 17-kDa protein that is secreted as a dimer by a restricted set of cells, predominantly activated human memory T cells. IL-17 exerts strong proinflammatory activities by enhancing T cell priming and stimulating myofibroblasts, endothelial cells, macrophages, and epithelial cells to produce multiple proinflammatory mediators, chemokines, and matrix metalloproteinases, thus inducing tissue damage (39). In contrast, no inhibitory effect was exerted by the CRAC inhibitor on IL-8, a chemokine produced mainly by

**FIGURE 4.** The CRAC inhibitor down-regulates the T cell cytokine release from biopsy samples grown ex vivo. IFN-γ, IL-2, IL-8, and IL-17 levels were detected by ELISA in the supernatants of organ culture biopsy samples taken from the inflamed gut of 12 patients with IBD and cultured for 24 h in the presence or absence of different concentrations of the CRAC inhibitor (10 and 1 μM) or 10 μM FK506. Cytokines production was measured (in picograms per milliliter). Error bar represents mean value (SD). †, p < 0.05 vs medium. *, p < 0.001 vs medium. **, p < 0.0001 vs medium.

Table II. Cytokine levels in the supernatants of purified CD3⁺ LPMC in IBD

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Medium</th>
<th>0.01 microM</th>
<th>0.1 microM</th>
<th>1 microM</th>
<th>10 microM</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
<td>12000 ± 352</td>
<td>1124 ± 391</td>
<td>764 ± 311</td>
<td>429 ± 108</td>
<td>1458 ± 457</td>
</tr>
<tr>
<td>IL-2</td>
<td>6848 ± 1842</td>
<td>8227 ± 1644</td>
<td>4724 ± 931</td>
<td>1567 ± 328</td>
<td>571 ± 184</td>
</tr>
<tr>
<td>IL-17</td>
<td>4266 ± 848</td>
<td>3941 ± 1128</td>
<td>2015 ± 887</td>
<td>1247 ± 358</td>
<td>532 ± 103</td>
</tr>
</tbody>
</table>

*a IFN-γ, IL-2, and IL-17 levels in the supernatants of purified CD3⁺ LPMC isolated from four patients with CD and four patients with UC and cultured with anti-CD3/CD28 Abs in the presence or absence of different concentrations of the selective CRAC inhibitor compound (0.01, 0.1, 1, and 10 microM). Results are mean ± SD.

b p < 0.05 vs medium.

c p < 0.001 vs medium.

d p < 0.0001 vs medium.
macrophages and other cell types such as epithelial cells (40), which is up-regulated in the mucosa of patients with IBD (1, 2).

We also explored the effect of CRAC blockade on T-bet, a well-known member of the T-box family of transcription factors, which is critical to Th1 polarization (41). T-bet is highly expressed in the inflamed gut of patients with CD (5, 42). It is rapidly induced when T cells recognize Ags in the presence of IFN-γ (41), and we have previously shown that IFN-γ enhances T-bet expression in normal mucosal T cells (43). The CRAC inhibitor dose-dependently inhibited T-bet expression both in LPMC and biopsy specimens from patients with IBD, and showed an efficacy comparable to that of FK506, which is known to act by blocking the calcineurin-dependent NFAT dephosphorylation essential for the transcription of IL-2 and other proinflammatory cytokine genes (9).

Activated T cells generally undergo apoptosis upon repeated stimulation. This activation-induced cell death is an important mechanism underlying T cell homeostasis after an immune response (44), particularly in the intestine where T cell activation through TCR by foreign and self-Ags is likely to occur constantly (45). It has been widely shown that T cells grown from CD lesions exhibit enhanced resistance to apoptosis (46). Hence, drugs that potentiate mucosal T cell apoptosis, such as anti-TNF Abs infliximab (47) and adalimumab (48), are useful for inducing clinical

**FIGURE 5.** The CRAC inhibitor down-regulates T-bet expression. Western blotting detection of T-bet in anti-CD3/CD28 (αCD3/CD8) Ab-stimulated LPMC (A) and mucosal biopsy specimens grown ex vivo (B), both taken from inflamed areas of patients with IBD and incubated for 48 h in the absence or presence of different concentrations of the CRAC inhibitor (10, 1, and 0.1 μM) or 10 μM FK506. Blots of mucosal homogenates and cell lysates were stripped and analyzed for β-actin as an internal loading control. Each example is representative of experiments performed in 10 patients with IBD for LPMC, and in 12 patients with IBD for organ culture experiments. Densitometry of T-bet expression normalized for β-actin is shown (right) and expressed in arbitrary units (a.u.). Results are mean and SD. †, p < 0.05; *, p < 0.001; **, p < 0.0001.

