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Expression of T-cell K_V 1.3 potassium channel correlates with pro-inflammatory cytokines and disease activity in ulcerative colitis☆

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Abstract

Background and aims—Potassium channels, K_V1.3 and K_{Ca}3.1, have been suggested to control T-cell activation, proliferation, and cytokine production and may thus constitute targets for anti-inflammatory therapy. Ulcerative colitis (UC) is a chronic inflammatory bowel disease characterized by excessive T-cell infiltration and cytokine production. It is unknown if K_V1.3 and K_{Ca}3.1 in the inflamed mucosa are markers of active UC. We hypothesized that K_V1.3 and K_{Ca}3.1 correlate with disease activity and cytokine production in patients with UC.

Methods—Mucosal biopsies were collected from patients with active UC (n = 33) and controls (n = 15). Protein and mRNA expression of K_V1.3 and K_{Ca}3.1, immune cell markers, and pro-inflammatory cytokines were determined by quantitative-real-time-polymerase-chain-reaction (qPCR) and immunofluorescence, and correlated with clinical parameters of inflammation. In-vitro cytokine production was measured in human $CD3^+$ T-cells after pharmacological blockade of K_V1.3 and K_{Ca}3.1.

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Conflict of interest

None.

Statement of authorship: LKH, TPH, TK, JK, HW, and RK designed the study. LKH, HW, TK, JK, and RK analyzed and interpreted the data. LKH, DL, LSM, HW, and MR conducted experiments. LKH, LK, TK, and JK collected human biopsies. TP performed all pathology assessments. LKH, HW, and RK drafted the manuscript. All authors took active part in revising the manuscript and all authors read and approved the final manuscript.

Results—Active UC $K_V1.3$ mRNA expression was increased 5-fold compared to controls. Immunofluorescence analyses revealed that $K_V1.3$ protein was present in inflamed mucosa in 57% of $CD4^+$ and 23% of $CD8^+$ T-cells. $K_V1.3$ was virtually absent on infiltrating macrophages. $K_V1.3$ mRNA expression correlated significantly with mRNA expression of pro-inflammatory cytokines TNF-α ($R^2 = 0.61$) and IL-17A ($R^2 = 0.51$), the mayo endoscopic subscore ($R^2 = 0.13$), and histological inflammation ($R^2 = 0.23$). In-vitro blockade of T-cell $K_V1.3$ and $K_{Ca}3.1$ decreased production of IFN-γ, TNF-α, and IL-17A.

Conclusions—High levels of $K_V1.3$ in CD4 and CD8 positive T-cells infiltrates are associated with production of pro-inflammatory IL-17A and TNF-α in active UC. $K_V1.3$ may serve as a marker of disease activity and pharmacological blockade might constitute a novel immunosuppressive strategy.

Keywords

Novel treatment strategy; Colitis ulcerosa; KCNN4; KCNA3; Interleukins; $K_{Ca}3.1$

1. Introduction

Ulcerative colitis (UC) is a relapsing chronic inflammatory bowel disease (IBD) characterized by bloody diarrhea. It impairs quality of life and can lead to life-threatening complications, e.g. toxic megacolon.[1] The present treatment options, such as corticosteroids, salicylates, and anti-TNF-α reagents, are unsatisfactory for many patients and there is a clear need to identify novel molecular targets for alternative treatment.

The etiology of UC is still under debate. Several hypotheses on the cause of IBD such as allergic disposition, hygiene conditions, infections, and nutrition have been presented.[2–4] It has also been speculated that the inflammation could be of heritable[5,6] and of autoimmune origin although the nature of this autoimmunity remains elusive.[7] After onset, mucosal inflammation is likely maintained by an abnormal activity of cytotoxic T-cells (T_C, $CD8^+$) and T helper cells (T_H, $CD4^+$),[8,9] and the concomitant cytotoxicity and excessive secretion of pro-inflammatory cytokines such as tumor necrosis factor α (TNF-α) and interleukin-17A (IL-17A), respectively.[10–12] The role of IFN-γ in UC is still ambiguous; some studies report an increased IFN-γ production when compared to controls while others do not find an increase.[13,14] It seems as if the immunological balance between T_H1, T_H2 and T_H17 responses is disturbed, and that an atypical T_H2 response occurs involving non-classical natural killer T cells.[13,15–18]

Ion channels in the T cell membrane play pivotal roles in T cell functions by maintaining intracellular Ca^{2+}-homeostasis, cytokine production, and clonal expansion after T-cell receptor (TCR) activation.[19] Of particular importance are the voltagegated potassium channel $K_V1.3$ (encoded by the KCNA3 gene) and the calcium-activated potassium channel $K_{Ca}3.1$ (encoded by the KCNN4 gene). Constitutively expressed $K_V1.3$ channels stabilize the membrane potential, thus keeping the electrical driving force for sustained Ca^{2+} influx through calcium release activated channels (CRAC) after TCR activation.[20–22] This longlasting increase in cytosolic Ca^{2+} triggers T-cell proliferation and cytokine production.[19,23] Increasing evidence also supports that $K_V1.3$ channels play a role in

autoimmune diseases such as type-1-diabetes, rheumatoid arthritis, multiple sclerosis, and glomerulonephritis.[24–28] Effector memory T-cells (T_{EM})($CD8^+$/ $CCR7^-$ and $CD4^+$/$CCR7^-$), a subset of terminally differentiated T cells, express high levels of $K_V1.3$ channels while activated T_{CM} and naïve T-cells primarily express and use the calciumactivated $K_{Ca}3.1$ channel to regulate their Ca^{2+} signaling.[23] In the light of these findings, small molecule blockers of $K_V1.3$ and $K_{Ca}3.1$ may be of therapeutic utility in inflammatory and autoimmune diseases.[23,29] $K_{Ca}3.1$ channels are generally upregulated in T cells following activation but are also found in macrophages and colonic crypts in which they are involved in the water and NaCl secretion to the intestinal lumen.[23,26,30,31] Whether Kv1.3 and $K_{Ca}3.1$ channels are also pathomechanistically linked to the inflammation in UC is not known and most data about T cell potassium channels are from in-vitro, or animal studies.

Therefore, our hypotheses were that (1) high expression of $K_V1.3$ and $K_{Ca}3.1$ in T cell infiltrates in the inflamed mucosa of UC patients correlated with disease activity and the synthesis of the pro-inflammatory cytokines TNF-α, IFN-γ, and IL-17A, and (2) that in vitro blockade of $K_V1.3$ and $K_{Ca}3.1$ on human $CD3^+$ T cells decreased production of TNF-α, IFN-γ, and IL-17A.

2. Materials and methods

2.1. Study design and patients

The study was a cross-sectional case–control study with follow-up. We included patients older than 18 years with established active UC. The control group consisted of patients scheduled for endoscopy at outpatient clinics with no inflammatory bowel disease (cancrophobia or control after previous polyp removal). Exclusion criteria were: (1) active treatment with anticoagulants, non-steroidal anti-inflammatory drugs (NSAID), or acetylsalicylic acid (ASA); (2) pregnancy or lactation; (3) stool cultures positive for pathogens; (4) contraindicated endoscopy. In the control group diarrhea was an exclusion criterion. Participants were included at Odense University Hospital and Hospital of South-West Jutland, Esbjerg, Denmark, after verbal and written informed consent. The study was approved by the Danish Ethics Committee (permit no. S-20110007) and the Danish Data Protection Agency (permit no. 2008-58-0035).

