Experimental colitis

ORIGINAL ARTICLE

The TRPA1 ion channel is expressed in CD4+ T cells and restrains T-cell-mediated colitis through inhibition of TRPV1

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ABSTRACT

Objective Transient receptor potential ankyrin-1 (TRPA1) and transient receptor potential vanilloid-1 (TRPV1) are calcium (Ca2+)-permeable ion channels mostly known as pain receptors in sensory neurons. However, growing evidence suggests their crucial involvement in the pathogenesis of IBD. We explored the possible contribution of TRPA1 and TRPV1 to T-cell-mediated colitis.

Design We evaluated the role of Trpa1 gene deletion in two models of experimental colitis (ie, interleukin-10 knockout and T-cell-adoptive transfer models). We performed electrophysiological and Ca2+ imaging studies to analyse TRPA1 and TRPV1 functions in CD4+ T cells. We used genetic and pharmacological approaches to evaluate TRPV1 contribution to the phenotype of Trpa1−/− CD4+ Tcells. We also analysed TRPA1 and TRPV1 gene expression and TRPA1+/−TRPV1− T cell infiltration in colonic biopsies from patients with IBD.

Results We identified a protective role for TRPA1 in T-cell-mediated colitis. We demonstrated the functional expression of TRPA1 on the plasma membrane of CD4+ T cells and identified that Trpa1+/− CD4+ T cells have increased T-cell receptor-induced Ca2+ influx, activation profile and differentiation into Th1-effector cells. This phenotype was abrogated upon genetic deletion or pharmacological inhibition of the TRPV1 channel in mouse and human CD4+ T cells. Finally, we found differential regulation of Trpa1 and Trpv1 gene expression as well as increased infiltration of Trpa1−/−TRPV1− T cells in the colon of patients with IBD.

Conclusions Our study indicates that TRPA1 inhibits TRPV1 channel activity in CD4+ T cells, and consequently restrains CD4+ T-cell activation and colitogenic responses. These findings may therefore have therapeutic implications for human IBD.

INTRODUCTION

IBDs are initiated by abnormal cell-mediated immune responses, primarily by CD4+ T cells to luminal microbial products in genetically susceptible hosts.1 The two most common forms of IBD are Crohn’s disease (CD) and UC.1,2 CD is characterised as a T helper (Th) 1-mediated inflammatory response with overproduction of interferon (IFN)-γ and tumour necrosis factor (TNF)-α, whereas UC is considered a Th2-mediated immune disease with massive production of interleukin (IL)-5, IL-9 and IL-13.2,3 Th17 cells and their related cytokines are also involved in the pathogenesis of CD and UC, but their function is more complex than originally thought as both proinflammatory and tissue-protective effects have been described.2,4 One of
the most advanced therapeutic strategies in patients with IBD is the inhibition of effector CD4+ T cell homing and functions, and hence intestinal inflammation.5 Despite recent progress in IBD treatment, many current approaches have undesirable side effects and new therapeutic targets aiming to suppress colitogenic CD4+ T cell responses are needed.

Growing evidence suggests that members of the transient receptor potential (TRP) family of ion channels are functionally expressed in immune cells, including T cells.6 7 Indeed, we recently demonstrated that the transient receptor potential vanilloid-1 (TRPV1) channel is expressed in CD4+ T cells and that it increases their proinflammatory properties in models of colitis.8 Mechanistically, we identified that TRPV1 contributes to T cell receptor (TCR)-induced calcium (Ca2+) influx and that phosphorylation by the lymphocyte-specific protein tyrosine kinase Lck is a possible gating mechanism for the TRPV1 channel in CD4+ T cells upon TCR stimulation.89 Like other TRPs, the TRPV1 channel functions as a homotetramer and heterotetramer with other TRP channel subunits.10 Since TRPV1 is predominantly coexpressed with the transient receptor potential ankyrin-1 (TRPA1) channel in sensory neurons11–13 and because these two channels can form heterotetrameric channel complexes,14–16 we evaluated in the present study TRPA1 function in CD4+ T cells and its potential role in T-cell-mediated colitis. TRPA1 is a Ca2+-permeable cation channel that is involved in various cellular processes.17 It was originally identified in sensory neurons to respond to noxious cold temperatures (<17°C) and certain pungent compounds such as allyl isothiocyanate (the pungent compound in mustard oil (MO)).11 18 Since neuronal TRPA1 is activated by a variety of exogenous and endogenous stimuli that cause pain and inflammation, it is considered to play a proinflammatory role.12 17 19 For example, intracolonic administration of the TRPA1-specific agonist MO or 2,4,6-trinitrobenzene sulfonic acid in mice leads to a severe colitis that is associated with activation of TRPA1 in gut sensory afferent nerves and with the subsequent release of neuropeptides that trigger neurogenic inflammation.20 21

Here, we report an unexpected anti-inflammatory role for the TRPA1 channel in CD4+ T cells. In contrast to the role of TRPV1,8 we found that TRPA1 decreases CD4+ T cell activation and proinflammatory properties. Indeed, TRPA1−/− CD4+ T cells display increased TCR-induced Ca2+ influx, CD4+ T cell activation and differentiation into Th1-effector cells. As presented below, we identified that TRPA1 restrains TRPV1 channel activity in CD4+ T cells and consequently controls CD4+ T cell activation and proinflammatory responses in two different models of T-cell-mediated colitis. Finally, we observed differential regulation of TRPA1 and TRPV1 gene expression as well as increased infiltration of TRPA1−/−TRPV1−/− T cells in colonic biopsies of patients with IBD.

