UNIVERSITY OF CALIFORNIA, SAN DIEGO

TRPA1 ion channel is functionally expressed in CD4 positive T cells and reduces T cell mediated colitis via inhibition of TRPV1

A thesis submitted in partial satisfaction of the requirements for the degree
Master of Science

in

Biology

by

Peter Kim

Committee in charge:

Professor Eyal Raz, Chair
Professor Li-fan Lu, Co-Chair
Professor Dong-Er Zhang

2016

The Thesis of Peter Kim is approved and it is acceptable in quality and form for publication on microfilm and electronically:

_____

_____

Co-Chair

_____

Chair

University of California, San Diego

2016

TABLE OF CONTENTS

SIGNATURE PAGE iii

TABLE OF CONTENTS iv

LIST OF ABBREVIATIONS vi

LIST OF FIGURES vii

ABSTRACT OF THE THESIS viii

1. INTRODUCTION 1

1.1. Inflammatory Bowel Disease 1

1.2. Transient Receptor Potential Channels 2

1.3. Transient Receptor Potential Ankyrin 1 2

1.4. The *IL10*$^{-/-}$ mouse model of colitis 3

2. METHODS 4

2.1. Antibodies and Reagents 4

2.2. Mice 5

2.3. *Il10*$^{-/-}$ Model of Spontaneous Colitis 5

2.4. T Cell Adoptive Transfer Model of Colitis 6

2.5. Histological Analysis 7

2.6. Human samples 7

2.7. Isolation of mRNA and PCR 8

2.8. Colonic Explants (CE) 9

2.9. Isolation and Stimulation of $CD4^+$ T cells 9

2.10. In vitro T Cell Differentiation Assays 10

2.11. Flow Cytometry Analysis 11
2.12. DCs and OT-II T Cells Co-culture 12
2.13. Immunofluorescence Staining and Confocal Microscopy Analysis 12
2.14. Calcium Imaging 12
2.15. TRPA1 and TRPV1 Knockdown 12
2.16. Immunoblotting 12
2.17. Statistical Analysis 12

3. RESULTS 5
3.1 TRPA1 channels are present in CD4+ T cells 5
3.2. *Trpa1*$^{-/-}$ CD4+ T cells are hyperactivated and display a Th1 bias 20
3.3. TRPA1 deficiency aggravates colitis in IL10 KO mice 21
3.4. *IL10*$^{-/-}$*Trpa1*$^{-/-}$ DKO naive CD4+ T cells display exacerbated colitogenic properties 23
3.5. *IL10*$^{-/-}$*Trpa1*$^{-/-}$ DKO mice display increased Th1-mediated inflammatory responses 24
3.6. TRPV1 hyperactivation is responsible for phenotype of *Trpa1*$^{-/-}$ CD4+ T cells 26
3.7. Increased infiltration of TRPA1+TRPV1+ T cells in inflamed colon sections of patients with active IBD. 29

4. DISCUSSION 31

REFERENCES 36

LIST OF ABBREVIATIONS

BM = Bone marrow

BMDC = Bone marrow-derived dendritic cells

CAP = Capsaicin

CD = Crohn’s Disease

CE = Colonic explants

DAI = Disease Activity Index

IBD = Inflammatory Bowel Disease

IL = Interleukin

Iono = Ionomycin

MLN = Mesenteric lymph nodes

MO = Mustard oil

OVA = Ovalbumin

SP = Spleen

TCR = T cell receptor

TRPA1 = Transient Receptor Potential Ankyrin 1

TRPV1 = Transient Receptor Potential Vanilloid 1

UC = Ulcerative Colitis

LIST OF FIGURES

Figure 1. *TRPA1 channels are present in CD4+ T cells* 19

Figure 2. *Trpa1$^{-/-}$ CD4^{+} T cells are hyperactivated and display a Th1 bias.* ... 20

Figure 3. *TRPA1 deficiency aggravates colitis in Il10$^{-/-}$ mice.* 22

Figure 4. *Il10$^{-/-}$Trpa1$^{-/-}$ naïve CD4^{+} T cells induce an exacerbated colitis in the adoptive transfer model* 24

Figure 5. *Il10$^{-/-}$Trpa1$^{-/-}$ mice display increased proinflammatory CD4^{+} T cell response*. 26

Figure 6. *TRPV1 hyperactivation is responsible for the phenotype of Trpa1$^{-/-}$ CD4^{+} T cells* 28

Figure 7. *Increased infiltration of TRPA1^{+}TRPV1^{+} T cells in inflamed colon sections of patients with active IBD* 30

ABSTRACT OF THE THESIS

TRPA1 ion channel is functionally expressed in CD4 positive T cells and reduces T cell mediated colitis via inhibition of TRPV1

by

Peter Kim

Master of Science in Biology

University of California, San Diego, 2016

Professor Eyal Raz, Chair
Professor Li-fan Lu, Co-Chair

Inflammatory Bowel Diseases (IBD) are characterized by an abnormal inflammation in the small intestine and colon and are mainly mediated by CD4+ T cells. This is why the primary strategy for treating IBD is by therapeutically targeting select CD4+ T cells. It is previously known that the Transient Receptor Potential Vanilloid-1 (TRPV1) ion channel is expressed in CD4+ T cells and functions to activate the proinflammatory characteristics. Furthermore, previous studies show that TRPV1 and TRP Ankyrin 1 (TRPA1) channels in sensory neurons interact with one another in order to affect the

level of activity or function of each ion channel. Here, we investigate the possible role of TRPA1 ion channel in CD4+ T cell mediated colitis. First, we show that the TRPA1 channel is infact expressed at the plasma membrane of CD4+ T cells. In addition, *Trpa1* gene deletion significantly intensified the T cell mediated colitis in murine models. We also found that TRPA1 inhibits TRPV1 channel which leads to a decrease in CD4+ T cell activation as well as colitogenic characteristics. In IBD patients, TRPA1+TRPV1+ T cell infiltration is significantly increased in the human colonic biopsies.

1. INTRODUCTION

1.1. Inflammatory Bowel Disease

Inflammatory Bowel Disease (IBD) is typically described as a systemic inflammation and damage to the gut mucosa (Funderburg et al., 2013). The two most prevalent IBDs are Ulcerative Colitis (UC) and Crohn's Disease (CD) (Hanauer, 2006). Patients with UC or CD usually show symptoms of abdominal pain, diarrhea, as well as weight loss (Conrad et al., 2014). IBD rate of prevalence has been increasing and is evolving into a global disease. In Europe, there were 505 per 100,000 persons with UC and 322 per 100,000 persons with CD. In North America, there were 249 per 100,000 persons with UC and 319 per 100,000 persons with CD (Molodecky et al., 2012).

Unfortunately, the exact molecular cause of IBD is not yet known (Geremia et al., 2014). However, it is evident that CD4+ T cells play a significant role in the induction of IBD. Although this provides a therapeutic strategy to relieve symptoms of IBD by inhibiting the functions of CD4+ T cells, there are detrimental side effects (Monteleone et al., 2010). This shows the necessity of a different therapeutic approach to inhibit the colitogenic properties of a CD4+ T cell.

1.2. Transient Receptor Potential Channels

The transient receptor potential (TRP) channels mediate cations through cell membranes. In addition, TRP channels have an important role in sensory and nonsensory processes (Caterina, 2015). However, recent evidence suggest that a subset family, TRP Vanilloid 1 (TRPV1) channel, is also expressed in immune CD4+ T cells. The elevated expression of TRPV1 in CD4+ T cells functions to increase its proinflammatory characteristics in colitis models (Bertin et al., 2014). More importantly, TRPV1 channel functions as a homo or heterotetramer with other TRP channels (Venkatachalam, 2007). It is already known that TRPV1 is mostly expressed as a heterotetrameric channel with TRP Ankyrin 1 channel in sensory neurons. For this reason, we assessed the function and role of TRPA1 in CD4+ T cells and its relations to T cell mediated colitis.

1.3. Transient Receptor Potential Ankyrin 1

Transient Receptor Potential Ankyrin 1 (TRPA1) channel is a calcium permeable cation channel that functions in a variety of cell processes. TRPA1 was initially found in sensory neurons to be activated with noxious cold temperatures and alkylative compounds (Nilius et al., 2012). TRPA1 found in neurons are classified to have a proinflammatory function due to its activation by stimuli which causes inflammation (Bautista et al., 2006). Stimuli that induce colitis in TRPA1 gut sensory nerves include TRPA1-specific agonist

mustard oil (MO) and 2,4,6-trinitrobenzene sulfonic acid (TNBS) (Engel et al., 2011). Conversely, this report suggests that TRPA1 channel in CD4+ T cells have a surprising anti-inflammatory role.

1.4. The IL10$^{-/-}$ mouse model of colitis

IL10$^{-/-}$ mouse model of colitis was used in order to study IBD. Interleukin-10 (IL-10) plays a crucial role in differentiation of hemopoietic cells and inhibition of both macrophage and T cell functions. Previously, *IL10$^{-/-}$* mice were shown to develop T cell-mediated colitis (Kuhn et al., 1993). Using this information to further study TRPA1 role in T cell mediated colitis, *IL10$^{-/-}$Trpa1$^{-/-}$* mice were generated by mating the two *IL10$^{-/-}$* and *Trpa1$^{-/-}$* strains together.