2.2. Collection of specimens

Blood sampling (C-reactive protein (CRP), leucocyte count (LEU)), fecal calprotectin, stool cultures, and endoscopy were performed the same day. Fecal samples were collected prior to endoscopy. Biopsies were obtained from rectal mucosa during endoscopic examination of healthy individuals and from patients with first-time attack or relapse of UC. Biopsies were immediately stored in RNAlater® (Ambion, Austin, TX, USA).

2.3. Scoring of inflammation and Mayo Score

Participants were scored according to the Mayo Score including the Mayo endoscopic subscore (0 none, 1 mild, 2 moderate, 3 severe).[32] Histological inflammation of the biopsies was scored according to Morson and Dawson's UC score for inflammation (0 none, 1 mild, 2 moderate, 3 severe) by a gastrointestinal pathologist.[33]

2.4. RNA preparation, DNase digestion, cDNA synthesis and quantitative Real Time PCR

RNA was isolated from biopsies using TRIZOL reagent® (Invitrogen, United Kingdom). The RNase-Free DNase Set (Qiagen, Germany) was used for DNase digestion. Complementary DNA (cDNA) was synthesized using iScript cDNA Synthesis Kit (Bio-Rad, CA, USA) and quantitative real-time PCR (qPCR) was performed according to the MIQE guidelines[34] (except from using only one reference gene) using SYBR Green Supermix (Bio-Rad, CA, USA) on a Stratagene MX3000P qPCR instrument (Agilent Technologies, Santa Clara, CA, USA). All primers were obtained from Sigma-Aldrich (St. Louis, MO, USA); efficiencies were 90–105% and reactions were run in duplicates.

Data exclusion criteria: Cq-value (formerly known as Ct-value) of GAPDH above 25 (indicating low amounts or bad quality of cDNA/mRNA); Cq-values in a double determination differed by more than 2 Cq-values (inconclusive data); non exponential amplification slopes (false-positive, incomplete reaction), or if melting curves of the product did not show one product of expected size. Expression levels are presented as a percentage of GAPDH (%GAPDH). No significant difference was found in GAPDH expression between UC and controls (data not shown).

2.5. Immunohistochemistry and immunofluorescence

Immunohistochemical (IHC) and immunofluorescent (IF) stainings were performed on the same paraffin-embedded biopsies cut in 5 μm serial sections as used for the pathological assessment. Tissue-Tek® Tissue-Clear® was used to deparaffinize the slides and then hydrated through an ethanol gradient from 99.9% ethanol to mQ water. After hydration the slides were put in TBS (Tris-Buffered Saline) with 1.5% H_2O_2. Heat-Induced Epitope Retrieval (HIER) was used to enhance the antibody/antigen binding capability. All antibodies were tested using three different buffers (Citrate (Dako #S2031), Tris-EGTA (TEG), and TRS (Dako #S1699) and in serial dilutions to find the best HIER buffer and the optimum concentration of the antibodies. Antibodies were checked for specificity with IHC before proceeding to IF stainings. We used the following antibodies: CD3 (AB Serotec, #MCA1477), CD4 (Thermo Scientific, #MA5–12259), CD8 (AB Serotec, #MCA1817), Macrophage/L1 molecule (MAC) (AB Serotec, #MCA874G), $K_V1.3$ (Novus Biologicals, #NBP1–19415) and $K_{Ca}3.1$ (Sigma-Aldrich, #AV35098). Primary antibodies were identified using the DAKO Envision™+ Kit (DAKO, Glostrup, Denmark). DAB+ (DAKO) was used as substrate-chromogen system. IHC slides were counterstained with Hematoxyline. IF stainings were performed accordingly using secondary antibodies combined with fluorochromes: Alexa Fluor 488, Alexa Flour 568, and 4’-6-Diamidino-2-phenylindole dihydrochloride (DAPI) for nuclei.

We used the FLoid® Cell Imaging Station (Life Technologies Europe, Nærum, Denmark.) to measure fluorescent signals. Three pictures of each patient were taken on the basis of the nuclei staining (blue) and afterwards the fluorescent signals were obtained (blue for nuclei, red for cell markers, and green for potassium channels). Light intensity and contrast were adjusted for better analyses of signals from T-cell infiltrates. Each fluorescent picture (red, green or blue) was analyzed individually and then quantified with the CellProfiler software

(Broad Institute, Boston, MA, USA)[35] for co-localization of fluorescent signal. This was done automated and blinded.

2.6. Proliferation and cytokine assays

CD3+ T cells were isolated from peripheral blood of a healthy volunteer with RosetteSep (StemCell Technologies, Vancouver, BC, Canada), washed and seeded at 8×10^4 cells per well into flat bottom 96-well plates in RPMI-1640 culture medium. $K_V1.3$ blockers: PAP-1 (concentrations: 100 nM, 250 nM, 1 μM) and ShK-L5 (concentrations: 1 nM, 10 nM),[23,36] $K_{Ca}3.1$ blocker: Senicapoc (concentrations: 100 nM, 250 nM, 1 μM),[37,38] or compound combinations were added at different concentrations and the cells then stimulated with 10 nM PMA + 175 nM ionomycin for 48 h. [^{3}H]-Thymidine (1 μCi per well) was added for the last 8 h. Plates were then frozen and later harvested onto glass fiber filters and radioactivity measured in a scintillation counter. Amounts of secreted cytokines were determined by removing 50 μl/well of supernatant from plates after 40 h of incubation (before the [^{3}H]-thymidine pulse) and frozen at −80 °C pending analysis. A cytokine panel (IL-1β, IL-2, IL-4, IL-10, IL-12, IL-17A, IL-17E/IL-25, IL-17F, IL-22, IFN-γ, and TNF-α) was then analyzed with a Millipore Milliplex magnetic bead human Th17 cytokine/chemokine kit and a Luminex 200™ reader according to the manufacturer's instructions. PAP-1 and Senicapoc were synthesized in the Wulff laboratory at the University of California, Davis. ShK-L5 was a generous gift from Michael Pennington at Peptides International (Louisville, KY).

2.7. Follow-up data

UC patients' records were studied to identify "days to relapse" after the date of inclusion. We defined a relapse as symptoms compatible with active UC and combined with alterations in treatment by the patient's gastroenterologist. Not all patients had endoscopies performed at relapse. We also studied the "days to relapse after initial remission was achieved".

2.8. Statistics

For comparison of datasets we used the unpaired Student's *t*-test or if applicable the non-parametric Mann–Whitney test. Results are presented as Mean ± SEM. To assess differences between two or more groups one or two-way ANOVA followed by the Tukey-post hoc test was used and presented with 95% confidence interval [CI]. All correlations were made using linear regression. The significance level was set as $p < 0.05$. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

3. Results

3.1. Analyses of mRNA expression of T-cell potassium channels, immune cell markers, and cytokines

We included 33 UC patients and 15 healthy controls (Table 1) and performed qPCR on mRNA extracts from mucosal biopsies. Primer sequences are shown in Table 2. First, we examined the expression of CD8 (T_C) and CD4 (T_H) and found that in UC patients the expression of CD8 was clearly 2.5-fold higher than in controls ($p < 0.01$, Fig. 1a). In contrast, UC patients did not show higher expression of CD4 ($p = 0.20$; Fig. 1b). In the UC group we found a 3-fold increase in mRNA-expression of CD14, a marker of monocytes (*p*

< 0.01; Fig. 1c) and a 14-fold increase of CD16, a marker of stimulated monocytes, phagocytic macrophages, and natural killer cells ($p < 0.01$; Fig. 1d).