RESULTS
TRPA1 deficiency aggravates colitis in Il10−/− mice
To investigate the role of TRPA1 in the development of T-cell-mediated colitis, we crossed Trpa1−/− mice with the Il10−/− strain.22 Between 8 and 10 weeks of age, Il10−/−Trpa1−/− mice developed severe spontaneous colitis (ie, body weight loss, diarrhea, rectal prolapse, shortening and thickening of the colon), whereas Il10−/− control mice developed very mild disease under the same housing conditions (figure 1A, B). The exacerbated colitis in Il10−/−Trpa1−/− mice was not transmissible to co-housed Il10−/− mice and was not likely the result of an altered gut microbiota composition (see online supplementary text and figure S1). Histological analysis of Il10−/−Trpa1−/− mice colons revealed epithelial hyperproliferation, massive crypt loss and marked infiltration of mononuclear cells in the mucosal layer (figure 1C). The gut barrier was also disrupted in these mice as shown by the increased levels of albumin detected in the faeces (figure 1D). Consistent with the aforementioned macroscopic alterations in the colon, we found increased transcript levels of proinflammatory cytokines (Il10, Il17a and Il1b), as well as T cell markers (Cd4 and Cd3d) in colon tissues from Il10−/−Trpa1−/− mice (figure 1E). Furthermore, ex vivo colonic explants (CEs) from Il10−/−Trpa1−/− mice produced significantly higher amounts of IFN-γ compared with Il10−/− control mice (figure 1F). These results indicate that TRPA1 deficiency accelerates the development of colitis and exacerbates intestinal inflammation in this model.

Il10−/−Trpa1−/− mice display increased Th1-mediated inflammatory responses
Since the spontaneous colitis in Il10−/− mice depends predominantly on CD4+ T cells,15 we analysed their inflammatory profile in Il10−/− and Il10−/−Trpa1−/− mice. Spleno- (SP) and mesenteric lymph nodes (MLNs) were enlarged in 12-week-old Il10−/−Trpa1−/− mice compared with age-matched Il10−/− control mice (figure 2A, overview insets). SP and MLN CD4+ T cells isolated from Il10−/−Trpa1−/− mice produced significantly higher levels of Th1-type cytokines (IFN-γ and IL-2) compared with those isolated from Il10−/− mice (figure 2A, B). To study the colonic T cells of these mice, we isolated lamina propria lymphocytes (LPLs) and analysed their inflammatory profile by q-PCR. Transcript levels of Il10 and Il2 as well as the transcription factors T-bet, Nfat1 and Nkfb were upregulated in Il10−/−Trpa1−/− LPLs compared with Il10−/− LPLs (figure 2C). We next analysed the cytokine production of naive SP CD4+ T cells isolated from 6-week-old mice without clinical signs of colitis. Interestingly, these cells also showed increased IFN-γ and IL-2 production compared with naive CD4+ T cells isolated from age-matched Il10−/− mice (figure 2D). These results indicate that CD4+ T cells isolated from Il10−/−Trpa1−/− mice have an increased proinflammatory profile and a Th1 bias.

Il10−/−Trpa1−/− naive CD4+ T cells display exacerbated colitogenic properties
To evaluate whether TRPA1 deficiency in CD4+ T cells was responsible for the phenotype of Il10−/−Trpa1−/− mice, we employed the T cell transfer model of colitis.24 We isolated naive (CD4+CD45RBhiCD25−) SP T cells from young (6 weeks old), colitis-free, Il10−/− and Il10−/−Trpa1−/− donor mice and adoptively transferred these cells to Rag1−/− recipient mice. As a control, we co-transferred wild-type C57BL/6 (WT) Tregs (CD4+CD45RBhiCD25+) together with WT-naïve CD4+ T cells and monitored the development of the colitis in the different experimental groups. Interestingly, naive CD4+ T cells isolated from Il10−/−Trpa1−/− mice induced a significantly greater body weight loss and a higher disease activity index (DAI) in the recipients compared with naive CD4+ T cells isolated from Il10−/− mice (figure 3A, B). As expected, the control group (WT-naïve T cells + Treg) did not show any clinical signs of colitis. Histological analysis of the colons confirmed the induction of severe colitis in the recipients of Il10−/−Trpa1−/− naive CD4+ T cells, whereas only a mild colitis was observed in the recipients of Il10−/− naive CD4+ T cells (figure 3C, D). The mRNA levels of several proinflammatory markers (eg, Cd4, T-bet, Il10) were also significantly increased in the colon homogenates of Il10−/−Trpa1−/− T cell recipients compared with Il10−/− T cell recipients (figure 3E). Finally, CD4+ T cells isolated both from the spleen and MLN of Il10−/−
Figure 1 Transient receptor potential ankyrin-1 (TRPA1) deficiency aggravates colitis in Il10−/− mice. (A) Time course of disease activity index (DAI; i.e., the combined score of weight loss and presence of blood in the stools) for Il10−/− and Il10−/−/Trpa1−/− mice. (B) Representative pictures of the rectal prolapse and the colon of Il10−/− and Il10−/−/Trpa1−/− mice. The percentage of mice that develop rectal prolapse at 12 weeks of age is indicated. (C) Left panel: representative pictures of colon sections stained with H&E. Scale bar=500 μm. Il10−/−/Trpa1−/− mice developed severe colonic inflammation as judged by colonic wall thickening (black arrowheads), massive crypt loss (black arrows). Right panel: colitis score of 12-week-old Il10−/− and Il10−/−/Trpa1−/− mice. (D) Albumin levels in the stools (faeces) analysed by ELISA. (E) The relative mRNA expression level of several proinflammatory cytokines (top panel) and cell markers (bottom panel) was analysed by q-PCR in colon homogenates from Il10−/−/Trpa1−/− and Il10−/− mice and was normalised to Gapdh housekeeping gene. The level in the Il10−/− control group was used as reference and assigned to 1. (F) Proinflammatory cytokine production by colonic explants after 24 hours of culture (ELISA). One representative experiment out of three is shown. Results are expressed as mean±SEM (n=9–10 (A and B) or 6–8 (C–F) mice/group). n.s., not significant; *p<0.05; **p<0.01; ***p<0.001 (two-way analysis of variance with post hoc Bonferroni t-test (B–F)).