2. METHODS

2.1. Antibodies and Reagents

For mouse T cell stimulation, we used hamster anti-mouse CD3 (145-2c11) and anti-mouse CD28 (PV-1, both from BioXcell) antibodies. A goat anti-hamster antibody (I1903-04P, US Biological) was used in cross-linking experiments. For human T cell stimulation, anti-human CD3 (UCHT1, BD Biosciences) and anti-human CD28 (CD28.2, eBioscience) antibodies were used. Other reagents included the following: PMA (phorbol 12-myristate 13-acetate) and capsaicin (M2028) were purchased from Sigma. Ionomycin (BP2527-1) was purchased from Fisher Scientific. The TRPV1 inhibitor BCTC was purchased from Tocris. The fluorescent dyes, CFSE (carboxyfluorescein diacetate succinimidyl ester) and Fura-2 AM were purchased from Invitrogen. For immunoblot analysis, we used the following antibodies: anti-TRPA1 (sc-32355; Santa Cruz), anti-TRPV1 (ACC-030, Alomone), anti-CD3(sc-1127, Santa Cruz) and anti-b-actin (AC-74, Sigma). For immunofluorescence studies, we used anti-TRPA1, anti-TRPV1 (Abs identified above), anti-PGP9.5 (MCA-BH7, Millipore), anti-mouse CD4 (GK1.5; eBioscience) and anti-human CD3 (UCHT1, eBioscience) primary antibodies, and AF488- (A11078), AF546- (A10040) and AF647- (A-21235) conjugated secondary antibodies (all from Invitrogen)

2.2. Mice

Initial breeding pairs of *Trpa1*$^{-/-}$ mice on the C57BL/6 background were kindly provided by Dr. David Julius (UCSF) and then bred in our vivarium. C57BL/6 (WT), *Trpv1*$^{-/-}$, *Il10*$^{-/-}$, *Rag1*$^{-/-}$ and OVA-transgenic (OT-II) mice on the C57BL/6 background (all from the Jackson Laboratories) were bred in our vivarium under specific pathogen-free (SPF) or enhanced-barrier SPF (*Rag1*$^{-/-}$ strains) conditions. For the generation of *Il10*$^{-/-}$*Trpa1*$^{-/-}$, *Rag1*$^{-/-}$*Trpa1*$^{-/-}$ and *Trpa1*$^{-/-}$ OT-II mice, *Trpa1*$^{-/-}$ mice were intercrossed with *Il10*$^{-/-}$, *Rag1*$^{-/-}$ or OT-II mice, respectively. All mice were bred in our vivarium for more than 6 months and were genotyped before they were used in any experiments. All experimental procedures were conducted in accordance with UCSD Institutional Animal Care and Use Committee (IACUC) guidelines.

2.3. Il10$^{-/-}$ *Model of Spontaneous Colitis*

Age- and sex-matched *Il10*$^{-/-}$ and *Il10*$^{-/-}$*Trpa1*$^{-/-}$ mice were randomly assigned to the different groups and wasting disease was monitored periodically. The Disease Activity Index (DAI; i.e., the combined score of weight loss and presence of blood in the stools) was determined as follows: for loss in body weight, 0 = no loss, 1 = 5-10%, 2 = 10-15%, 3 = 15-20%, 4 = over 20%; for hemoccult, 0 = no blood, 2 = positive, and 4 = gross blood; as

previously described. For co-housing experiments, 4-week old *Il10*$^{-/-}$ and *Il10*$^{-/-}$ *Trpa1*$^{-/-}$ mice were co-housed in the same cage to allow colonization with the same microflora. Mice were sacrificed at 12-13 weeks of age for analysis as previously described.

2.4. T Cell Adoptive Transfer Model of Colitis

SP naïve WT, *Trpa1*$^{-/-}$, *Trpa1*$^{-/-}$*Trpv1*$^{-/-}$, *Il10*$^{-/-}$ or *Il10*$^{-/-}$*Trpa1*$^{-/-}$ CD4^{+} T cells were enriched by immunomagnetic negative selection, stained with CD4-APC, CD25-AF488 and CD45RB-PE (eBioscience) and sorted into naïve (CD4^{+}CD45RB^{high}CD25^{-}) and regulatory (CD4^{+}CD45RB^{low}CD25^{+}) populations (usual purity >98%) with a BD FACSAria II Flow Cytometer. Eight to 10-week old *Rag1*$^{-/-}$ or *Rag1*$^{-/-}$*Trpa1*$^{-/-}$ sex and age-matched recipients were reconstituted by i.p. injection with 3x10^{5} naïve CD4^{+} T cells from sex-matched donor mice from the different genotypes. For the co-transfer experiments, 1.5x10^{5} regulatory T cells from WT mice were co-injected with the naïve CD4^{+} T cell population. After reconstitution, mice were monitored weekly for signs of intestinal inflammation such as weight loss and diarrhea. The DAI was determined as indicated above. Diseased animals were sacrificed for analysis as previously described.

2.5. Histological Analysis

Histological analysis was performed as previously described. Briefly, entire colons were excised, opened longitudinally, rolled onto a wooden dowel and fixed with 10% buffered formalin. Paraffin sections (5μm) were stained with H&E. Colonic epithelial damage was scored blindly as follows: 0 = normal; 1 = hyperproliferation, irregular crypts, and goblet cell loss; 2 = mild to moderate crypt loss (10–50%); 3 = severe crypt loss (50–90%); 4 = complete crypt loss, surface epithelium intact; 5 = small to medium sized ulcer (<10 crypt widths); 6 = large ulcer (>10 crypt widths). Infiltration with inflammatory cells was scored separately for mucosa (0 = normal; 1 = mild; 2 = modest; 3 = severe), submucosa (0 = normal; 1 = mild to modest; 2 = severe), and muscle/serosa (0 = normal; 1 = moderate to severe). Scores for epithelial damage and inflammatory cell infiltration were added, resulting in a total colitis scoring range of 0-12.

2.6. Human samples

For TRPA1 and TRPV1 knockdown experiments, blood samples were collected from healthy blood donors. For immunofluorescence staining of human tissue sections, archived specimens of human colonic biopsies from healthy controls and patients with active UC and CD who had undergone routine colonoscopy for clinical indications were used. The study was approved by the Institutional Review Board of the VA San Diego Healthcare

System. Prospective specimens of colon were obtained from patients undergoing colonoscopy for routine clinical indications after giving informed consent as previously described. The clinical status was confirmed with chart review, and all specimens were de-identified prior to use. The study was approved by the Institutional Review Board of the University of California, San Diego.

2.7. Isolation of mRNA and PCR

Isolation of total RNA from mouse cells or colonic tissues was carried out with the RNeasy Mini Kit (Qiagen) following the manufacturers protocol. Total RNA was extracted from human colonic biopsies as previously described. Two hundred fifty nanograms to 1 mcg of RNA sample was used for reverse transcription and cDNA synthesis using qScript cDNA superMix (Quanta Biosciences). Quantitative real-time PCR (q-PCR) was performed on an AB7300 (Applied Bisosytems) using PerfeCTa SYBR Green FastMix (Quanta Biosciences) as previously described. q-PCR primers for specific target genes were designed based on their reported sequences and synthesized by IDT Technologies. For TRPA1 expression analysis, PCR products from q-PCR reactions were run on a 2% agarose gel stained with SYBR Safe DNA (Invitrogen).

2.8. Colonic Explants (CE)

Colonic explants were processed as previously described. Briefly, colonic longitudinal sections (≈ 3-4 mm wide) were weighed and washed in RPMI 1640 medium containing 100 μg/mL streptomycin and 100 U/mL penicillin. The CE were cultured for 24h in complete RPMI medium at 37°C and 5% CO_2. Culture supernatants were then collected and cytokine levels measured (ELISA).

2.9. Isolation and Stimulation of $CD4^+$ T cells

$CD4^+$ T cells were isolated from the SP or MLN using a $CD4^+$ T cell negative selection kit (Stemcell #19852). Purity of the enriched populations was measured by FACS staining for CD4 and TCR and was typically > 94% for spleen and >97% for MLN derived $CD4^+$ T cells. For some experiments, naïve $CD4^+$ T cells were isolated using a naïve $CD4^+$ T cell negative selection kit (Stemcell #19765). For ELISA, $CD4^+$ T cells were stimulated with 10 □g/mL plate-bound anti-CD3 and 1 μg/mL soluble anti-CD28 (Abs identified above) in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/mL penicillin and 100 μg/mL streptomycin (complete RPMI medium) unless mentioned otherwise. In some experiments, cells were preincubated for 30 min with BCTC (10 μg/mL) before

and during the stimulation with anti-CD3/28 Abs. Twenty-four- and 48-hour culture supernatants were collected for cytokine production analysis (ELISA kits, eBioscience). For flow cytometry analysis, $CD4^+$ T cells were stimulated with 1 μg/mL plate-bound goat anti-hamster and 2 μg/mL soluble hamster anti-mouse CD3 and anti-mouse CD28 (Abs identified above) in complete RPMI 1640 for 5h (intracellular cytokine staining) or 24h (surface activation markers) as previously described.

