Expression of T-cell Kv1.3 potassium channel correlates with pro-inflammatory cytokines and disease activity in ulcerative colitis☆

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Received 21 January 2014; received in revised form 18 March 2014; accepted 7 April 2014

KEYWORDS

Novel treatment strategy; Colitis ulcerosa; KCNN4; KCNA3; Interleukins; KCa3.1

Abstract

Background and aims: Potassium channels, Kv1.3 and KCa3.1, 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 Kv1.3 and KCa3.1 in the inflamed mucosa are markers of active UC. We hypothesized that Kv1.3 and KCa3.1 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 Kv1.3 and KCa3.1, 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 Kv1.3 and KCa3.1.

☆ Conference presentation: Part of this data was presented at the 8th Congress of ECCO, Vienna, Austria, February 2013.

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http://dx.doi.org/10.1016/j.crohns.2014.04.003

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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. 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. It has also been speculated that the inflammation could be of heritable and of autoimmune origin although the nature of this autoimmunity remains elusive. After onset, mucosal inflammation is likely maintained by an abnormal activity of cytotoxic T-cells (T_C, CD8+) and T helper cells (TH, CD4+), 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. 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. It seems as if the immunological balance between TH1, TH2 and TH17 responses is disturbed, and that an atypical TH2 response occurs involving non-classical natural killer T cells.

Ion channels in the T cell membrane play pivotal roles in T cell functions by maintaining intracellular Ca2+-homeostasis, cytokine production, and clonal expansion after T-cell receptor (TCR) activation. Of particular importance are the voltage-gated potassium channel Kv1.3 (encoded by the KCNA3 gene) and the calcium-activated potassium channel KCa3.1 (encoded by the KCNN4 gene). Constitutively expressed Kv1.3 channels stabilize the membrane potential, thus keeping the electrical driving force for sustained Ca2+ influx through calcium release activated channels (CRAC) after TCR activation. This long-lasting increase in cytosolic Ca2+ triggers T-cell proliferation and cytokine production. Increasing evidence also supports that Kv1.3 channels play a role in autoimmune diseases such as type-1 diabetes, rheumatoid arthritis, multiple sclerosis, and glomerulonephritis. Effector memory T-cells (TEM)(CD8+/CCR7 and CD4+/CCR7), a subset of terminally differentiated T cells, express high levels of Kv1.3 channels while activated TEM and naive T-cells primarily express and use the calcium-activated KCa3.1 channel to regulate their Ca2+ signaling.

In the light of these findings, small molecule blockers of Kv1.3 and KCa3.1 may be of therapeutic utility in inflammatory and autoimmune diseases. Whether Kv1.3 and KCa3.1 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 Kv1.3 and KCa3.1 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 Kv1.3 and KCa3.1 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). Histological inflammation of the biopsies was scored according to Morson and Dawson’s grading (0 none, 1 mild, 2 moderate, 3 severe) by a gastrointestinal pathologist.

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 (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% H2O2. Heat-Induced Epitope Retrieval (HIER) was used to enhance the antibody/antigen binding capability. All antibodies were tested using three different buffers (Citrate (Dako #52031), Tris-EGTA (TEG), and TRS (Dako #51699) 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), K01.3 (Novus Biologicals, #NB1P-19415) and K03.1 (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 Fluor 568, and 4’-6-Diamidino-2-phenylindole dihydrochloride (DAPI) for nuclei.

We used the Fluidigm Cell Imaging Station (Life Technologies Europe, Naerum, 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) 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 8x10^4 cells per well into flat bottom 96-well plates in RPMI-1640 culture medium. K03.1 blockers: PAP-1 (concentrations: 100 nM, 250 nM, 1 μM) and ShK-L5 (concentrations: 1 μM, 10 nM),23,36 K03.1 blocker: Senicapoc (concentrations: 100 nM, 250 nM, 1 μM) or compound combinations were added at different concentrations and the cells then stimulated with 10 nM PMA + 175 nM ionomycin for 48 h. [3H]-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 [3H]-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
Expression of T-cell KV1.3 potassium channel

Table 1 Baseline characteristics of controls and patients with ulcerative colitis (UC) at inclusion. 5ASA = Mesalazine, GC = Glucocorticoids, IFX = Infliximab, AZA = Azathioprine.