Il10−/− T cell recipients produced higher levels of IFN-γ compared with Il10−/− T cell recipients (figure 3F). These results indicate that Il10−/−/Trpa1−/− CD4+ T cells display increased colitogenic properties and suggest that TRPA1 deficiency in CD4+ T cells is responsible for the exacerbated colitis observed in Il10−/−/Trpa1−/− mice.

CD4+ T cells express a functional TRPA1 channel

To evaluate TRPA1 expression in CD4+ T cells, we isolated spinal cord, SP CD4+ T cells and CD11c+ bone marrow-derived dendritic cells (BMDCs) from WT and Trpa1−/− mice and analysed Trpa1 mRNA expression by q-PCR. Trpa1 transcripts were found in WT spinal cord and WT CD4+ T cells but not in WT BMDCs or in Trpa1−/− samples (figure 4A, B). We confirmed TRPA1 protein expression in CD4+ T cells by immunofluorescence staining and identified by confocal microscopy that TRPA1 is mainly expressed at the plasma membrane of CD4+ T cells (figure 4C). Indeed, the fluorescence signals of TRPA1 and CD4 (a transmembrane protein) were largely co-localised (Pearson’s correlation coefficient, r=0.86) (figure 4D).

We next demonstrated TRPA1 channel functionality in CD4+ T cells by recording whole-cell currents evoked by MO (a specific TRPA1 channel agonist) at the holding potential (V_holding) of −60 mV in the perforated patch clamp configuration, which leaves the cell intracellular environment intact.25 MO-gated currents (I_MO) were recorded in WT CD4+ T cells (10.5±1.2 pA; n=12) but not in Trpa1−/− CD4+ T cells (1.6±0.3 pA; n=5) (figure 5A, B). We confirmed these findings by recording MO-gated currents from pieces of plasma membrane of CD4+ T cells using the cell attached single-channel configuration at V_holding=−60 mV (see online supplementary figure S2).

Finally, we performed single-cell Ca2+ imaging to further demonstrate TRPA1 channel functionality in CD4+ T cells. We found that MO significantly increased the intracellular Ca2+ concentration ([Ca2+]i) in WT but not in Trpa1−/− CD4+ T cells (figure 5C). In contrast, equal rises in [Ca2+]i were observed in WT and Trpa1−/− CD4+ T cells following stimulation with ionomycin, a Ca2+ ionophore used as positive control (figure 5D). Collectively,
**Figure 2**  
$\text{Il10}^{-/-}\text{Trpa1}^{-/-}$ 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 $\text{Il10}^{-/-}$ and $\text{Il10}^{-/-}\text{Trpa1}^{-/-}$ mice (overview insets). Cytokine production by SP or MLN CD4$^+$ T cells isolated from each group, 24 hours after re-stimulation with anti-CD3/28 Abs (ELISA). (B) SP CD4$^+$ T cells were stimulated or not with anti-CD3/28 Abs for 5 hours. Cells were then stained for CD4 and intracellular staining was performed for interferon (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). (C) The relative mRNA expression level of several proinflammatory cytokines (top panel) and transcription factors (bottom panel) was analysed in lamina propria lymphocytes (LPLs) isolated from the colon of $\text{Il10}^{-/-}\text{Trpa1}^{-/-}$ and $\text{Il10}^{-/-}$ control mice and was normalised to Gapdh housekeeping gene. The level in the $\text{Il10}^{-/-}$ control group was used as reference and assigned to 1. (D) Naïve CD4$^+$ T cells cytokine production. Naive CD4$^+$ T cells were isolated from the spleen of 6-week-old $\text{Il10}^{-/-}\text{Trpa1}^{-/-}$ mice that did not develop colitis yet and did not have any signs of colonic inflammation and from age-matched $\text{Il10}^{-/-}$ control mice. IFN-$\gamma$, interleukin (IL)-2, IL-17A and tumour necrosis factor (TNF)-$\alpha$ production was analysed 24 hours after re-stimulation with anti-CD3/28 Abs (ELISA). One representative experiment out of three is shown. Results are expressed as mean±SEM (n=6 (A) or 4 (B–D) mice/group). n.s., not significant; *p<0.05; **p<0.01; ***p<0.001 (two-tailed Student’s t-test).
these results indicate that the TRPA1 channel is functionally expressed on the plasma membrane of CD4+ T cells.