2.10. In vitro T Cell Differentiation Assays

Naïve $CD4^+$ T cells were isolated from the spleen of WT or *Trpa1*$^{-/-}$ mice and stimulated ($1x10^6$ cells/well in flat-bottom 24 well plates) with goat anti-hamster (1 μg/mL, plate-bound), hamster anti-mouse CD3 (2 μg/mL, soluble) and anti-mouse CD28 (2 mcg/mL, soluble) (Abs identified above) in presence of: recombinant murine (rm) IL-12 (10 ng/mL, eBioscience) and neutralizing anti-IL-4 Ab (BVD6-24G2, BioXcell, 10 μg/mL) in complete RPMI medium for Th1 differentiation or rm IL-6 (20 ng/mL) and TGF-β (4 ng/mL, both from eBioscience), neutralizing anti-IFN-gamma (XMG1.2) and anti-IL-4 Abs (BVD6-24G2, both from BioXcell) both at 10 μg/mL in complete IMDM medium for Th17 differentiation. At day 2, rm IL-2 (10 ng/mL, eBioscience) was added into the Th1 cultures. After 5 days, $CD4^+$ T cells were recovered and re-stimulated with 10 μg/mL plate-bound anti-CD3 and 1 μg/mL soluble

anti-CD28 Abs (Abs identified above) in the presence of GolgiStop (BD Biosciences) for 5h. Intracellular cytokine staining (ICS) was performed as described in the Flow cytometry analysis section below.

2.11. Flow Cytometry Analysis

TRPV1 staining was performed as previously described. For surface activation markers staining, $CD4^+$ T cells were isolated from the spleen of WT or *Trpa1*$^{-/-}$ mice and stimulated with goat anti-hamster (1 μg/mL, plate-bound), hamster anti-mouse CD3 (2 μg/mL, soluble) and CD28 (2 μg/mL, soluble) (Abs identified above) for 24h. Cells were then recovered and stained with anti-mouse CD4 (GK1.5), CD25 (PC61.5), CD69 (H1.2F3), OX40 (OX86), or CD44 (IM7) Abs (all from eBioscience) for 30 min at 4ºC. For intracellular cytokine staining, $CD4^+$ T cells were stimulated as indicated above in the presence of GolgiStop (BD Biosciences) for 5h. After CD4 surface staining, cells were fixed, permeabilized and stained with IFN-gamma (XMG1.2), IL-2 (JES6-5H4), or IL-17A (17B7) Abs according to the manufacturer's instructions (eBioscience). Cells were analyzed on an Accuri C6 flow cytometer and data were computed using FlowJo software (TreeStar).

2.12. DCs and OT-II T Cells Co-culture

Bone marrow derived DCs (BMDCs) were cultured and harvested as previously described. $CD11c^+$ BMDCs were then isolated using a $CD11c^+$ positive selection kit (Stemcell #18758) and loaded with 10 μg/mL of $I\text{-}A^d$-restricted OVA peptide ($OVA_{323\text{-}339}$: ISQAVHAAHAEINEAGR, PeptidoGenic Research) for 2h before the addition of *Trpa1*$^{-/-}$ OT-II or control OT-II $CD4^+$ T cells to the culture (DC/T cell ratio of 1:2). After three days of co-culture, $CD4^+$ T cells were recovered and re-stimulated with anti-CD3/28 Abs for 5 (ICS) or 24h (ELISA).

2.13. Immunofluorescence Staining and Confocal Microscopy Analysis

SP $CD4^+$ T cells were isolated as described above and cytocentrifuged (Cytospin 2, Shandon) onto microscope slides for 3 min at 500 rpm. The air-dried cytospin preparations were fixed in 4% paraformaldehyde/PBS for 10 min at room temperature and permeabilized with 0.2% Triton X-100 for 15 min at 4ºC. Cells were washed, blocked with 5% BSA / 0.2% Triton X-100 in PBS for 30 min at 4ºC and stained with a goat anti-TRPA1 Ab (sc-32355, Santa Cruz, 1:50 dilution) and with a rat anti-CD4-PE (GK1.5, eBioscience, 1:100 dilution) or with a rabbit anti-TRPV1 Ab (ACC-030, Alomone, 1:100 dilution) overnight at 4ºC. Cells were then washed and incubated with anti-goat-AF-488 (A11078, Invitrogen, 1:200 dilution) and anti-rabbit-AF-546 (A10040,

Invitrogen, 1:200 dilution) Abs for 1h at room temperature. One cytospin preparation served as a negative control staining using the secondary Abs alone. Cells were finally counterstained with Hoechst 33258 (50 ng/mL, Invitrogen) for 10 min at RT, rinse with deionized water and slides were mounted in ProLong Gold antifade reagent (Invitrogen). For immunofluorescence staining of human colon sections, colonic biopsies from healthy controls and patients with active UC or CD were fixed in 10% buffered formalin and embedded in paraffin using standard methods. Immunofluorescence staining of 4- to 6-µm paraffin-embedded sections was performed as described above with primary Abs against human CD3 (UCHT1, eBioscience, 1:100 dilution), PGP9.5 (MCA-BH7, Millipore, 1:1000 dilution), TRPA1 and TRPV1 (Abs identified above) and detection with AF488-, AF546- (Abs identified above) and AF647-conjugated (A-21235, Invitrogen, 1:200 dilution) secondary antibodies. The fluorescence images were acquired using a 60x or 100x oil-immersion objectives on a confocal laser-scanning microscope (Olympus IX81) with Fluoview software. Co-localization studies were performed using Velocity® software.

2.14 Calcium Imaging

$[Ca^{2+}]_i$ levels in $CD4^+$ T cells were measured by Fura-2 fluorescence ratio digital imaging as previously described.[8] Briefly, $CD4^+$ T cells were isolated from the spleen of WT or *Trpa1*$^{-/-}$ mice, loaded with 5 μM Fura-2 acetoxymethyl ester (AM) (Molecular Probes) [dissolved in 0.01% Pluronic F-127 plus 0.1% DMSO in normal physiological salt solution (PSS)] in culture medium for 50 min, and then washed in PSS. Cells were attached on BD Cell-Tek™ coated coverslips. Thereafter, the coverslips with the $CD4^+$ T cells were mounted in a perfusion chamber on a Nikon microscope stage and Fura-2 fluorescence ratio (510-nm light emission excited by 340- or 380-nm illuminations), as well as background fluorescence, was collected with the use of a ×40 Nikon UV-Fluor objective and an intensified CCD camera (ICCD200). The fluorescence signals emitted from the cells were monitored continuously every 5-s interval using a MetaFluor Imaging System (Universal Imaging, Downingtown, PA) and recorded for later analysis. PSS used in digital Ca^{2+} measurement contained the following (in mM): 140 Na^+, 5.0 K^+, 2 Ca^{2+}, 147 Cl^-, 10 HEPES, and 10 glucose, pH 7.4. For the Ca^{2+}-free PSS solution, Ca^{2+} was omitted, and 0.5 mM EGTA was added to prevent possible Ca^{2+} contamination. The osmolality for all solutions were ~300 mosM/L. Cells were stimulated with 100 μM of mustard oil, 10 μM capsaicin, 1 □M ionomycin, 1 mcM thapsigargin, or with 10 mcg/mL of biotinylated anti-CD3 Ab (clone 145-2C11, eBioscience) and 10 mcg/mL of streptavidin (eBioscience).

In some experiments, cells were preincubated for 10 min with BCTC (10 μg/mL) before and during the stimulation with capsaicin. Peak Ca^{2+} ratios were calculated as the maximal amount of Ca^{2+} influx. For each experiment, 40-50 individual cells were analyzed using OriginPro (Originlab) analysis software. All experiments were performed at room temperature (22-25°C).

2.15 TRPA1 and TRPV1 Knockdown

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density-gradient centrifugation from healthy blood donors. $CD4^+$ T cells were further enriched from PBMCs using a negative selection kit (Stemcell #19052) and transfected with 200 nM of TRPA1, TRPA1+V1 siRNAs (sc-44780 and sc-36826, Santa Cruz Biotechnology) or a control siRNA (Non-Targeting siRNA #1; Dharmacon) at a cell density of 10×10^6 cells per 100 μl of human T cell nucleofector solution (VPA-1002; Lonza) with the Amaxa Nucleofector II device using program U-014 (Lonza). After nucleofection, cells were immediately transferred into prewarmed complete RPMI medium and cultured in a 24-well plate at 37°C in a 5% CO2 humidified incubator. Six hours after transfection cells were stimulated with anti-human CD3 and anti-human CD28 (Abs identified above), 0.01 and 1 μg/mL, respectively. After a total of 16 and 48 hours, the cells were analyzed for cytokine expression levels (IL-2 [q-PCR], IFN-gamma [ELISA]) and TRPA1 and TRPV1 knockdown efficiency (immunoblot), respectively.

2.16 Immunoblotting

To evaluate TRPA1 and TRPV1 knockdown efficiency, $CD4^+$ T cells ($5x10^6$) were lysed 48h post-transfection with RIPA buffer (Teknova) supplemented with protease inhibitors (Roche) for 5 min on ice. The protein concentration was determined with a protein-quantification kit (Bio-Rad). Protein samples (10 mcg/lane) were separated through SDS polyacrylamide gel electrophoresis (4-12% gradient, Invitrogen) and then transferred to PVDF membranes (Millipore). The blots were blocked by 5% BSA/0.3% Tween 20 in PBS for 45 min at RT, and incubated with anti-TRPA1 (1:200 dilution), anti-TRPV1 (1:200 dilution), anti-CD3 (1:1000 dilution) or anti-b-actin (1:10.000 dilution) (Abs identified above) overnight at 4ºC. The blots were washed and incubated with their corresponding HRP-conjugated secondary Abs from Jackson Lab (1:5000 to 1:10.000 dilution) for 45 min at RT and developed in ECL solution (Pierce).

2.17 *Statistical Analysis*

Data are presented as mean ± SEM. The statistical significance between two groups was determined using unpaired Student t-test with two-tailed *p*-values. The statistical significance between more than two groups was determined using one-way ANOVA with post hoc Bonferroni's test. For time course experiments *in vivo*, two-way ANOVA with post hoc Bonferroni's test was used. All statistics were computed using PRISM software (GraphPad).