**FIGURE 6.** The CRAC inhibitor does not affect T cell apoptosis. A, Apoptosis of IBD LPMC stimulated with anti-CD3/CD28 Abs and cultured for 48 h with or without increasing concentrations of the CRAC inhibitor (0.1–10 μM). In control experiments, anti-CD3/CD28-stimulated LPMC were cultured with 10 μg/ml infliximab or its isotype-matched control (human IgG1). Apoptosis was assessed in gated CD3+ cells by using Annexin V/propidium iodide staining. Percentages shown within the dot plot represents apoptotic cells count (Annexin V-positive/propidium iodide-negative). Data are representative of experiments performed in 10 patients with IBD. B, Percentage of apoptosis in mucosal T cells from 10 patients with IBD, cultured in the absence or presence of different stimuli. Results are mean with SD. *, p < 0.0005.
remission in patients with CD (49, 50). We were therefore interested to determine the role of the CRAC inhibitor in this phenomenon. The comparable rate of apoptosis that we observed in IBD T cell samples treated with different concentrations of the CRAC inhibitor clearly indicates that the compound does not affect T cell survival. We can therefore conclude that the anti-inflammatory properties of the CRAC inhibitor are related to its capacity of inhibiting CRAC channels on T cells, rather than to a modulatory influence on apoptosis. However, we found that the highest concentration of the CRAC inhibitor was capable of reducing T cell proliferation. Hence, we cannot exclude that the down-regulatory effect exerted by the compound on proinflammatory cytokine production could be a consequence of the effect exerted on T cell proliferation, at least for the highest dose of the inhibitor.

Following the discovery that CRAC channels are essential for T cell function, new CRAC inhibitor compounds have been developed to modulate T cell activation in disease. Although the complexity of the CRAC channel activation mechanism offers a large number of target loci, few inhibitors exist (15). The best known are the imidazole antimycotics (e.g., econazole and SKF-96365), 2-amino-ethyldiphenyl borate, and the pyrazole derivative YM-58483 (BTP2) (17). The latter has been reported to potently inhibit both Ca\(^{2+}\) influx through CRAC channels and NFAT-driven IL-2 production in activated T cells (21), and to prevent Ag-induced airway eosinophilia and late phase asthmatic responses via Th2 cytokine inhibition in animal models (51).

The ultimate therapeutic value of a CRAC channel modulator will depend on how specifically it affects Ca\(^{2+}\) signaling in T cells as opposed to SOC entry in all other cells. The degree of specificity will in turn depend on whether the subunit composition of CRAC channels and other SOCs, or the auxiliary proteins involved in their activation, are cell-specific; but this is not yet known for any endogenous SOC, including the CRAC channel. The prospect for a specific immune modulator are promising; however, given that the loss-of-function mutation in Orai-1, a pore-forming subunit of the CRAC channels (30), causes immunodeficiency in human patients without serious functional deficits in organs and tissues outside the immune system that are known to express SOCs (52).

In conclusion, we provide evidence that the suppression of CRAC channel function may dampen the increased T cell response in the inflamed gut, thus suggesting a promising role for CRAC inhibitor drugs in the therapeutic management of patients with IBD. Much work remains to be done to bring CRAC channel blockers to the clinic. However, the complex scenario by which store depletion leads to CRAC channel opening reveals a number of potential sites for controlling Ca\(^{2+}\) signaling in T cells (53) and, hence, for fine modulating the immune response in inflammatory disorders.

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Disclosures

The authors have no financial conflict of interest.

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