**Trpa1−/−** CD4+ T cells are hyperactivated and display a Th1 bias

We next evaluated whether **Trpa1** gene deletion affects TCR-induced Ca2+ influx and the subsequent activation of CD4+ T cells. Interestingly, TCR stimulation induced a higher and more sustained Ca2+ influx in **Trpa1−/−** CD4+ T cells compared with WT CD4+ T cells (figure 5E). Similarly to what was observed with ionomycin (figure 3D), no differences in Ca2+ influx were identified between WT and **Trpa1−/−** CD4+ T cells following stimulation with the sarcoplasmic reticulum Ca2+-ATPase (SERCA) pump inhibitor, thapsigargin (figure 5F), which bypasses proximal TCR signalling and induces store-operated Ca2+ entry.28 In line with their increase in TCR-induced Ca2+ influx and analogous to our observations for **Il10−/−** CD4+ T cells (figure 2A), **Trpa1−/−** CD4+ T cells produced higher amounts of the Th1-type cytokines IFN-γ and IL-2 (figure 6A) and showed increased T-bet expression (see online supplementary figure S3A) after stimulation with anti-CD3/28 Abs compared with WT CD4+ T cells. However, TCR-independent activation of **Trpa1−/−** and WT CD4+ T cells with phorbol 12-myristate 13-acetate (PMA)/ionomycin resulted in a similar secretion of these cytokines (figure 6B). **Trpa1−/−** CD4+ T cells stimulated with anti-CD3/28 Abs also showed enhanced expression of surface activation markers (ie, CD25, CD69, OX40, and CD44) (see online supplementary figure S3B, D) compared with WT CD4+ T cells. As shown in figure 6C, **Trpa1−/−** naive CD4+ T cells displayed an increased capacity to differentiate in vitro into Th1 effectors, but not Th17 effectors or Tregs (data not shown), compared with WT-naive CD4+ T cells. We confirmed this increased Th1-cytokine production by using an antigen-specific model and found that **Trpa1−/−** OT-II CD4+ T cells stimulated with ovalbumin-loaded WT BMDCs display significantly increased IFN-γ and IL-2 production compared with control (**Trpa1−/−** OT-II CD4+ T cells) (figure 6D–F). Importantly, as observed for **Il10−/−** naive CD4+ T cells (figure 3), we found that
TRpa1−/− naïve CD4+ T cells have increased colitogenic properties in vivo (see online supplementary figure S4).

Since visceral afferent sensory neurons and intestinal epithelial cells express functional TRPA1 channels that could affect the severity of the colitis,20 27–30 we generated Rag1−/−TRpa1−/− mice and compared the colitis induced by the transfer of WT-naïve CD4+ T cells into Rag1−/− and Rag1−/−TRpa1−/− recipients. We found that both recipient groups had comparable body weight loss, colonic inflammation and displayed similar CD4+ T cell responses (see online supplementary figure S5). Collectively, these data suggest that cell-intrinsic expression of TRPA1 in CD4+ T cells regulates their activation, their differentiation into Th1-effectors and their colitogenic properties in an adoptive transfer model.