3. RESULTS

3.1. TRPA1 channels are present in CD4+ T cells

The presence of TRPA1 in CD4+ T cells was confirmed by utilizing q-PCR to measure the *Trpa1* mRNA expression levels from isolated spinal cord, CD4+ T cells (SP) and CD11c+ BMDCs from both WT and *Trpa1*$^{-/-}$ mice. *Trpa1* transcripts were evident in the WT spinal cord and WT CD4+ T cells (SP), but not in the WT CD11c+ BMDCs (Fig. 1A, B). To verify the expression of TRPA1 protein in CD4+ T cells, immunofluorescence staining and confocal microscopy was performed. The result displayed that in CD4+ T cells, TRPA1 is heavily expressed in the plasma membrane (Fig. 1C).

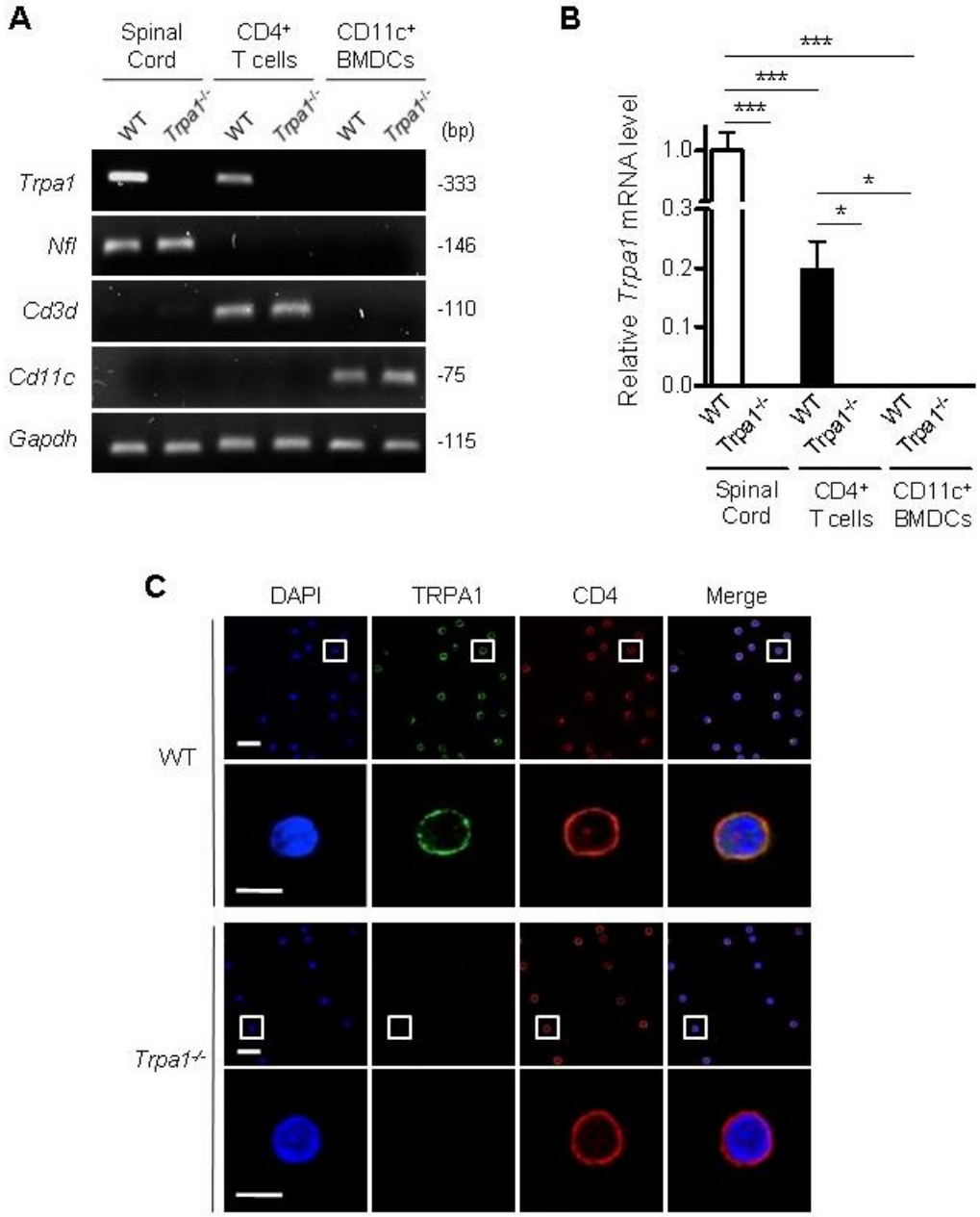

Figure 1. *TRPA1 channels are present in CD4+ T cells.*

(A) Spinal cord, SP CD4$^+$ T cells and bone marrow-derived dendritic cells (BMDCs) were isolated from C57BL/6 (i.e., WT) and *Trpa1*$^{-/-}$ mice, and *Trpa1* mRNA expression was analyzed by q-PCR. PCR products were separated on a 2% agarose gel. (B) Comparative expression of *Trpa1* in spinal cord, SP CD4$^+$ T cells and CD11c$^+$ BMDCs by q-PCR. Mean ± SEM of 6 mice is shown *p <0.05; ***p <0.001 (one-way ANOVA with post hoc Bonferroni's test). (C) Immunofluorescence labeling of TRPA1 and CD4 in WT and *Trpa1*$^{-/-}$ SP CD4$^+$ T cells. Scale bars = 20 um (overview insets), 5 um (high-power field

3.2. Trpa1$^{-/-}$ CD4+ T cells are hyperactivated and display a Th1 bias

To see the effect of *Trpa1* gene knockout, the cytokines from *Trpa1*$^{-/-}$ and WT CD4+ T cells were analyzed when stimulated with anti-CD3/28 Abs. Interestingly, there was a significant increase in IFN-gamma and IL-2 cytokines in the *Trpa1*$^{-/-}$ CD4+ T cells compared to the WT CD4+ T cells (Fig. 2A). This suggests that *Trpa1*$^{-/-}$ CD4+ T cells has a Th1 bias. This finding was confirmed with the use of PMA/IONO in both WT and *Trpa1*$^{-/-}$ CD4+ T cells that reversed the phenotype of *Trpa1* gene knockout for IFN-gamma and IL-2 (Fig. 2B).

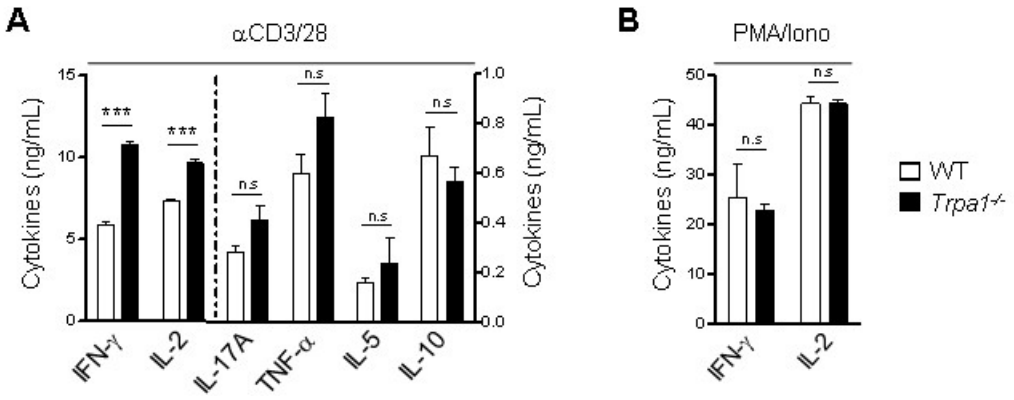

Figure 2. *Trpa1*$^{-/-}$ *CD4*$^{+}$ *T cells are hyperactivated and display a Th1 bias*.

(A) WT and *Trpa1*$^{-/-}$ SP CD4^{+} T cells were stimulated with anti-CD3/28 Abs for 48h and cytokine production was assessed (ELISA). Mean ± SEM (n = 4 mice/group) is shown. (B) Cells were stimulated with PMA (25 ng/mL) and ionomycin (500 nM) for 48h and cytokine production was assessed (ELISA) as A. n.s: not significant; **p* <0.05; ***p* <0.01; ****p* <0.001; *****p* <0.0001(two-tailed Student t-test).

3.3. *TRPA1 deficiency aggravates colitis in IL10 KO mice*

To investigate the role of TRPA1 in vivo during T cell mediated colitis , *Trpa1*$^{-/-}$ and *IL10*$^{-/-}$ mice were crossed to create a *IL10*$^{-/-}$*Trpa1*$^{-/-}$ DKO strain. It is already known that *IL10*$^{-/-}$ mice develop T cell mediated colitis. *IL10*$^{-/-}$*Trpa1*$^{-/-}$ mice had a significant increase in the Disease Activity Index (DAI) which measures the level of spontaneous colitis compared to *IL10*$^{-/-}$ mice during 11 to 13 weeks of age, with the trend starting at 8 weeks of age (Fig. 3A,B). In addition, histological analysis of the *IL10*$^{-/-}$*Trpa1*$^{-/-}$ mice colons compared to the *IL10*$^{-/-}$ mice colons revealed a significant colitis score that takes in consideration of crypt loss, increase proliferation of epithelial cells, and an infiltration into the mucosal layer by mononuclear cells (Fig. 3C). IFN-gamma was also found to be increased in *ex vivo* colonic explants in *IL10*$^{-/-}$*Trpa1*$^{-/-}$ mice compared to *IL10*$^{-/-}$ mice (Fig. 3D).