$K_V1.3$ was increased 5-fold in UC patient biopsies compared to the very low levels of controls ($p < 0.01$; Fig. 1e). In contrast, expression of $K_{Ca}3.1$ was not significantly different (*ns*; Fig. 1f).

UC patients exhibited a 28-fold increase in expression of IFN-γ, a 3-fold increase of TNF-α, and a 200-fold increase of IL-17A compared to the very low levels detected in controls ($p < 0.01$, $p < 0.05$, and $p < 0.01$, respectively; Fig. 1g, h, and i).

3.2. Correlations with clinical scores and blood samples

In keeping with the hypothesis that these genes are markers of disease activity we pooled all data from UC patients and controls and tested whether mRNA expression correlated positively with clinical scores (Mayo score, Mayo endoscopic subscore, and histology score) and laboratory test results (fecal calprotectin, LEU and CRP).

As shown in Fig. 2, mRNA expression of $K_V1.3$ was found to correlate very well, and much better than IFN-γ, TNF-α and IL-17A, with the Mayo endoscopic subscore and the histology score. $K_V1.3$ also showed borderline significant correlations with Mayo-score ($p = 0.06$) and LEU ($p = 0.05$; Fig. 2). The median level of calprotectin, LEU and CRP were 173.5 mg/kg, 8.0×10^9/l, and 2.0 mg/l, respectively. In contrast, $K_{Ca}3.1$ did not correlate with any of the clinical scores or laboratory findings (Table 3).

Subsequently, $K_V1.3$ and $K_{Ca}3.1$ mRNA expression was correlated with the mRNA expression of CD8, CD4, CD14 and CD16, and pro-inflammatory cytokines: IFN-γ, TNF-α and IL-17A (Table 4). Expression of $K_V1.3$ correlated significantly with the expression of CD14, CD16, TNF-α, and IL-17A. Similarly, $K_{Ca}3.1$ expression correlated with those of CD14, CD16 and TNF-α, but not with IL-17A. Additional correlations of CD4, CD8, CD14, CD16, IFN-γ, TNF-α, and IL-17A can be found in the supplement.

UC patients were stratified according to treatment, receiving either 5ASA or 5ASA+ one of the following immunosuppressants: glucocorticoids, infliximab, and azathioprine. However, we did not find significant differences or significant correlations in gene expression levels (data not shown).

In keeping with the idea that $K_V1.3$ and $K_{Ca}3.1$ mRNA expression in the individual UC patient could be indicative of relapse probability and thus of prognostic value, we correlated mRNA levels of channels at inclusion with “days to relapse” (n = 10). Here, we found no correlation with $K_V1.3$ ($R^2 = 0.11$, $p = 0.35$). However, there was a trend towards a negative correlation with $K_{Ca}3.1$ ($R^2 = 0.34$, $p = 0.08$). Examining the “days to relapse after initial remission was achieved” (n = 9), we found no correlations with either $K_V1.3$ ($R^2 = 0.21$, $p = 0.22$) or $K_{Ca}3.1$ ($R^2 = 0.26$, $p = 0.16$).

3.3. Immunostainings

To identify the cell types in the colonic mucosa expressing $K_V1.3$ and/or $K_{Ca}3.1$ channels, we performed immunohistochemical (IHC) and immunofluorescent stainings (IF) (Figs. 3 and 4). IHC revealed the presence of $K_V1.3$ protein only in infiltrating cells in the inflamed mucosa but not in the non-inflamed samples from controls (Fig. 3). In contrast, $K_{Ca}3.1$ protein was found in both colonic crypts of UC and controls as described previously[30,39] and in infiltrating cells present in the inflamed mucosa. In the inflamed mucosa these cells were positive for CD4, CD8, or MAC identifying them as T_H, T_C, or macrophages, respectively. $CD4^+$, $CD8^+$, or MAC^+ cells were only present at normal low levels in controls. In the standard IHC stainings, we did not quantitate the cells and ion channels further as this was done in IF.

Immunofluorescent cell markers were studied for colocalization with $K_V1.3$ and $K_{Ca}3.1$ in the UC patients (Fig. 4 for representative images and Fig. 5 for quantification) and we found that $K_V1.3$ co-localized with CD4 in 57% [CI: 45–70] of cells and with 23% [CI: 5–41] of CD8 cells. Moreover, we found that 80% [CI: 66–95] of the $K_V1.3^+$ cells were chemokine receptor 7 (CCR7) positive (Fig. 5). $K_V1.3$ immunofluorescence was only detectable in 4% [CI: 1–7]of the macrophages. $K_{Ca}3.1$ co-localized with CD4 in 49% [CI: 31–68] of the cells and to a minor extent with 4% [CI: 2–6] of CD8. In macrophages, we found a similar weak co-localization in 6% of the cells [CI: 1–11]. As for controls, immunostainings were also performed, but there were only very few infiltrating T cells. Therefore, we preferred to not compare the relative colocalization between UC and control samples.

This analysis demonstrates that T_H cells ($CD4^+$) showed more abundant expression of both $K_V1.3$ and $K_{Ca}3.1$ proteins compared to T_C cells ($CD8^+$) and macrophages (Fig. 5).

3.4. Cytokine production and cell proliferation assay

To study if $K_V1.3$ and $K_{Ca}3.1$ play a role in T cell cytokine production, we stimulated $CD3^+$ T cells with PMA+ ionomycin to drive a strong, Ca^{2+}-dependent T cell activation.[40] Successful stimulation was verified by incorporation of [^{3}H]-thymidine (Fig. 6), which is a measure of proliferation.

Blockade of the $K_V1.3$ channel with 1 μM PAP-1 revealed a decrease in cell proliferation and a decrease in production of inflammatory cytokines: IL-2, IFN-γ, TNF-α, IL-12, and IL17-A. In contrast, the anti-inflammatory cytokine IL-10 was increased. Using another $K_V1.3$ blocker, ShK-L5, similar results were found (Fig. 6).

Blockade of $K_{Ca}3.1$ with 1 μM Senicapoc showed decreased production of IL-2, IFN-γ, IL-12, and IL-17A, but not of TNF-α and IL-10. Using the $K_V1.3$ and $K_{Ca}3.1$ blockers in combination (at concentrations of 1 μM), the secretion of all the above-mentioned cytokines and proliferation were suppressed almost to the same levels of non-stimulated cells. The cytokines IL-1β, IL-4, IL-17E, IL-17 F and IL-22 were below detection limit.

4. Discussion

The main outcome of our study is that mRNA expression and protein expression of $K_V1.3$ in infiltrating T_H and T_C in the inflamed mucosa are characteristic features of active ulcerative colitis. Moreover, we found that pharmacological blockade of $K_V1.3$ and $K_{Ca}3.1$ in isolated human T cells, in vitro, led to decreased production of pro-inflammatory cytokines, including TNF-α, IFN-γ, and IL-17A. Furthermore, increased mRNA expression of $K_V1.3$, in vivo, was linked to increased expression of the inflammatory cytokines TNF-α and to IL-17A.