TRPV1 hyperactivation is responsible for the phenotype of TRpa1−/− CD4+ T cells

We then investigated the mechanism underlying the phenotype of TRpa1−/− CD4+ T cells. Since we have previously shown that CD4+ T cells express the TRPV1 channel8 and because TRPA1 and TRPV1 can regulate each other’s activities,13,16 we evaluated TRPV1 expression and function in WT and TRpa1−/− CD4+ T cells. We first performed TRPA1 and TRPV1 double immunofluorescence staining and assessed the potential co-localisation of these two proteins by confocal microscopy. Interestingly, the staining showed a high degree of TRPA1 and TRPV1 co-localisation at the plasma membrane of WT CD4+ T cells (figure 7A, B), suggesting a potential direct interaction between these two channels. However, the expression of Trpv1 mRNA or TRPV1 protein in either total cell lysates (ie, both active and inactive TRPV1 channels) or plasma membrane extracts (ie, active TRPV1 channels) was similar in WT and TRpa1−/− CD4+ T cells (see online supplementary figure S6). In contrast, TRPV1 channel activity was increased in TRpa1−/− CD4+ T cells. As shown in figure 7C, D, the whole-cell perforated patch current evoked by capsaicin (ICAP), a specific TRPV1 agonist,31 was significantly higher in TRpa1−/− CD4+ T cells (18.7±1.9 pA; n=8) compared with WT CD4+ T cells (9.9±1.2 pA; n=8). As expected, no ICAP could be recorded in TRpa1−/− CD4+ T cells (0±0.3 pA; n=6) and in vehicle-treated WT CD4+ T cells (1.6±0.3 pA; n=5). Consequently, CAP-induced Ca2+ influx was significantly higher in TRpa1−/− CD4+ T cells compared with WT cells (see online supplementary figure S7A, B). To verify that this effect was mediated by TRPV1, we treated TRpa1−/− CD4+ T cells with N-(4-Tertiarybutylphenyl)-4-(3-chlorophyrindin-2-yl)tetrahydroprazine -1(2H)-carbox-amide (BCTC), a selective TRPV1 antagonist.32 BCTC almost completely inhibited CAP-induced Ca2+ influx in these cells (see online supplementary figure S7A, B). In line with their increased TCR-induced Ca2+ influx (figure...
Figure 5  CD4+ T cells express a functional transient receptor potential ankyrin-1 (TRPA1) channel. (A) Representative traces of whole-cell recordings in wild-type (WT) and \textit{Trpa1\(^{-/-}\)} SP CD4+ T cells after application of mustard oil (MO, 100 \(\mu\)M) or its vehicle (Veh, 0.01% dimethyl sulfoxide (DMSO)) in presence of 2 mM extracellular calcium concentration ([Ca\(^{2+}\)]\(_{e}\)). (B) Statistical analysis of MO-gated currents (I\(_{MO}\)) in WT (n=12), \textit{Trpa1\(^{-/-}\)} (n=6) and Veh-treated WT (n=5) CD4+ T cells as shown in (A). (C--F) WT and \textit{Trpa1\(^{-/-}\)} SP CD4+ T cells were loaded with Fura-2 AM. Changes in [Ca\(^{2+}\)] were monitored by confocal imaging and were calculated as the ratio of Fura-2 emission at 340 vs 380 nm wavelength excitation. (C) Representative Ca\(^{2+}\) influx profiles in WT and \textit{Trpa1\(^{-/-}\)} CD4+ T cells after application of MO (100 \(\mu\)M) in [Ca\(^{2+}\)]\(_{e}\)=2 mM (2Ca). Right panel: statistical analysis of the Ca\(^{2+}\) influx peak as indicated by a dashed line in the left panel. (D) Cells were stimulated with ionomycin (Iono, 1 \(\mu\)M) in Ca\(^{2+}\)-free medium (0Ca) and Ca\(_{Cl}\) (2 mM) was added to the extracellular medium as indicated. Right panel: statistical analysis of the sustained T-cell receptor (TCR)-induced Ca\(^{2+}\) influx as indicated by a dashed line in the left panel. (E) Cells were stimulated with soluble biotinylated anti-CD3 (10 \(\mu\)g/mL) and cross-linked with streptavidin (10 \(\mu\)g/mL) in Ca\(^{2+}\)-free medium and Ca\(_{Cl}\) (2 mM) was added to the extracellular medium as indicated. Right panel: statistical analysis of the sustained Ca\(^{2+}\) influx as indicated by a dashed line in the left panel. Statistical analyses of the Ca\(^{2+}\) influx profiles (C–F) are shown as the mean \pm SEM of 40–50 individual cells. n.s., not significant; **\(p<0.001\); ****\(p<0.0001\) (one-way analysis of variance with post hoc Bonferroni’s test (B) or two-tailed Student’s t-test (C–F)). Data are representative of three independent experiments.

\textit{Trpa1\(^{-/-}\)} CD4+ T cells also displayed increased TCR-gated currents compared with WT CD4+ T cells (figure 7E, F). Importantly, both genetic deletion and pharmacological inhibition of TRPV1 in \textit{Trpa1\(^{-/-}\)} CD4+ T cells (ie, \textit{Trpa1\(^{-/-}\}/Trpv1\(^{-/-}\)} CD4+ T cells and BCTC treatment, respectively) significantly decreased their elevated TCR-gated currents (figure 7E, F) and IFN-\(\gamma\) production (see online supplementary figure S7C).

To evaluate the role of TRPV1 in the proinflammatory phenotype of \textit{Il10\(^{-/-}\)/Trpa1\(^{-/-}\)} CD4+ T cells, we treated \textit{Il10\(^{-/-}\)/Trpa1\(^{-/-}\)} mice with SB366791, a specific TRPV1 inhibitor effective in vivo, \(^8, 33\) and compared the spontaneous colitis occurrence with vehicle-treated \textit{Il10\(^{-/-}\)/Trpa1\(^{-/-}\)} mice or untreated \textit{Il10\(^{-/-}\)} control mice. SB366791 treatment significantly decreased the colitis severity in \textit{Il10\(^{-/-}\)/Trpa1\(^{-/-}\)} mice (figure 8A, B). To confirm the cell-intrinsic role of TRPV1 in \textit{Trpa1\(^{-/-}\)} CD4+ T cells, we adaptively transferred \textit{Trpa1\(^{-/-}\)} and \textit{Trpa1\(^{-/-}\}/Trpv1\(^{-/-}\)} naive CD4+ T cells to Rag1\(^{-/-}\) recipients and compared the severity of the colitis induced in the different groups. As expected, \textit{Trpa1\(^{-/-}\)} naive T cells induced severe colitis in the recipients, as reflected by clinical and histological signs of colonic inflammation. However, a significantly delayed and attenuated inflammatory response was observed in the recipients of \textit{Trpa1\(^{-/-}\}/Trpv1\(^{-/-}\)} naive CD4+ T cells (figure 8C–F).