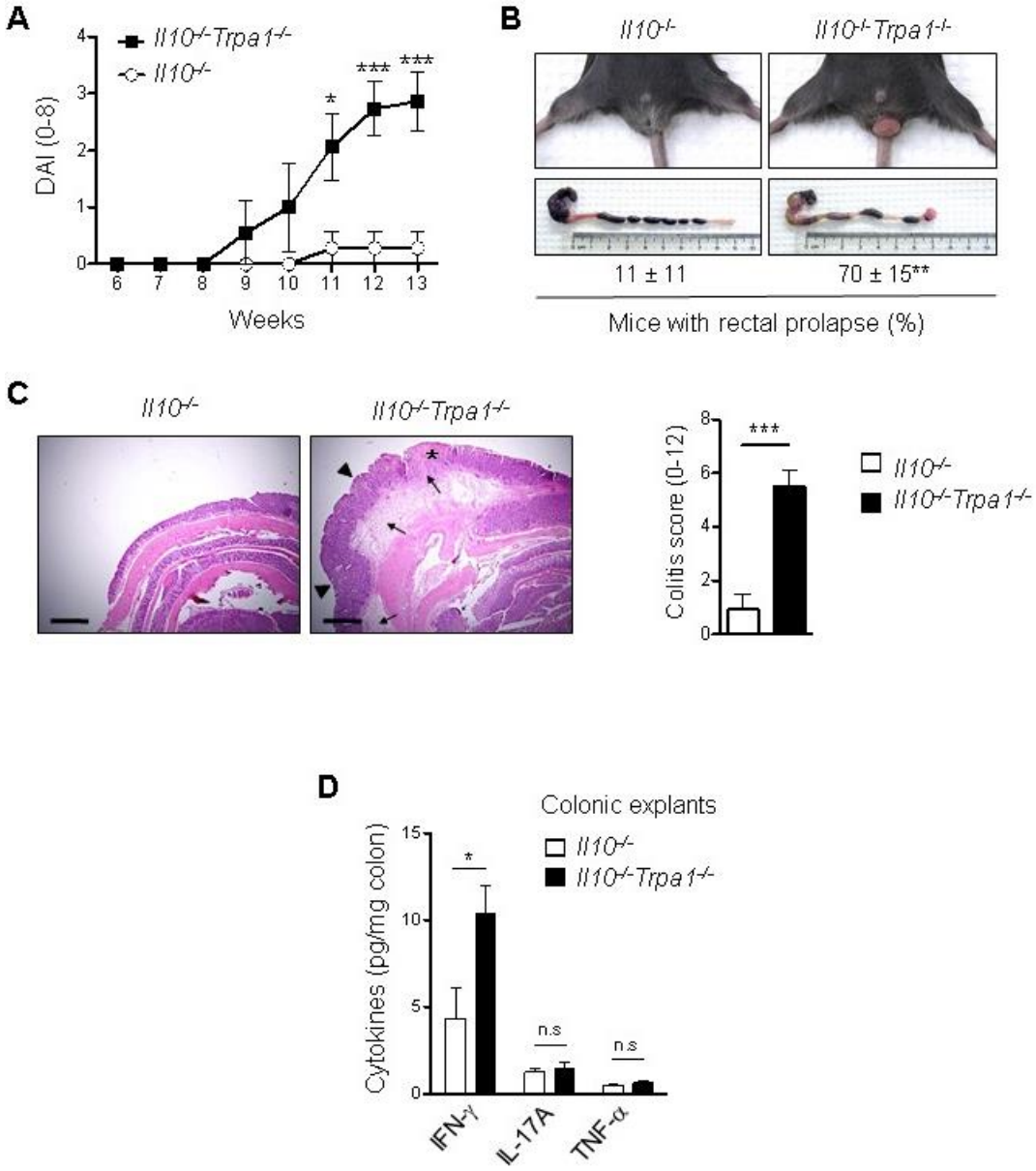

Figure 3. *TRPA1 deficiency aggravates colitis in Il10$^{-/-}$ mice.*

(A) Time course of Disease Activity Index for *Il10$^{-/-}$* and *Il10$^{-/-}$Trpa1$^{-/-}$* mice. (B) Representative pictures of the rectal prolapse and the colon of *Il10$^{-/-}$* and *Il10$^{-/-}$Trpa1$^{-/-}$* mice. (C) Left panel: Representative pictures of colon sections stained with H&E. Scale bar = 500 um. *Il10$^{-/-}$Trpa1$^{-/-}$* mice developed severe colonic inflammation as judged by colonic wall thickening (black arrowheads), massive crypt loss (black asterisk), and marked infiltration of mononuclear cells in the mucosa and lamina propria (black arrows). Right panel: Colitis score of 12-week old *Il10$^{-/-}$* and *Il10$^{-/-}$Trpa1$^{-/-}$* mice. (D) Proinflammatory cytokine production by colonic explants after 24h of culture (ELISA). One representative experiment out of three is shown. Results are expressed as mean ± SEM (n = 9-10 [A,B] or 6-8 [C,D] mice/group). *p <0.05; **p <0.01; ***p <0.001 (two-way ANOVA with post hoc Bonferroni's test [A] or two-tailed Student t-test [B-F]).

3.4. IL10$^{-/-}$Trpa1$^{-/-}$ DKO naive CD4+ T cells display exacerbated colitogenic properties

To see if the absence of TRPA1 in CD4+ T cells cause the observed phenotype in *IL10$^{-/-}$Trpa1$^{-/-}$* mice, a model of colitis by T cell transfer was performed. First, naive SP T cells from *IL10$^{-/-}$* and *IL10$^{-/-}$Trpa1$^{-/-}$* donor mice were isolated to perform an adoptive transfer to Rag1-/ - recipient mice. WT C57BL Tregs were also co-transferred with WT naive CD4+ T cells to observe as a control sample. By 30 days post transfer, there was a significant decrease in body weight and increase in Disease Activity Index in mice that had *IL10$^{-/-}$Trpa1$^{-/-}$* T cells transferred compared to the *IL10$^{-/-}$* T cells (Fig 4A,B). To verify the severity of colitis, histological analysis of the colons were done and results were consistent in that the recipients of *IL10$^{-/-}$Trpa1$^{-/-}$* T cells had a higher colitis score compared to recipients of *IL10$^{-/-}$* T cells (Fig 4C,D). Additionally, the CD4+ T cells that were isolated from the MLN or spleen of *IL10$^{-/-}$Trpa1$^{-/-}$* T cells recipients compared to *IL10$^{-/-}$* T cell recipients had a significant increase in IFN-gamma (Fig. 4E).

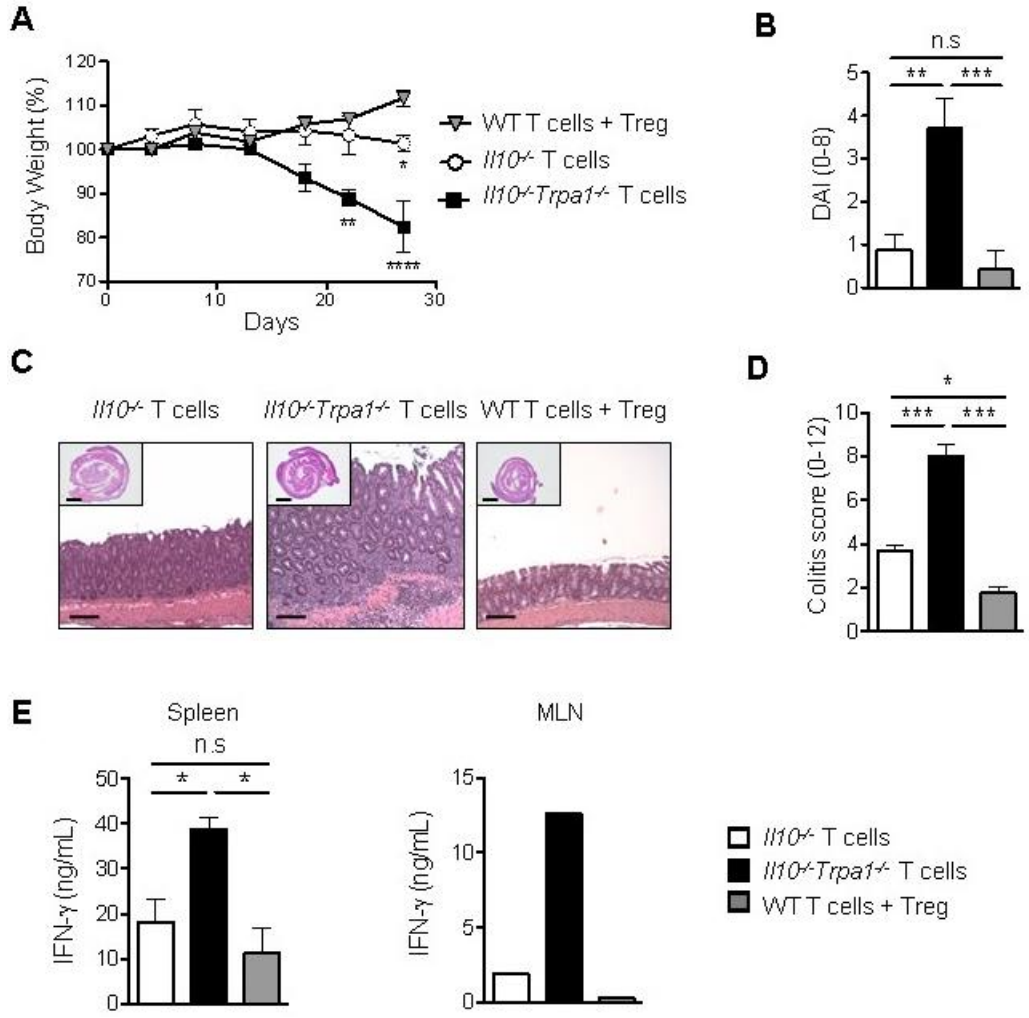

Figure 4. *Il10$^{-/-}$Trpa1$^{-/-}$ naïve CD4^{+} T cells induce an exacerbated colitis in the adoptive transfer model.*