<table>
<thead>
<tr>
<th>Control</th>
<th>N</th>
<th>Mean age years</th>
<th>Male N (%)</th>
<th>MayoScore Mean [CI]</th>
<th>5ASA N (%)</th>
<th>GC N (%)</th>
<th>IFX N (%)</th>
<th>AZA N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>15</td>
<td>55</td>
<td>7 (47%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>UC</td>
<td>33</td>
<td>44</td>
<td>13 (39%)</td>
<td>8.3 [7.4–9.2]</td>
<td>28 (85%)</td>
<td>8 (24%)</td>
<td>5 (15%)</td>
<td>2 (1%)</td>
</tr>
</tbody>
</table>

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 (Tc) and CD4 (Th) 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).

$K_{V}1.3$ 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-$\gamma$, a 3-fold increase of TNF-$\alpha$, 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. 1 g, 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 $K_{V}1.3$ was found to correlate very well, and much better than IFN-$\gamma$, TNF-$\alpha$, and IL-17A, with the Mayo endoscopic subscore and the histology score. $K_{V}1.3$ 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, $K_{V}1.3$ and $K_{Ca}3.1$ mRNA expression was correlated with the mRNA expression of CD8, CD4, CD14 and CD16, and pro-inflammatory cytokines: IFN-$\gamma$, TNF-$\alpha$, and IL-17A (Table 4). Expression of $K_{V}1.3$ correlated significantly with the expression of CD14, CD16, TNF-$\alpha$, and IL-17A. Similarly, $K_{Ca}3.1$ expression correlated with those of CD14, CD16 and TNF-$\alpha$, but not with IL-17A. Additional correlations of CD4, CD8, CD14, CD16, IFN-$\gamma$, TNF-$\alpha$, 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 $K_{V}1.3$ 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 $K_{V}1.3$ ($R^2 = 0.11$, $p = 0.35$). 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 $K_{V}1.3$ ($R^2 = 0.21$, $p = 0.22$) or $K_{Ca}3.1$ ($R^2 = 0.26$, $p = 0.16$).