Finally, to explore the relevance of the data generated in the mouse to the human condition, we performed siRNA-mediated knockdown of TRPA1 and TRPV1 in human primary CD4+ T cells isolated from healthy blood donors. Similarly to our observations with mouse \textit{Trpa1\(^{-/-}\)} and \textit{Trpa1\(^{-/-}\}/Trpv1\(^{-/-}\)} CD4+
**Figure 6** *Trpa1<sup>−/−</sup>* CD4+ T cells are hyperactivated and display a Th1 bias. (A) wild-type (WT) and *Trpa1<sup>−/−</sup>* SP CD4+ T cells were stimulated with anti-CD3/28 Abs for 48 hours and cytokine production was assessed (ELISA). Mean±SEM (n=4 mice/group) is shown. (B) Cells were stimulated with phorbol 12-myristate 13-acetate (PMA) (25 ng/mL) and ionomycin (500 nM) for 48 hours and cytokine production was assessed (ELISA) as (A). (C) Spleen (SP)-naive CD4+ T cells were isolated from WT and *Trpa1<sup>−/−</sup>* mice, cultured for 5 days under Th1 and Th17-polarising conditions and re-stimulated as described in the 'Materials and methods' section. Representative panels of intracellular cytokine production in the different differentiated subsets as determined by flow cytometry on gated CD4+ T cells are shown. Right panel: percentage of interferon (IFN)-γ<sup>+</sup> and interleukin (IL)-17A<sup>+</sup> CD4+ T cells in the Th1-inducing and Th17-inducing conditions, respectively. (D–F) Cytokine production by OT-II or *Trpa1<sup>−/−</sup>* OT-II CD4+ T cells co-cultured with ovalbumin (OVA)-loaded WT bone marrow-derived dendritic cells (BMDCs) for 3 days and re-stimulated with anti-CD3/28 Abs for 6 (intracellular staining, ICS) or 24 hours (ELISA). (D) Representative panels of intracellular cytokine production in gated CD4+ T cells and (E) mean %±SEM (3 mice/group) of IL-2<sup>+</sup>+IFN-γ<sup>+</sup> and IFN-γ<sup>+</sup> CD4+ T cells are shown. (F) For ELISA, supernatants were collected and IFN-γ and IL-2 levels were measured. n.s., not significant; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 (two-tailed Student’s t-test (A, B and E) or one-way analysis of variance with post hoc Bonferroni’s test (F)). Data are representative of two (D–F) or three (A–C) independent experiments.

Figure 7  Transient receptor potential vanilloid-1 (TRPV1) hyperactivation is responsible for the phenotype of Trpa1−/− CD4+ T cells. (A) Transient receptor potential ankyrin-1 (TRPA1) and TRPV1 co-localisation in CD4+ T cells as determined by double immunofluorescence labelling. Resting wild-type (WT) spleen (SP) CD4+ T cells were stained with TRPA1 and TRPV1 Abs and analysed by confocal microscopy. Hoechst 33258 (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 colour in the merge panel indicates areas of TRPA1 and TRPV1 co-localisation at the plasma membrane. Scale bar=5 μm. (B) TRPA1 and TRPV1 co-localisation scatter plot was generated using Velocity software. r, Pearson’s correlation coefficient. (C) Representative traces of whole-cell perforated patch recordings for capsaicin (CAP, 1 μM) or its vehicle (Veh, 0.01% dimethyl sulfoxide (DMSO)) in WT, Trpa1−/− and Trpv1−/− CD4+ T cells in [Ca2+]i=2 mM. (D) Statistical analysis of CAP-gated currents (ICAP) in WT (n=8), Trpa1−/− (n=8), Trpv1−/− (n=6) and Veh-treated WT (n=5) CD4+ T cells as shown in C. (E) Representative traces of whole-cell recordings for anti-CD3 Ab (10 μg/mL) in WT, Trpa1−/−+BCTC and Trpa1−/− Trpv1−/− CD4+ T cells in [Ca2+]i=2 mM. (F) Statistical analysis of CD3-gated currents (ICD3) in unstimulated WT (n=7) and in anti-CD3 Ab-stimulated WT (n=12), Trpa1−/− (n=9), Trpa1−/−+BCTC (n=7), and Trpa1−/− Trpv1−/− (n=11) CD4+ T cells as shown in E. n.s., not significant; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 (one-way analysis of variance with post hoc Bonferroni’s test). Data are representative of three independent experiments.

T cells, TRPA1 knockdown increased IFN-γ and IL-2 production by human CD4+ T cells upon stimulation with anti-CD3/28 Abs, and this effect was abolished by double knockdown of TRPA1 and TRPV1 genes (see online supplementary figure S8). To evaluate the clinical significance of these findings, we analysed TRPA1 and TRPV1 transcript levels in colonic biopsies from patients with IBD. We found that TRPV1 gene expression was significantly downregulated in patients with active UC and CD compared with healthy controls (see online supplementary figure S9). Interestingly, TRPA1 gene expression showed the opposite trend and was upregulated in patients with IBD, although the differences observed did not reach statistical significance with the sample size used in this study. To determine whether this effect resulted from a differential infiltration by TRPA1+ and TRPV1+ T cells in the colonic mucosa of patients with IBD, we performed triple immunofluorescence staining of colon sections stained with H&E (right panel). Scale bars=1 mm (overview insets), 100 μm (high-power fields). (B) Colitis score in the three different groups (n=6–10 mice/group). (C-F) Rag1−/− mice were transferred with 3×105 Trpa1−/− or Trpa1−/−Trpv1−/− fluorescence-activated cell sorting (FACS)-sorted naive (CD4+CD45RBlowCD25+) T cells, or as control with 3×10⁵ wild-type (WT) naive CD4+ T cells+1.5×10⁵ WT Treg (CD4+CD45RBlowCD25+). (C) Percentage of initial body weight in the different recipient groups (n=6 mice/group). (D) Disease activity index (DAI) in the three recipient groups. (E) Representative pictures of colon sections stained with H&E. Scale bars=1 mm (overview insets), 100 μm (high-power fields). (F) Colitis score in the three recipient groups. n.s., not significant; *p<0.05; **p<0.01; ***p<0.001 (one-way (B, D, F) or two-way (C) analysis of variance with post hoc Bonferroni’s test).