(A) Percentage of initial body weight of *Rag1$^{-/-}$* recipient mice 4 weeks post-adoptive transfer with 3x10^{5} *Il10$^{-/-}$* or *Il10$^{-/-}$Trpa1$^{-/-}$* FACS-sorted naïve (CD4^{+}CD45RB^{high}CD25^{-}) T cells, or with 3x10^{5} WT naïve CD4^{+} T cells + 1.5x10^{5} WT Treg (CD4^{+}CD45RB^{low}CD25^{+}). Statistical analysis compared *Il10$^{-/-}$Trpa1$^{-/-}$* T cell group to *Il10$^{-/-}$* T cell group and *Il10$^{-/-}$* T cell group to WT T cells + Treg group. (B) Disease Activity Index (DAI) in the three recipient groups. (C) Representative pictures of colon sections stained with H&E. Scale bars = 1 mm (overview insets), 100 μm (high-power fields). (D) Colitis score in the three recipient groups. (E) Cytokine production by SP or pooled MLN CD4^{+} T cells isolated from *Rag1$^{-/-}$* recipients, 24h after re-stimulation with anti-CD3/28 Abs (ELISA). Mean ± SEM (n = 7 mice/group [*Il10$^{-/-}$* or *Il10$^{-/-}$Trpa1$^{-/-}$* T cell groups] or 4 mice/group [WT T cells + Treg group]) of one representative experiment out of two is shown. n.s: not significant; **p* <0.05; ***p* <0.01; ****p* <0.001; *****p* <0.0001 (one- [B-F] or two-way [A] ANOVA with post hoc Bonferroni's test).

3.5. IL10$^{-/-}$Trpa1$^{-/-}$ DKO mice display increased Th1-mediated inflammatory responses

To develop a better understanding of the inflammatory profile of *IL10$^{-/-}$ Trpa1$^{-/-}$* T cells, spleen and MLN from *IL10$^{-/-}$Trpa1$^{-/-}$* were analyzed compared to *IL10$^{-/-}$* mice. By 12 weeks of age, it was clear that both the spleen and MLN were larger in *IL10$^{-/-}$Trpa1$^{-/-}$* mice compared to *IL10$^{-/-}$* mice (Fig. 5A). The CD4+ T cells that were isolated from *IL10$^{-/-}$Trpa1$^{-/-}$* mice were found to have a significant increase in IFN-gamma and IL-2 levels compared to the CD4+ T cells isolated from *IL10$^{-/-}$* mice, indicating a Th1 bias (Fig 5B).

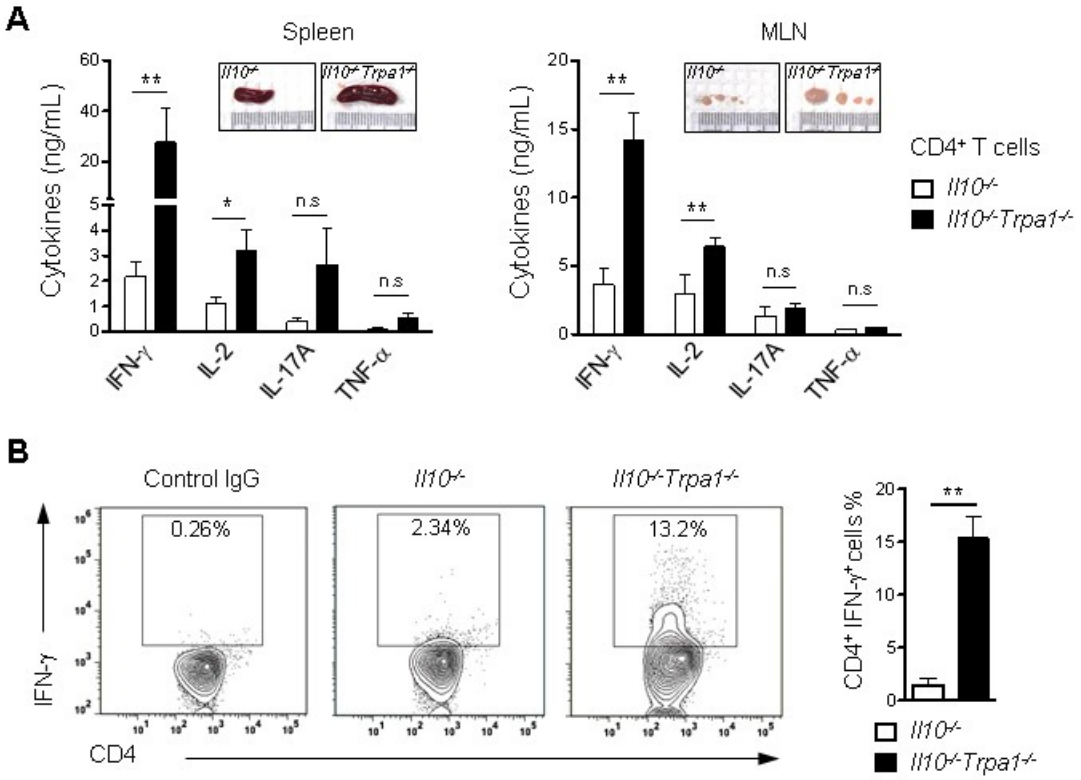

Figure 5. *Il10$^{-/-}$Trpa1$^{-/-}$ mice display increased proinflammatory CD4^{+} T cell response.*

(A) Representative pictures of the spleen (SP) and the mesenteric lymph nodes (MLN) harvested from 12-week old *Il10$^{-/-}$* and *Il10$^{-/-}$Trpa1$^{-/-}$* mice (overview insets). Cytokine production by SP or MLN CD4^{+} T cells isolated from each group, 24h after re-stimulation with anti-CD3/28 Abs (ELISA). (B) SP CD4^{+} T cells were stimulated or not with anti-CD3/28 Abs for 5h. Cells were then stained for CD4 and intracellular staining was performed for IFN-gamma. Representative panels of intracellular cytokine production in the different conditions are shown. Mean % ± SEM of CD4^{+}IFN-gamma^{+} cells is indicated (right panel). Results are expressed as mean ± SEM (n = 6 [A] or 4 [B] mice/group). n.s: not significant; *p <0.05; **p <0.01; ***p <0.001 (two-tailed Student t-test).

3.6. TRPV1 hyperactivation is responsible for phenotype of Trpa -/- CD4+ T cells

To develop a better understanding of the *Trpa1*$^{-/-}$ CD4+ T cells, the mechanism responsible for the phenotype was investigated. Due to the previous studies that show TRPV1 channel expression in CD4+ T cells and also the direct interaction between TRPA1 and TRPV1 channels, TRPV1 function and expression was investigated in both WT and *Trpa1*$^{-/-}$ CD4+ T cells. First, confocal microscopy was performed by TRPV1 and TRPA1 double immunofluorescence staining to determine the possibility of co-localization of the two TRP channels. The results show that there is a close degree of localization between TRPV1 and TRPA1 in the plasma membrane (Fig 6A).

Interestingly in *Trpa1*$^{-/-}$ CD4+ T cells, the activity of TRPV1 channel is increased, as shown by the whole-cell perforated patch current. A TRPV1 agonist, capsaicin, was used to induce the activity of the channels. With the use of capsaicin, the current was significantly increased in *Trpa1*$^{-/-}$ CD4+ T cells compared to WT CD4+ T cells. A TRPV1 antagonist, BCTC, was used to treat *Trpa1*$^{-/-}$ CD4+ T cells to confirm that the observed effect was caused by TRPV1. BCTC treatment reversed the effect of the CAP-induced calcium influx in the TRPA1$^{-/-}$ cells as well as reversed the increased levels of IFN-gamma (Fig. 6B,C).