To our knowledge, we provide the first evidence that T-cell $K_V1.3$ potassium channels could serve as a marker of disease activity and as a potential treatment target in active UC in vivo.

$K_V1.3$ contributes to T-cell function by setting and keeping the membrane potential and thus maintains the driving force for calcium influx after T-cell stimulation.[19] Moreover, $K_V1.3$ channels are highly expressed in effector memory T-cells (T_{EM}) in autoimmune diseases and are believed to contribute to the pathology of multiple sclerosis, type-1 diabetes and rheumatoid arthritis by sustaining the activity of $CCR7^{-}T_{EM}$ cells.[24–28,41] Although UC is not considered a pure T cell disease, but also may be related to barrier problems etc., our study still suggests that $K_V1.3$ channels play a role in the inflammation of patients with active UC. Here, we report that $K_V1.3$ is present on T-cell infiltrates of inflamed mucosa and particularly in most T_H (80%) and to a lesser extent in T_C (23%) cells in UC. However, the majority of the $Kv1.3^{+}$ cells are $CCR7^{+}$. This suggests that $K_V1.3$ is a feature of general T-cell infiltration and activity of T cells in active UC, but unlike in inflammatory infiltrates in MS brain,[24] we do not have evidence that the $Kv1.3^{+}$ cells are classical $CCR7^{-}$ T_{EM} cells.

Considering the channel as a molecular marker of disease activity, high $K_V1.3$ expression correlates better with the degree of inflammation than the expression of TNF-α and IL-17A, which are known mediators of the inflammatory response in both UC and CD.[11,12,42,43] Interestingly, up-regulation of IL-17A mRNA expression has been associated with treatment success in infliximab treatment of active UC.[44] However, IL-17A's pathomechanistic roles are still unclear since IL-17A secretion by activated T-cells has been suggested to be pro-inflammatory[10,11] – by recruiting other immune cells – as well as protective in UC as IL17-deficient T-cells produce more T_{H1} cytokines in a model of T-cell driven colitis.[45]

Interestingly, $K_V1.3$ mRNA expression distinguishes more clearly between UC and controls than the classical markers of T_H ($CD4^{+}$) and T_C ($CD8^{+}$) but equally well as a marker of stimulated monocytes, phagocytic macrophages, and natural killer cells (CD16). Importantly, $K_V1.3$ expression correlated positively with the clinical parameters and non-molecular markers of disease activity thus further supporting the idea that this channel is a strong molecular marker of UC activity.

Mechanistically, our T cells stimulation assay revealed that $K_V1.3$ and $K_{Ca}3.1$ are indeed involved in cell proliferation and in the secretion of inflammatory cytokines. In other studies, $K_V1.3$ channel inhibition or knockout in mice subjected to experimental autoimmune encephalomyelitis has also been associated with decreased production of pro-inflammatory cytokines, IFN-γ and IL-17A.[27] Additionally, pharmacological inhibition of

$K_V1.3$ in rats by PAP-1 ameliorated oxazolone-induced allergic contact dermatitis in vivo and decreased production of IFN-γ and IL17-A.[46]

In our study, we also demonstrated that $K_V1.3$ correlated strongly with expression levels of TNF-α and IL-17A suggesting a link between the function of the channels and the syntheses of these two cytokines in particularly T_H ($CD4^+$) cells. But in keeping with the presence of the channel in a subset of T_C ($CD8^+$) cells we cannot exclude that the channel may also drive cytotoxic functions of T_C ($CD8^+$) cells. While CD8-mRNA expression was significantly increased almost 3-fold, surprisingly, the 1.5-fold difference in mRNA expression of CD4 between the two groups did not reach statistical significance despite the fact that both $CD4^+$ and $CD8^+$ T cells infiltrated the mucosa of the UC patients in the present study and of those of previous studies.[9,47]

Moreover, our study showed increased mRNA expressions of IFN-γ, TNF-α, and IL-17A in these UC patients compared to controls, which suggests both T_H1 and T_H17 responses. However, it is also well established that an atypical T_H2 response is involved in the pathogenesis of UC in contrast to CD, which has been suggested to be T_H1 driven with IFN-γ as the major cytokine.[13,15,16,18,48] Our results do not allow us to distinguish between a T_H1 and a T_H2 response.

The precise mechanism, by which $K_V1.3$ channels contribute to the functions of T-cells in the inflamed mucosa of UC patients, remains unclear. However, $K_V1.3$ probably stabilizes a negative membrane potential that in turn maintains the electrical driving force for calcium influx through store-operated calcium channels in the activated T_H and T_C as reported previously.[21,22,49–51] $K_V1.3$ mRNA expression also correlated with CD14 and CD16 expression. This suggests that high expression of $K_V1.3$ in T-cells, but not in the macrophages themselves, is involved in the recruitment of macrophages into the mucosa presumably by its above mentioned control of TNF-α synthesis in T-cells. Together, the above findings support that $K_V1.3$ modulation is affecting mechanisms up-stream of the synthesis of these inflammatory cytokines.

Regarding the other T-cell potassium channel $K_{Ca}3.1$, a steep up-regulation of the channel in activated T-cells (from very low basal expression levels in naïve unstimulated T-cells) is found in in vitro experimentation and is suggested to drive re-stimulated T-cell function, expansion, and migration by having a strong positive feedback on calcium influx and thus maintaining elevated intracellular calcium levels.[19,52–54] Here, we found that $K_{Ca}3.1$ mRNA expression did not differentiate between UC and controls. This is apparently due to the constitutive expression of the channels in colonic crypts in both UC and control group, which has been described previously[39,55] thus masking potential differences in T-cell expression levels between the two. Moreover, a down-regulation of $K_{Ca}3.1$ functions and unchanged mRNA expression has been reported to occur in crypts of UC patients.[30] Together, these circumstances may explain the lack of correlation of $K_{Ca}3.1$ mRNA expression with clinical parameters of UC. Nonetheless, expression levels correlated positively with the expression of TNF-α, CD14 and CD16 suggesting that $K_{Ca}3.1$ is involved in the recruitment of monocytes, macrophages, and possibly natural killer cells to the inflammation site. Despite the fact that we did not find a difference between controls and

UC patients, studies investigating the role of $K_{Ca}3.1$ in two mouse models and a rat model of UC showed that inhibition of $K_{Ca}3.1$ led to decreased inflammation, thus suggesting $K_{Ca}3.1$ as a potential pharmacological target.[56,57]

Although $K_V1.3$ mRNA expression correlated well with disease activity in our UC cohort, we did not find significant correlations between either of the two potassium channels and "days to relapse" from the inclusion date or after remission was achieved. This indicates that in this small patient cohort and the relatively short follow-up period (range: 4 to 26 months) the observed expression levels of the channel are not indicative of relapse probability. Nonetheless, we observed a trend ($p = 0.08$) towards a negative correlation of $K_{Ca}3.1$ expression and "days to relapse", which could indicate that high expression of $K_{Ca}3.1$ increases relapse probability. However, longer follow-up intervals and more patient data are needed to clarify whether channel expression levels are of potential prognostic value.