Figure 8 Genetic deletion or pharmacological inhibition of transient receptor potential vanilloid-1 (TRPV1) rescues the proinflammatory phenotype of Il10−/− and Trpa1−/−CD4+ T cells in vivo. (A and B) Il10−/−Trpa1−/− mice were treated three times per week with the TRPV1 inhibitor SB366791 (3 mg/kg of body weight, intraperitoneally) or with its vehicle (Veh, 10% dimethyl sulfoxide (DMSO)/90% saline) starting at 6 weeks of age and until they reached 12 weeks old. Age-matched and sex-matched untreated Il10−/− mice were used as controls. (A) Representative pictures of colon sections stained with H&E (right panel). Scale bars=1 mm (overview insets), 100 μm (high-power fields). (B) Colitis score in the three different groups (n=6–10 mice/group). (C-F) Rag1−/− mice were transferred with 3×10⁵ Trpa1−/− or Trpa1−/−Trpv1−/− FACS-sorted naive T cells, or as control with 3×10⁵ wild-type (WT) naive CD4+ T cells+1.5×10⁵ WT Treg (CD4+CD45RBlowCD25+). (C) Percentage of initial body weight in the different recipient groups (n=6 mice/group). Statistical analysis compared Trpa1−/− and Trpa1−/−Trpv1−/− naive T cells groups. (D) Disease activity index (DAI) in the three recipient groups. (E) Representative pictures of colon sections stained with H&E. Scale bars=1 mm (overview insets), 100 μm (high-power fields). (F) Colitis score in the three recipient groups. n.s., not significant; *p<0.05; **p<0.01; ***p<0.001 (one-way (B, D, F) or two-way (C) analysis of variance with post hoc Bonferroni’s test).

In summary, these results suggest that TRPV1 channel hyperactivation is responsible for the increased TCR-gated currents, the Th1 bias and the exacerbated colitogenic properties of Trpa1−/−CD4+ T cells. Moreover, the differential regulation of TRPA1 and TRPV1 gene expression and the increased infiltration of TRPA1+TRPV1+ T cells in inflamed colon sections of patients with UC and CD suggest that TRPA1 and TRPV1 channels may play important roles in the pathogenesis of IBD.
Growing evidence suggests the crucial involvement of the TRPA1 and TRPV1 ion channels in the inflammatory response observed in IBD. Most studies have focused on the role played by these two channels in peripheral neurons innervating the gut and on their contribution to neurogenic inflammation. However, TRPA1 and TRPV1 have a wide tissue distribution and therefore can play diverse roles in a variety of non-neuronal cells. For example, TRPA1 mRNA and protein were detected in many organs and tissues, including brain, heart, small intestine, lung, skeletal muscle, pancreas and spleen. In this study, we demonstrated for the first time the functional expression of TRPA1 in skeletal muscle, pancreas and spleen. In accordance with the higher and more sustained Ca^{2+} influx required in Th1 cells compared with other Th subsets, we identified that T_{rpa1}^{+/−} CD4+ T cells have increased TCR-gated currents and subsequently TCR-induced Ca^{2+} influx. Furthermore, we found that T_{rpa1}^{+/−} CD4+ T cells have increased TRPV1 channel activity and that genetic deletion or pharmacological inhibition of TRPV1 in T_{rpa1}^{+/−} CD4+ T cells (ie, T_{rpa1}^{+/−} T_{rpa1}^{+/−} and T_{rpa1}^{+/−}) OT-II) and the T-cell-intrinsic role of TRPA1 was confirmed by employing the T-cell-adoptive transfer model of colitis. However, in contrast to T_{rpa1}^{+/−} mice, T_{rpa1}^{+/−} and T_{rpa1}^{+/−} OT-II mice housed under specific pathogen-free conditions do not develop spontaneous colitis, presumably due to proper regulatory T cell function.