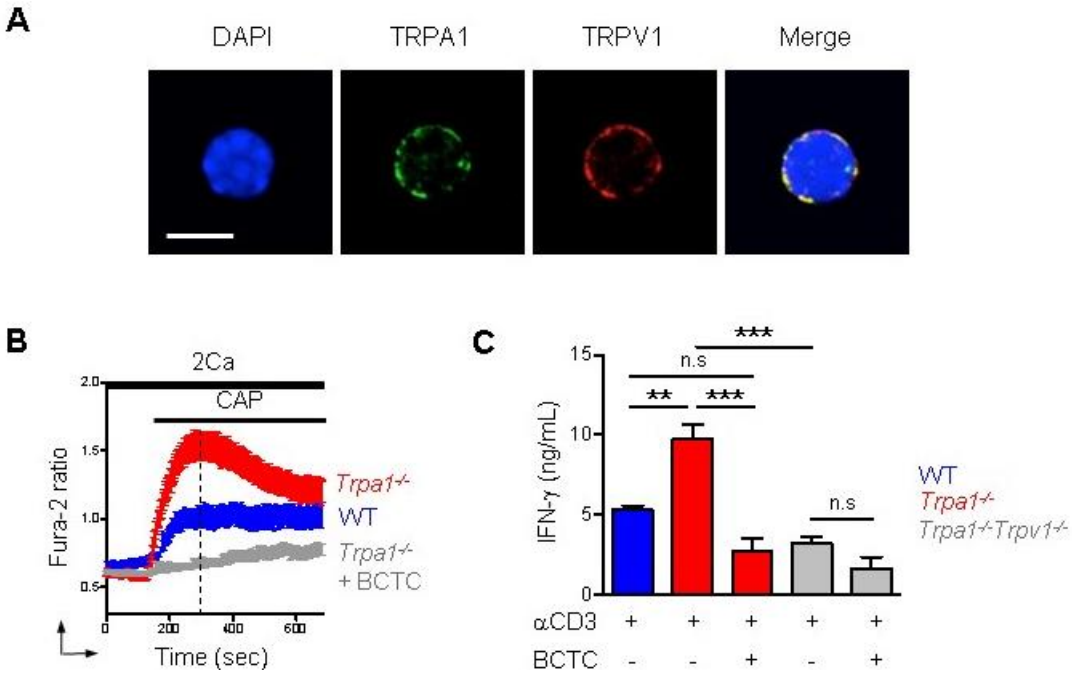

Figure 6. *TRPV1 hyperactivation is responsible for the phenotype of Trpa1$^{-/-}$ CD4^{+} T cells.*

(A) TRPA1 and TRPV1 co-localization in CD4^{+} T cells as determined by double immunofluorescence labeling. Resting WT SP CD4^{+} T cells were stained with TRPA1 and TRPV1 Abs and analyzed by confocal microscopy. DAPI (left panel) was used as a nuclear counterstain. TRPA1-AF488 (mid-left panel), TRPV1-AF546 (mid-right panel) and the merge (right panel) are shown. The yellow color in the merge panel indicate areas of TRPA1 and TRPV1 co-localization at the plasma membrane. Scale bar = 5 μm. (B) WT and *Trpa1$^{-/-}$* SP CD4^{+} T cells were loaded with Fura-2 AM and changes in $[Ca^{2+}]_i$ after capsaicin (CAP, 10 μM) stimulation were monitored by confocal imaging. *Trpa1$^{-/-}$* CD4^{+} T cells are hyperresponsives to CAP and pretreatment with a specific TRPV1 inhibitor, BCTC (10 μM), completely inhibits CAP-induced Ca^{2+} influx in these cells. (C) WT, *Trpa1$^{-/-}$* and *Trpa1$^{-/-}$Trpv1$^{-/-}$* SP CD4^{+} T cells were pretreated with BCTC (10 μM) or its vehicle and stimulated with anti-CD3/28 Abs for 24h. IFN-□□production was then analyzed by ELISA. Mean ± SEM (n = 3 mice/group) is shown. n.s: not significant; **p* <0.05; ***p* <0.01; ****p* <0.001 (one-way ANOVA with post hoc Bonferroni's test).

3.7. Increased infiltration of TRPA1+TRPV1+ T cells in inflamed colon sections of patients with active IBD.

To utilize the data generated from a murine model into a human model, colonic samples were analyzed from patients with IBD. Triple immunofluorescence staining was performed for colon sections for TRPA1, TRPV1, and CD3. Interestingly, a majority of the infiltrating CD3+ cells were both TRPA1+ and TRPV1+. Furthermore, the amount of these cells was significantly greater in both the CD and UC patients when compared to control subjects (Fig. 7A,B).

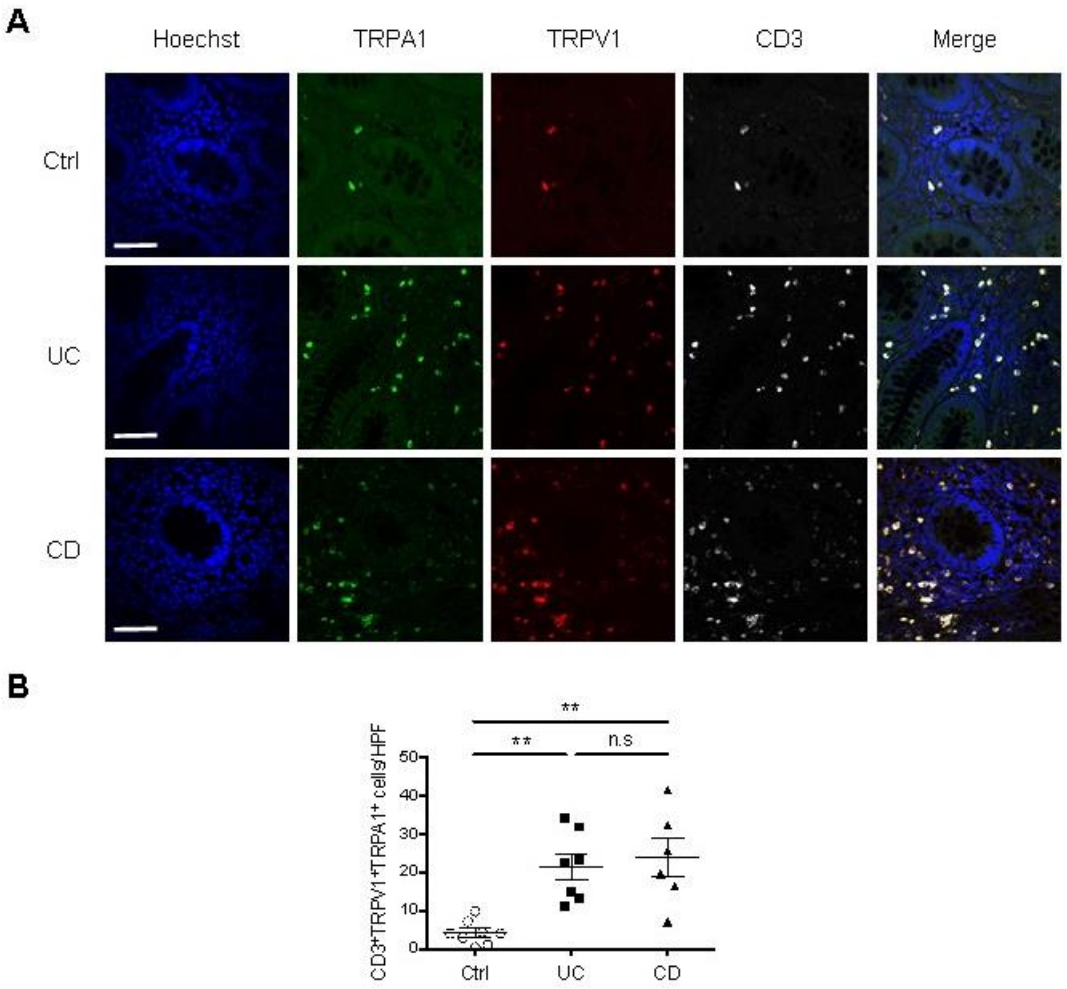

Figure 7. *Increased infiltration of TRPA1⁺TRPV1⁺ T cells in inflamed colon sections of patients with active IBD.*

(A) Representative pictures of immunofluorescence staining for TRPA1, TRPV1 and CD3 in colon sections from healthy controls (Ctrl), patients with active UC and patients with active CD. Nuclei were stained with Hoechst 33258 (blue). TRPA1 (green), TRPV1 (red), CD3 (white) and merged image are shown. Scale bars = 50 μm. (B) Quantification of $CD3^+TRPA1^+TRPV1^+$ cells per high-power field (HPF, average of 3-6 different 600x visual fields) in healthy controls (n = 8), patients with active UC (n = 7) and patients with active CD (n = 6). Each symbol represents an individual subject; horizontal lines indicate the mean ± SEM. n.s: not significant; ***p* <0.01 (one-way ANOVA with post hoc Bonferroni's test).

4. DISCUSSION

It is well known that TRPA1 ion channel is functionally expressed in sensory neurons in order to induce inflammation. Additionally, there is evidence that TRPA1 is abundant in all over the human body. In the sensory neurons, TRPA1 is co-localized with TRPV1. Our lab has previously found that TRPV1 is expressed in CD4+ T cells and plays a critical role in T-cell mediated colitis. Using these findings, our lab sought to find whether or not TRPA1 activity plays an essential role in CD4+ T cells.

Initial *in vitro* experiments found that there was indeed *Trpa1* protein found in WT CD4+ T cells by western blotting technique. As expected, the WT spinal cord cells did have *Trpa1* expression as a positive control. Through transcriptional analysis with q-PCR, it was found that WT CD4+ T cells had expression of *Trpa1* mRNA, whereas Trpa1 KO CD4+ T cells' expression of *Trpa1* mRNA was absent. To further confirm the expression of TRPA1 ion channel in CD4+ T cells, an immunofluorescent labeling and confocal microscopy of TRPA1 in WT and *Trpa1*$^{-/-}$ spleen CD4+ T cells was performed. This verified the expression of *Trpa1* in the plasma membrane of CD4+ T cells. These results suggest that TRPA1 channels exist in CD4+ T cells.