In conclusion, the present study identified, for the first time, $K_V1.3$ in T-cell infiltrates in active UC as a novel molecular marker of disease activity and more interestingly, showed that $K_V1.3$ and to some extent also $K_{Ca}3.1$ play a role in the production of inflammatory cytokines and thus may serve as a new pharmacological target upstream of TNF-α and IL17-A for the treatment of IBD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.crohns.2014.04.003.

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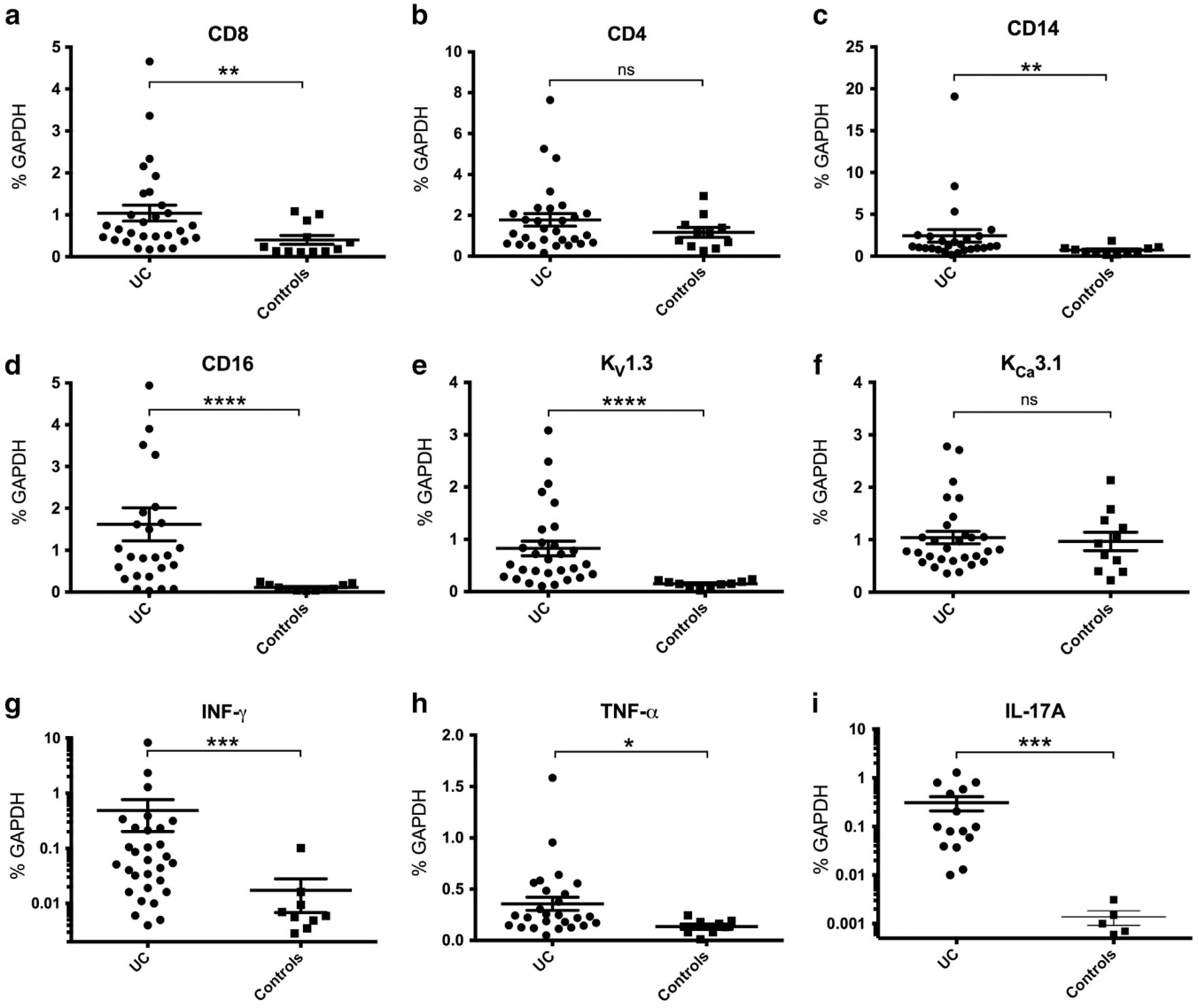

Figure 1.

mRNA expression of cell markers, pro-inflammatory cytokines, and potassium channels in mucosal biopsies of UC patients and controls. Data from individual patients are also given as means ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

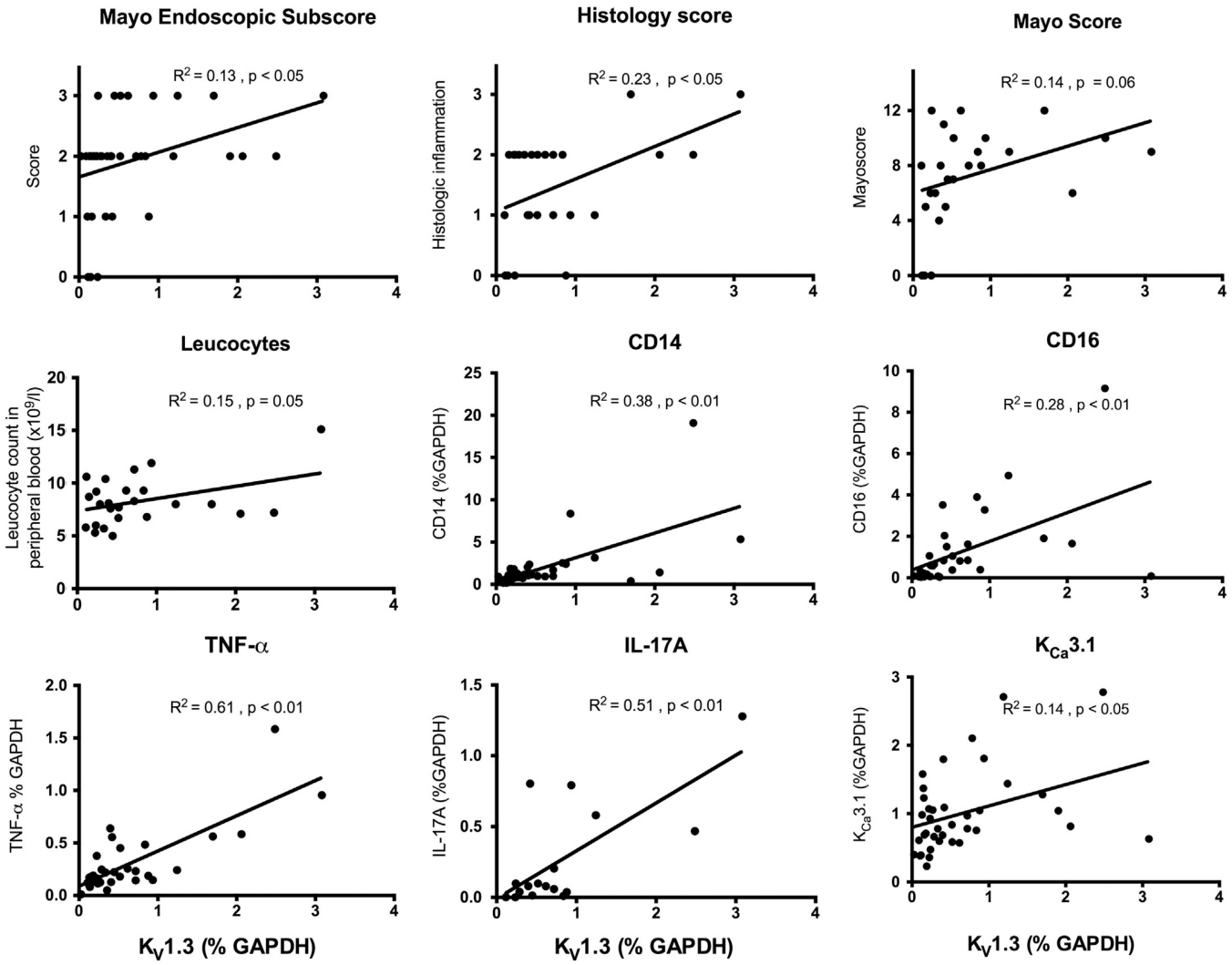

Figure 2.