Table 2 Primer specifications.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Size (bp)</th>
<th>NCBI reference sequence</th>
<th>Sense</th>
<th>Anti-sense</th>
</tr>
</thead>
<tbody>
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<td>GAPDH</td>
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<td>NM_002046.3</td>
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<td>gccccacactgatattttgaga</td>
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<td>CD8</td>
<td>112</td>
<td>NM_001145873.1</td>
<td>gctggtctgctgtatctcttc</td>
<td>acgtctcggctctgcttcgtt</td>
</tr>
<tr>
<td>CD4</td>
<td>90</td>
<td>NM_000616.4</td>
<td>cctcttggacgccactccttc</td>
<td>gacccacactctttcccttgatg</td>
</tr>
<tr>
<td>CD14</td>
<td>165</td>
<td>NM_001174105.1</td>
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<td>ttcctactgctcagctcacaag</td>
</tr>
<tr>
<td>CD16</td>
<td>156</td>
<td>NM_001127595.1</td>
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<td>agacccttgacctctatgag</td>
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<td>K_{V}1.3</td>
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<td>NM_002250.2</td>
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<td>K_{Ca}3.1</td>
<td>175</td>
<td>NM_002232.3</td>
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<td>cttctactgctcagctcacaag</td>
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<tr>
<td>IFN-$\gamma$</td>
<td>236</td>
<td>NM_000619.2</td>
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<td>acctgctctgcgcttggt</td>
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<tr>
<td>TNF-$\alpha$</td>
<td>151</td>
<td>NM_000594.2</td>
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</tr>
<tr>
<td>IL-17A</td>
<td>174</td>
<td>NM_002190.2</td>
<td>catcactaaccggaaatacacta</td>
<td>tagtccacagttccccatcagc</td>
</tr>
</tbody>
</table>
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 Kv1.3 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.
Table 3  Correlations between mRNA expression of Kv1.3 and KCa3.1 potassium channels and clinical parameters. Statistical analyses were performed using linear regression. (PB = peripheral blood). *p < 0.05; () p = 0.05–0.1.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Kv1.3</th>
<th>R²</th>
<th>p value</th>
<th>KCa3.1</th>
<th>R²</th>
<th>p value</th>
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<tr>
<td>Mayo-score</td>
<td>0.142</td>
<td>0.058</td>
<td>Mayo-score</td>
<td>0.004</td>
<td>0.130</td>
<td>0.001</td>
</tr>
<tr>
<td>Mayo endoscopic subscore</td>
<td>0.230</td>
<td>0.015</td>
<td>History score</td>
<td>0.036</td>
<td>0.230</td>
<td>0.022</td>
</tr>
<tr>
<td>Histology Score</td>
<td>0.007</td>
<td>0.622</td>
<td>Age</td>
<td>0.008</td>
<td>0.590</td>
<td></td>
</tr>
<tr>
<td>Fecal Calprotectin (mg/kg)</td>
<td>&lt;0.001</td>
<td>0.985</td>
<td>Fecal Calprotectin (mg/kg)</td>
<td>0.015</td>
<td>0.230</td>
<td></td>
</tr>
<tr>
<td>Leucocytes in PB (×10⁹/l)</td>
<td>0.153</td>
<td>0.054</td>
<td>Leucocytes in PB (×10⁹/l)</td>
<td>0.001</td>
<td>0.863</td>
<td></td>
</tr>
<tr>
<td>C-reactive protein in PB (mg/l)</td>
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<td>0.495</td>
<td>C-reactive protein in PB (mg/l)</td>
<td>0.006</td>
<td>0.712</td>
<td></td>
</tr>
</tbody>
</table>

3.3. Immunostainings

To identify the cell types in the colonic mucosa expressing Kv1.3 and/or KCa3.1 channels, we performed immunohistochemical (IHC) and immunofluorescent stainings (IF) (Figs. 3 and 4). IHC revealed the presence of Kv1.3 protein only in infiltrating cells in the inflamed mucosa but not in the non-inflamed samples from controls (Fig. 3). In contrast, KCa3.1 protein was found in both colonic crypts of UC and controls as described previously 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 Th, Tc, 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 co-localization with Kv1.3 and KCa3.1 in the UC patients (Fig. 4 for representative images and Fig. 5 for quantification) and we found that Kv1.3 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 Kv1.3⁺ cells were chemokine receptor 7 (CCR7) positive (Fig. 5). Kv1.3 immunofluorescence was only detectable in 4% [CI: 1–7] of the macrophages. Kv3.1 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 not to compare the relative co-localization between UC and control samples.

This analysis demonstrates that Tc cells (CD8⁺) showed more abundant expression of both Kv1.3 and KCa3.1 proteins compared to Th cells (CD4⁺) and macrophages (Fig. 5).

3.4. Cytokine production and cell proliferation assay

To study if Kv1.3 and KCa3.1 play a role in T cell cytokine production, we stimulated CD3⁺ T cells with PMA + ionomycin to drive a strong, Ca²⁺-dependent T cell activation. Successful stimulation was verified by incorporation of [³H]-thymidine (Fig. 6), which is a measure of proliferation.

Blockade of the Kv1.3 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 Kv1.3 blocker, ShK-L5, similar results were found (Fig. 6).