Next, to note the role of TRPA1 in CD4+ T cells, a *Trpa1* gene knockout was created and analyzed with a stimulating anti-CD3/28 Abs. Compared to the WT, *Trpa1*$^{-/-}$ T cells produced significantly higher levels of IFN-gamma and IL-2, which suggests a Th1 bias. Other pathways were investigated such as

Th2 and Th17, but there was no significant difference between WT and *Trpa1*$^{-/-}$ T cells in IL-10 and IL-17a cytokines. This observed effect of increase in Th1 cytokines was due to the knockout of TRPA1 because it was further confirmed that when cells are stimulated with PMA and ionomycin,a calcium ionophore, the effect is reversed and there is no longer a significant difference in IFN-gamma and IL-2 cytokine production between WT and *Trpa1*$^{-/-}$ T cells. Combining these results, *Trpa1*$^{-/-}$ CD4+ T cells are hyperactivated and display a Th1 bias.

In addition to the *in vitro* experiments, there must be an *in vivo* model to support our findings. In previous studies of T-cell mediated colitis, *IL10*$^{-/-}$ mice models were used as a standard. In this study, we created *IL10*$^{-/-}$*Trpa1*$^{-/-}$ double knockout to see the effects of TRPA1. Interestingly, it is found that the *IL10*$^{-/-}$*Trpa1*$^{-/-}$ mice, when compared to *IL10*$^{-/-}$ single knockout mice, show a significant increase in the Disease Activity Index (DAI) and colitis score, which accounts for the loss in body weight and positive hemocculs. A colonic explant was done and the cytokines were analyzed to find that indeed, IFN-gamma is still significantly increased, whereas IL-17a and TNF-a remains not significant. This gives evidence to see that TRPA1 deficiency in *IL10*$^{-/-}$mice results in an increase in colitis.

To still see if the *in vitro* finding of Th1 bias is still intact in the *in vivo* model, spleen and MLN from *IL10*$^{-/-}$*Trpa1*$^{-/-}$ mice were analyzed. It was obvious that both the spleen and MLN were enlarged in the *IL10*$^{-/-}$*Trpa1*$^{-/-}$ mice

compared to the *IL10*$^{-/-}$ mice, suggesting an increase in inflammatory properties in the *IL10*$^{-/-}$*Trpa1*$^{-/-}$ mice. Additionally, the CD4+ T cells were found to have a significant increase in IFN-gamma and IL-2 in *IL10*$^{-/-}$*Trpa1*$^{-/-}$ mice compared to *IL10*$^{-/-}$ mice. These results indicate an *in vivo* Th1 bias of TRPA1 deficiency in IL10 knockout mice.

It is previously known that TRPA1 and TRPV1 channels co-localize and interact with one another in neuronal cells. However, our lab proposes that this also exists at the CD4+ T cell level and that TRPV1 hyperactivation is responsible for the phenotype of *Trpa1*$^{-/-}$ CD4+ T cells. This was shown by a confocal microscopy of CD4+ T cells that displayed that both TRPA1 and TRPV1 appear to be centralized at the plasma membrane. The two ion channels also appear to be present at the same place in the plasma membrane of the CD4+ T cell. The effect of TRPV1 relating to TRPA1 was studied by utilizing BCTC, a TRPV1 antagonist. When WT and *Trpa1*$^{-/-}$ spleen CD4+ T cells were stimulated with capsaicin and monitored by confocal imaging, *Trpa1*$^{-/-}$ CD4+ T cells show a hyperresponsiveness to CAP. However, when BCTC was added to *Trpa1*$^{-/-}$ CD4+ T cells, this effect was negated. To further investigate this interesting finding, IFN-gamma production was analyzed from *Trpa1*$^{-/-}$ with and without BCTC, as well as *Trpa1*$^{-/-}$*Trpv1*$^{-/-}$ with and without BCTC. IFN-gamma was not increased in *Trpa1*$^{-/-}$ with BCTC and in *Trpa1*$^{-/-}$*Trpv1*$^{-/-}$ with or without BCTC. This suggests that there is the same decrease in IFN-gamma production when TRPV1 is inhibited. This suggests

that TRPV1 hyperactivation is responsible for the *Trpa1*$^{-/-}$ CD4+ T cells phenotype. Finally, to have a clinical impact, human samples of UC and CD were immunofluorescence stained for TRPA1, TRPV1, and CD3. The findings verify the increased levels of TRPA1+TRV1+ T cells in the colonic sections of patients with IBD. In both the UC and CD patients, the majority of infiltrating CD3+ cells were both TRPA1 and TRPV1+. Furthermore, the quantity of these double positive cells are significantly greater in both UC and CD patients when compared to the control subjects.

To summarize, our lab first established that there are infact functional TRPA1 channels in CD4+ T cells *in vitro*. Without these channels, the CD4+ T cells become hyperactivated and undergo a Th1 bias. Furthermore, a TRPA1 deficiency *in vivo* produces a significantly increased level of colitis in IL10 KO mice. The observed effect of the *IL10*$^{-/-}$*Trpa1*$^{-/-}$ mice was confirmed with T cell transfer. *IL10*$^{-/-}$*Trpa1*$^{-/-}$ donor mice had the naive CD4+ T cells isolated and then adoptively transferred into *Rag1*$^{-/-}$ recipient mice. By day 30, the recipient mice that had the *IL10*$^{-/-}$*Trpa1*$^{-/-}$ CD4+ T cells displayed a significant increase in colitogenic characteristics. This indicates that the observed phenotype is due to the effect of CD4+ T cells. In addition,CD4+ T cells from *IL10*$^{-/-}$*Trpa1*$^{-/-}$ mice also had a significant increase in IL-2 and IFN-gamma, suggesting a Th1 bias that is similar to the *in vitro* experiments. Further studies indicated that the phenotype of the *Trpa1*$^{-/-}$ CD4+ T cells is due to the hyperactivation of another family of TRP channels, TRPV1. This colocalization of TRPV1 and

TRPA1 in CD4+ T cells indicate a direct interaction that mediates the effects of colitis. The data suggests that as TRPV1 exacerbates colitis characteristics, TRPA1 acts as an inhibitor to the colitogenic TRPV1 effect. This is further supported in clinical subjects where patients with IBD (UC and CD) have an increased level of double positive TRPA1+TRPV1+ cells compared to the control subjects in the colon.

REFERENCES

Bautista D.M., Jordt S.E., Nikai T., Tsuruda P.R., Read A.J., Poblete J., Yamoah E.N., Basbaum A.I., and Julius D. (2006). TRPA1 mediates the inflammatory actions of environmental irritants and proalgesic agents. Cell *124*,1269-82.

Bertin S., Aoki-Nonaka Y., de Jong P.R., Nohara L.L., Xu H., Stanwood S.R., Srikanth S., Lee J., To K., Abramson L., Yu T., Han T., Touma R., Li X., Gonzalex-navajas J.M., Herdman S., Corr M., Fu G., Dong H., Gwack Y., Franco A., Jefferoes W.A., and Raz E. (2014). The ion channel TRPV1 regulates the activation and proinflammatory properties of CD4(+) T cells. *Nat* Immunol ,15,1055-63.

Caterina, M. J. (2015). An Introduction to Transient Receptor Potential Ion Channels and Their Roles in Disease. In TRP Channels as Therapeutic Targets: From Basic Science to Clinical Use. (pp. 1-12).

Conrad, K., Roggenbuck, D., and Laass, M.W. (2014). Diagnosis and classification of ulcerative colitis. Autoimmunity Reviews *13*, 4-5.

Engel M.A., Leffler A., Niedermirtl F., Babes A., Zimmermann K., Filipovic MR., Izydorczyk I., Eberhardt M., Kichko T.I., Mueller-Tribbensee S.M., Kahli M., Siklosi N., Nau C., Ivanovic-Burmazovic I., Neuhuber W.L., Becker C., Neurath M.F., and Reeh P.W. (2011). TRPA1 and substance P mediate colitis in mice. Gastroenterology, *141*,1346-58.

Funderburg, N. T., Stubblefield Park, S. R., Sung, H. C., Hardy, G., Clagett, B., Ignatz-Hoover, J., Harding, C. V., Fu, P., Katz, J. A., Lederman, M. M. and Levine, A. D. (2013), Circulating $CD4^+$ and $CD8^+$ T cells are activated in inflammatory bowel disease and are associated with plasma markers of inflammation. Immunology *140*, 87–97.

Geremia, A., Biancheri, P., Allan, P., Corazza, G.R.,and Sabatino, A.D. (2014). Innate and adaptive immunity in inflammatory bowel disease. Autoimmunity Reviews *13*, 3-10.

Hanauer, S. B. (2006), Inflammatory bowel disease: Epidemiology, pathogenesis, and therapeutic opportunities. Inflamm Bowel Dis *12*, S3–S9.

Kuhn R., Lohler J., Rennick D., Rajewsky K., and Muller W. (1993). Interleukin-10-deficient mice develop chronic enterocolitis. Cell *75*, 263-74.

Molodecky, N., Rabi, D., Ghali, W., Ferris, M., Chernoff, G., Benchimol, E., Panaccione, R., Ghosh, S., Barkema, H., and Kaplan, G. (2012). Increasing Incidence and Prevalence of the inflammatory bowel diseases with time, based on systematic review. Gastroenterology *142*, 46-54.

Monteleone G. and Caprioli F. (2010). T-cell-directed therapies in inflammatory bowel diseases*.* Clin Sci (Lond) *118,* 707-15.

Nilius B., Appendino G., and Owsiankik G. (2012). The transient receptor potential channel TRPA1: from gene to pathophysiology. *Pfugers Archiv-* European Journal of Physiology *464***,** 425-58.

Venkatachalam K, Montell C. TRP channels*. (2007).* Annu Rev Biochem *76*, 387-41.