Significant and borderline significant correlations of $K_V1.3$ mRNA expression (in percentage of GAPDH) with clinical scores, cell markers and cytokines. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

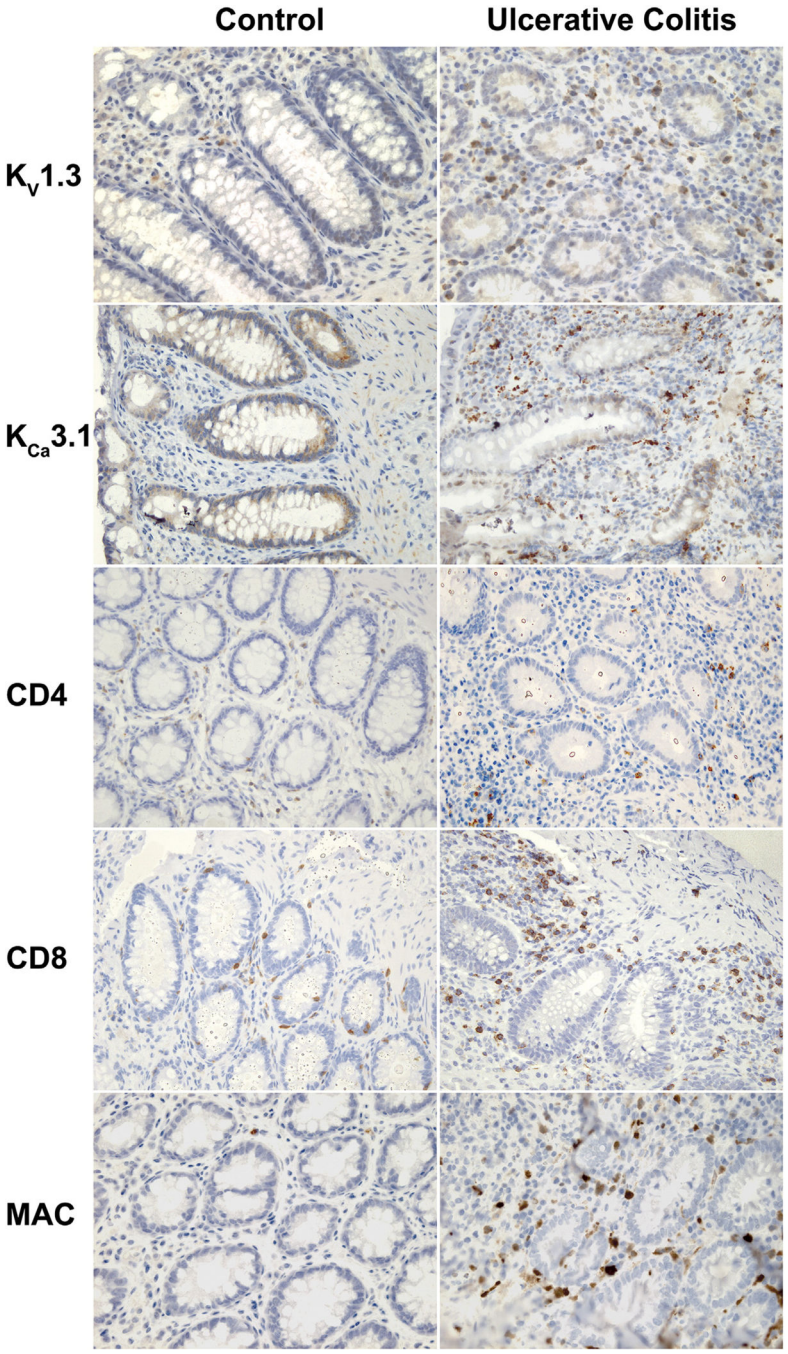

Figure 3.

Immunohistochemical stainings visualizing the potassium channels and cell types in the normal mucosa of controls and the inflamed mucosa of patients with active ulcerative colitis. Note that the crypts in UC are more irregular and contain less mucus. $K_V1.3^+$ cells are located in the interstitial tissue around crypts in UC patients. Crypts in both patient groups are positive for $K_{Ca}3.1$ and immunoreactivity is also detected in cell infiltrates in UC. Both $CD4^+$ and $CD8^+$ cells clearly infiltrate the tissue in UC. Infiltration of macrophages (MAC) is also evident in the inflamed mucosa of UC.