Blockade of KCa3.1 with 1 μM Senicapoc showed decreased production of IL-2, IFN-γ, IL-12, and IL17-A, but not of TNF-α and IL-10. Using the Kv1.3 and KCa3.1 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 Kv1.3 in infiltrating Th and Tc in the inflamed mucosa are characteristic features of active ulcerative colitis. Moreover, we found that pharmacological blockade of Kv1.3 and KCa3.1 in isolated human T cells, in vitro, led to decreased production of pro-inflammatory cytokines, including TNF-α, IFN-γ, and IL17-A. Furthermore, increased mRNA expression of Kv1.3, 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 Kᵥ1.3 potassium channels could serve as a marker of disease activity and as a potential treatment target in active UC in vivo.

Kᵥ1.3 contributes to T-cell function by setting and keeping the membrane potential and thus maintains the

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. Kᵥ1.3+ cells are located in the interstitial tissue around crypts in UC patients. Crypts in both patient groups are positive for KᵥCa3.1 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 Kv1.3 and in the lower row Kv3.1.
driving force for calcium influx after T-cell stimulation.\textsuperscript{19}
Moreover, \(K_{\text{v}1.3}\) channels are highly expressed in effector memory T-cells (\(T_{\text{EM}}\)) 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\(T_{\text{EM}}\) cells.\textsuperscript{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 \(K_{\text{v}1.3}\) channels play a role in the inflammation of patients with active UC. Here, we report that \(K_{\text{v}1.3}\) is present on T-cell infiltrates of inflamed mucosa and particularly in most \(T_{\text{H}}\) (80\%) and to a lesser extent in \(T_{\text{C}}\) (23\%) cells in UC. However, the majority of the \(K_{\text{v}1.3}\) expressing cells are CCR7\(^+\). This suggests that \(K_{\text{v}1.3}\) is a feature of general T-cell infiltration and activity of T cells in active UC, but unlike in inflammatory infiltrates in MS brain,\textsuperscript{24} we do not have evidence that the \(K_{\text{v}1.3}\) expressing cells are classical CCR7\(^+\) TEM cells.

Considering the channel as a molecular marker of disease activity, high \(K_{\text{v}1.3}\) expression correlates better with the degree of inflammation than the expression of TNF-\(\alpha\) and IL-17A, which are known mediators of the inflammatory response in both UC and CD.\textsuperscript{11,12,42,43} Interestingly, up-regulation of IL-17A mRNA expression has been associated with treatment success in infliximab treatment of active UC.\textsuperscript{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\textsuperscript{10,11} – by recruiting other immune cells – as well as protective in UC as IL17-deficient T-cells produce more \(T_{\text{H}1}\) cytokines in a model of T-cell driven colitis.\textsuperscript{45}

Interestingly, \(K_{\text{v}1.3}\) mRNA expression distinguishes more clearly between UC and controls than the classical markers of \(T_{\text{H}}\) (CD4\(^+\)) and \(T_{\text{C}}\) (CD8\(^+\)) but equally well as a marker of stimulated monocytes, phagocytic macrophages, and natural killer cells (CD16). Importantly, \(K_{\text{v}1.3}\) 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 \(K_{\text{v}1.3}\) and \(K_{\text{Ca}3.1}\) are indeed involved in cell proliferation and in the secretion of inflammatory cytokines. In other studies, \(K_{\text{v}1.3}\) channel inhibition or knockout in mice subjected to experimental autoimmune encephalomyelitis has also been associated with decreased production of pro-inflammatory cytokines, IFN-\(\gamma\) and IL-17A.\textsuperscript{27} Additionally, pharmacological inhibition of \(K_{\text{v}1.3}\) in rats by PAP-1 ameliorated allergic contact dermatitis in vivo and decreased production of IFN-\(\gamma\) and IL17-A.\textsuperscript{46}