In accordance with the higher and more sustained Ca^{2+} influx required in Th1 cells compared with other Th subsets, we identified that T_{rpa1}^{+/−} CD4+ T cells have increased TCR-gated currents and subsequently TCR-induced Ca^{2+} influx. Furthermore, we found that T_{rpa1}^{+/−} CD4+ T cells have increased TRPV1 channel activity and that genetic deletion or pharmacological inhibition of TRPV1 in T_{rpa1}^{+/−} CD4+ T cells (ie, T_{rpa1}^{+/−} T_{rpa1}^{+/−} CD4+ T cells and BCTC treatment, respectively) rescued their elevated TCR-gated Ca^{2+} currents and their increased Th1 cytokine production. Since BCTC has also been shown to block TRPM8, we have confirmed the data generated with BCTC in vitro and the contribution of TRPV1 to the phenotype of T_{rpa1}^{+/−} mice by using another TRPV1 antagonist (ie, SB366791) in vivo. We found that treatment with SB366791 significantly decreased the colitis severity in T_{rpa1}^{+/−} mice. However, we could not exclude that inhibition of TRPV1 in other cell types (eg, visceral sensory neurons) and its consequences (eg, inhibition of neuro-peptide release) may also have contributed to this effect when...
SB366791 is given systemically. Therefore, we employed the T-cell-adoptive transfer model to address the effect of cell-intrinsic inhibition of TRPV1 in Traf1−/− CD4+ T cells. We found that Traf1−/− TRPV1−/− CD4+ T cells have decreased colitogenic properties compared with Traf1−/− CD4+ T cells. Furthermore, we identified that genetic deletion of Traf1 or Traf1 in the Rag1−/− recipients (ie, Rag1−/− Traf1−/− or Rag1−/− TRPV1−/− mice, respectively) did not affect the course of the colitis in this model. Taken together, these findings suggest that TRPV1 hyperactivation is responsible for the phenotype of Traf1−/− CD4+ T cells and that cell-intrinsic expression and function of TRPA1 and TRPV1 in CD4+ T cells regulates the colitis severity in this model.

The TRPV1 hyperactivation observed in TRPA1-deficient CD4+ T cells can be achieved by different mechanisms leading to increased (i) TRPV1 mRNA and/or protein expression, (ii) TRPV1 recruitment to the plasma membrane or (iii) TRPV1 phosphorylation that reduces its activation threshold.14–16 Our data suggest that TRPV1 hyperactivation is not likely due to increased TRPV1 expression or increased TRPV1 trafficking to the plasma membrane. Therefore, modification of TRPV1 phosphorylation status or TRPV1 quaternary structure (eg, channel multimerisation) might account for the observed TRPV1 channel hyperactivation and the resultant phenotype of Traf1−/− CD4+ T cells. In line with this hypothesis, we and others previously reported that TRPA1 and TRPV1 can form heterotetrameric channel complexes14–16 in which TRPA1 functionally inhibits TRPV1.15 Hence, the increased formation of TRPV1 homotetramers in the absence of TRPA1 could potentially result in the increased TRPV1 channel activity observed in Traf1−/− CD4+ T cells.

The phenotype of Traf1−/− CD4+ T cells is not likely the result of a developmental defect in Traf1−/− mice since it was reproduced by knockdown of TRPA1 in primary, mature human CD4+ T cells. Indeed, the increased Th1 cytokine production was also observed after TRPA1 gene knockdown in human primary CD4+ T cells and was abolished by double knockdown of TRPA1 and TRPV1 genes. In addition, we identified reciprocal regulation of TRPA1 and TRPV1 gene expression in colonic biopsies from patients with active IBD. These findings are consistent with previous reports, which showed differential expression of TRPV1 and TRPA1 in inflamed colon sections of patients with IBD.29–44 However, some of these studies suggested an increase in TRPV1 expression by sensory neurons innervating the colon of IBD patients.44–45 In agreement with our findings, Kim et al recently reported a decrease in TRPV1 expression and an increase in TRPA1 expression in colon biopsies from patients with IBD. Interestingly, the authors of this study also observed strong TRPA1-immunopositivity and TRPV1-immunopositivity within mononuclear cells infiltrating the colonic mucosa of patients with IBD.45 In line with these observations, we found an increased infiltration of TRPA1+TRPV1+ T cells in the colonic mucosa of both patients with UC and CD. Collectively, these results suggest that changes in gene expression of TRPA1 and TRPV1 and/or in infiltration of TRPA1+TRPV1+ T cells in the colon may contribute to the pathophysiology of IBD.

In summary, we demonstrated in this study the functional expression of TRPA1 in CD4+ T cells and reported an unexpected anti-inflammatory role for this channel in T-cell-mediated colitis. These findings therefore challenge the current dogma that TRPA1 plays a detrimental role in inflammatory responses that occur in colitis and suggest that TRPA1 in CD4+ T cells has an opposite function to TRPA1 in visceral sensory neurons.19–20 While neuronal TRPA1 increases acute (eg, neurogenic) inflammation, TRPA1 in CD4+ T cells appears to decrease the severity of chronic (eg, T-cell-mediated) colitis.

Our results are in agreement with another recent study reporting a protective role for TRPA1 in colitis.29 and with our previous observations regarding the role of TRPV1 in CD4+ T cell activation and its proinflammatory function. Therefore, additional studies will be necessary to determine the relative contribution of TRPA1 signalling in visceral sensory neurons and in CD4+ T cells, respectively to the pathogenesis of human IBD. Finally, our study further reinforces the idea that Ca2+- is a critical regulator of T cell inflammatory responses in colitis and suggests that Ca2+-permeable channels, such as TRPA1 and TRPV1, could represent new therapeutic targets in IBD and potentially in other T-cell-mediated diseases.

MATERIALS AND METHODS

See online supplementary materials.
Experimental colitis


The TRPA1 ion channel is expressed in CD4+ T cells and restrains T-cell-mediated colitis through inhibition of TRPV1

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