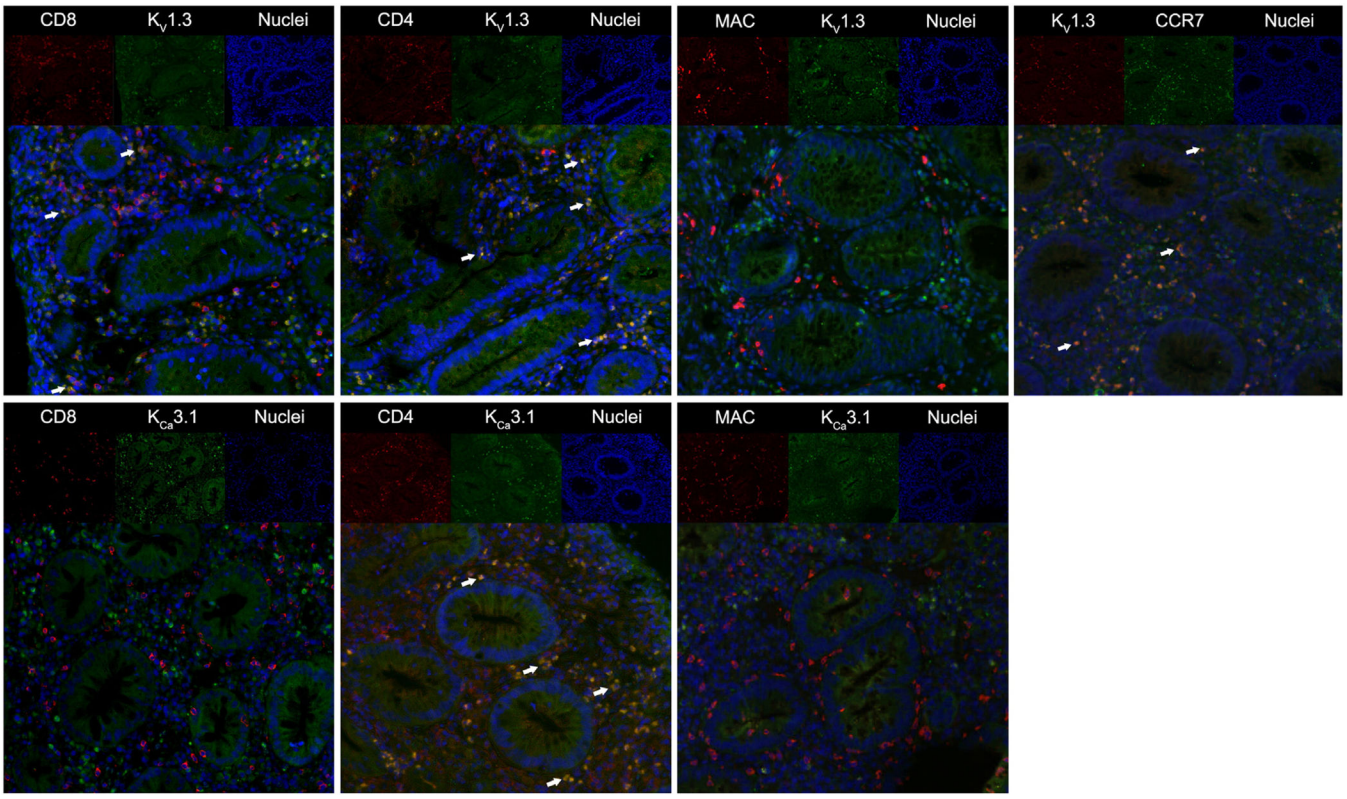

Figure 4.

Immunofluorescent stainings of potassium channels, cell markers, and chemokine-receptor-7 (CCR7). In the upper part of each picture the individual fluorochromes are shown. The lower part shows the merged pictures visualizing co-localized immunofluorescence as a yellow signal. Arrows indicates co-localized immunofluorescence. The top row displays immunostainings of $K_V1.3$ and in the lower row $K_{Ca}3.1$.

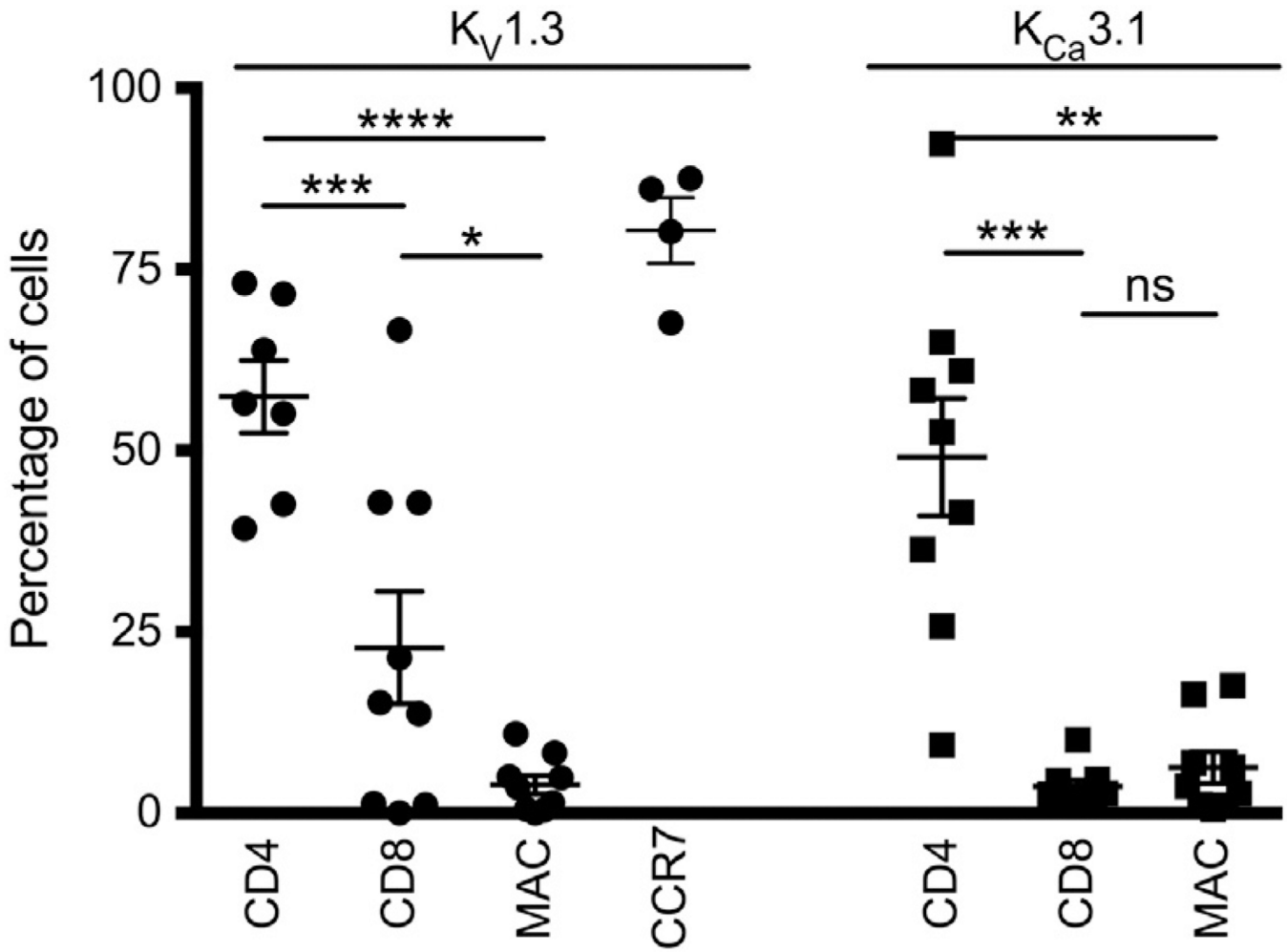

Figure 5.

The left side of the graph shows the percentage of $K_V1.3$ immunofluorescence that co-localize with CD8, CD4, macrophages and Chemokine Receptor 7 (CCR7) after analyses with the Cell Profiler software. The right side shows the percentage of $K_{Ca}3.1$ immunofluorescence co-localizing with CD4, CD8 and macrophages. Error bars show mean ± SEM.