In our study, we also demonstrated that \(K_{\text{v}1.3}\) correlated strongly with expression levels of TNF-\(\alpha\) and IL-17A suggesting a link between the function of the channels and the synthesis of these cytokines in particularly \(T_{\text{H}}\) (CD4\(^+\)) cells. But in keeping with the presence of the channel in a subset of \(T_{\text{C}}\) (CD8\(^+\)) cells we cannot exclude that the channel may also drive cytotoxic functions of \(T_{\text{C}}\) (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.\textsuperscript{9,47}

Moreover, our study showed increased mRNA expressions of IFN-\(\gamma\), TNF-\(\alpha\), and IL-17A in these UC patients compared to controls, which suggests both \(T_{\text{H}1}\) and \(T_{\text{H}17}\) responses. However, it is also well established that an atypical \(T_{\text{H}1}\) response is involved in the pathogenesis of UC in contrast to CD, which has been suggested to be \(T_{\text{H}1}\) driven with IFN-\(\gamma\) as the major cytokine.\textsuperscript{13,15,16,18,48} Our results do not allow us to distinguish between a \(T_{\text{H}1}\) and a \(T_{\text{H}17}\) response.

The precise mechanism, by which \(K_{\text{v}1.3}\) channels contribute to the functions of T-cells in the inflamed mucosa of UC patients, remains unclear. However, \(K_{\text{v}1.3}\) 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 \(T_{\text{H}}\) and \(T_{\text{C}}\) as reported previously.\textsuperscript{21,22,49–51} \(K_{\text{v}1.3}\) mRNA expression also correlated with CD14 and CD16 expression. This suggests that high expression of \(K_{\text{v}1.3}\) 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-\(\alpha\) synthesis in T-cells. Together, the above findings support that \(K_{\text{v}1.3}\) modulation is affecting mechanisms up-stream of the synthesis of these inflammatory cytokines.

Regarding the other T-cell potassium channel \(K_{\text{Ca}3.1}\), 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.\textsuperscript{19,52–54} Here, we found that \(K_{\text{Ca}3.1}\) 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\textsuperscript{19,55} thus masking potential differences in T-cell expression levels between the two. Moreover, a down-regulation of \(K_{\text{Ca}3.1}\) functions and unchanged mRNA expression has been reported to occur in crypts of UC patients.\textsuperscript{30} Together, these circumstances may explain the lack of correlation of \(K_{\text{Ca}3.1}\) 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.\textsuperscript{56, 57}
Although KCa3.1 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 KCa3.1 expression and “days to relapse”, which could indicate that high expression of KCa3.1 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_{v}1.3 in T-cell infiltrates in active UC as a novel molecular marker of disease activity and more interestingly, showed that K_{v}1.3 and to some extent also KCa3.1 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.

Conflict of interest

None.

Acknowledgment

We would like to thank the clinical staff of Odense University Hospital, Hospital of South-West Jutland Esbjerg, and Institute of Molecular Medicine at the University of Southern Denmark, Odense. The study received funding from the Regional Research Foundation of Southern Denmark (to JK, TK, and RK), the National Institute of Health (RO1 GM076063 to HW), the Fondo de Investigación Sanitaria (Red HERACLES RD12/0042/0014 to RK), the Danish Colitis–Crohn Society, Institute of Regional Health Science, the Karola Jørgensen’s Research Foundation, the Edith and Vagn Hedegaard Jensen’s Foundation, the Jens Lysholts Efff. Ltd, the Johannes M. Klein and Spouse’s Foundation, the A.P. Møller Foundation for the Advancements in Medical Science, the A.J. Andersen and Spouse’s Foundation, and the Foundation of CEO Jacob Madsen and his spouse Olga Madsen (to LKH). DL received a studentship from the Vibeke Binder and Povl Riis’s Foundation.

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.

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.

References


Figure 6 Effect of K_{v}1.3 and KCa3.1 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_{v}1.3 blockers; Senicapoc is a KCa3.1 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.