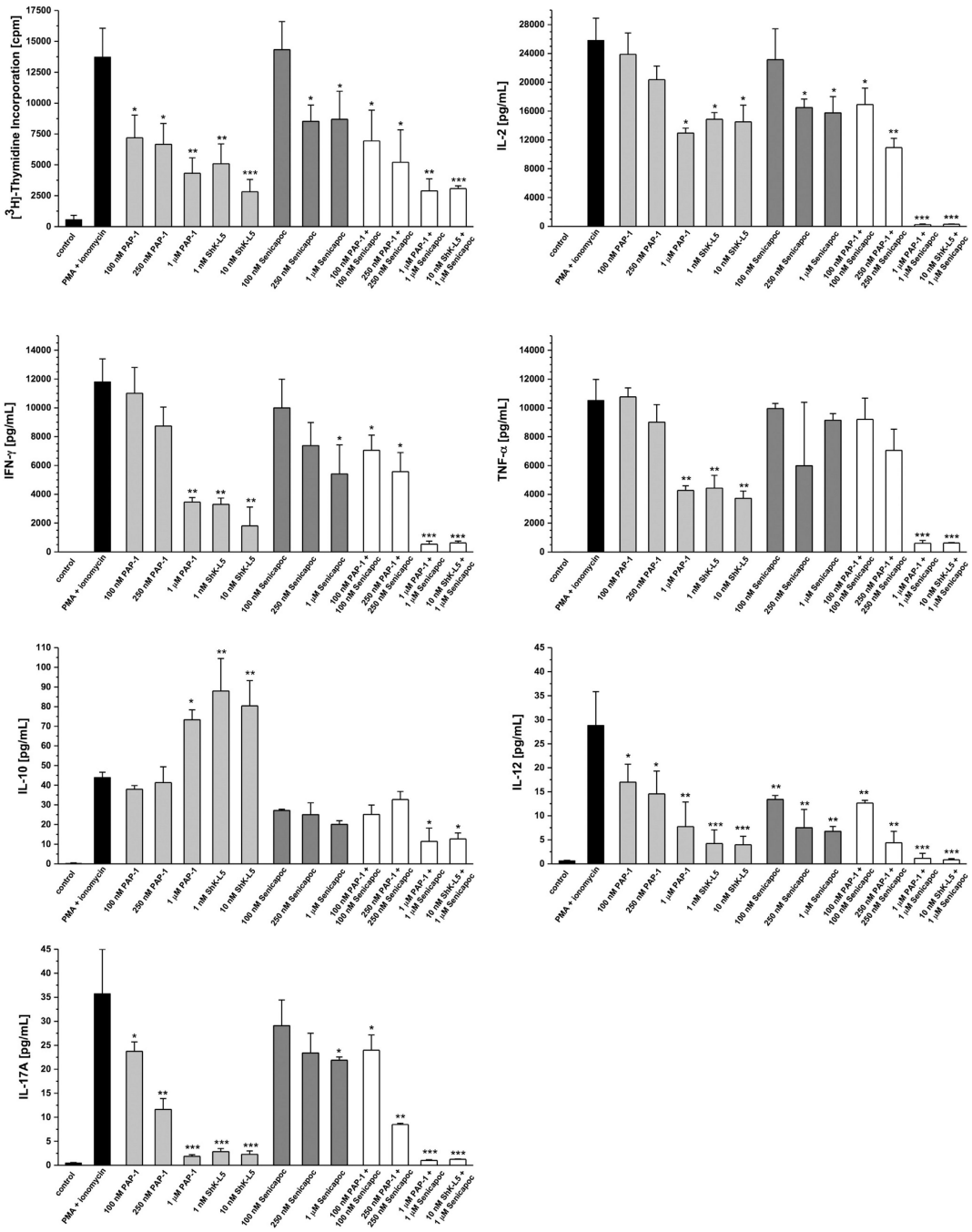

Figure 6.

Effect of $K_V1.3$ and $K_{Ca}3.1$ blockage in PMA (phorbol-12-myristate-13-acetate) + Ionomycin-stimulated human $CD3^+$ T cells. First figure shows $[^3H]$-thymidine incorporation, which is a measure of cell proliferation. Additional figures visualize production of inflammatory cytokines. PAP-1 and ShK-L5 are $K_V1.3$ blockers; Senicapoc is a $K_{Ca}3.1$ blocker. The blockers were tested at different concentrations, and in the right-hand side of each graph combinations of blockers are presented. Individually, the blockers

decrease the level of cell proliferation and cytokine secretion and when used in combination the effect is even more prominent.

Table 1

Baseline characteristics of controls and patients with ulcerative colitis (UC) at inclusion. 5ASA = Mesalazine, GC = Glucocorticoids, IFX = Infliximab, AZA = Azathioprine.

	N	Mean age years	Male N (%)	Mayoscore Mean [CI]	5ASA N (%)	GC N (%)	IFX N (%)	AZA N (%)
Controls	15	55	7 (47%)	0	0	0	0	0
UC	33	44	13 (39%)	8.3 [7.4–9.2]	28 (85%)	8 (24%)	5 (15%)	2 (1%)

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Table 2

Primer specifications.

Primer	Size (bp)	NCBI reference sequence	Sense	Anti-sense
GAPDH	91	NM 002046.3	caccatcaaggctgagaacg	gccccacttgattttggagg
CD8	112	NM 001145873.1	gctggacttcgcctgtgatatc	acgtcttcggttcctgtggtt
CD4	74	NM 000616.4	ccctttaggcacttgcttctg	gcaccactttctttccctgagt
CD14	165	NM 001174105.1	gccgctgtgtaggaaagaag	ttcatcgtccagctcacaag
CD16	156	NM_001127595.1	gctccggatatctttggtga	agcaccctgtaccattgagg
$K_{Ca}3.1$	159	NM_002250.2	catcacattcctgaccatcg	acgtgcttctctgccttgtt
$K_V1.3$	175	NM_002232.3	tctggtgggcagtggtaacc	ccttctgtctcccggtggta
IFN-γ	236	NM_000619.2	tgaccagagcatccaaaaga	ctcttcgacctcgaaacagc
TNF-α	151	NM_000594.2	tcttctcgaaccccgagtga	cctctgatggcaccaccag
IL-17A	174	NM_002190.2	catccataaccggaataccaata	tagtccacgttcccatcagc

Table 3

Correlations between mRNA expression of $K_V1.3$ and $K_{Ca}3.1$ potassium channels in mucosal biopsies and clinical parameters. Statistical analyses were performed using linear regression.

$K_V1.3$	R^2	*p* value	$K_{Ca}3.1$	R^2	*p* value
Mayo-score	0.142	(*) 0.058	Mayo-score	0.004	0.773
Mayo endoscopic subscore	0.130	* 0.024	Mayo endoscopic subscore	0.001	0.825
Histology Score	0.230	* 0.015	Histology score	0.036	0.361
Age	0.007	0.622	Age	0.008	0.590
Fecal Calprotectin (mg/kg)	<0.001	0.985	Fecal calprotectin (mg/kg)	0.015	0.723
Leucocytes in PB ($\times 10^9$/l)	0.153	(*) 0.054	Leucocytes in PB ($\times 10^9$/l)	0.001	0.863
C-reactive protein in PB (mg/l)	0.020	0.495	C-reactive protein in PB (mg/l)	0.006	0.712

(PB = peripheral blood).

* $p < 0.05$;

(*) $p = 0.05–0.1$.

Table 4

Correlations between mRNA expression of $K_V1.3$ and $K_{Ca}3.1$ potassium channels and pro-inflammatory cytokines and cell markers. Data are shown as Goodness of Fit (R^2) and P value. Significant correlations are highlighted.

GENE	$K_{Ca}3.1$	IFN-γ	TNF-α	IL-17A	CD4	CD8	CD14	CD16
$K_V1.3$	R^2= 0.14 p = 0.20	R^2 = 0.02 p = 0.39	***R^2 = 0.61 p < 0.01***	***R^2 = 0.51 p < 0.01***	R^2 = 0.07 p = 0.10	R^2 = 0.08 p = 0.10	***R^2 = 0.38 p < 0.01***	***R^2 = 0.28 p < 0.01***
$K_{Ca}3.1$		R^2 = 0.05 p = 0.19	***R^2 = 0.15 p = 0.03***	R^2 = 0.05 p = 0.39	R^2 < 0.01 p = 0.69	R^2 < .01 p = .89	***R^2 = 0.38 p < 0.01***	***R^2 = 0.31 p < 0.01***