Histol Histopathol (2011) 26: 735-745
DOI: 10.14670/HH-26.735

http://www.hh.um.es

Histology and Histopathology
Cellular and Molecular Biology

Role of cannabinoid receptors and RAGE in inflammatory bowel disease

Sebastian Stintzing[1]*, Till Th. Wissniowski[2]*, Christina Lohwasser[2], Beate Alinger[3], Daniel Neureiter[3] and Matthias Ocker[2,4]

[1]Department of Medicine III, University Hospital Grosshadern, Munich, Germany, [2]Department of Medicine 1, University Hospital Erlangen, Germany, [3]Institute of Pathology, Landeskliniken Salzburg, Paracelsus Medical University, Austria and [4]Institute for Surgical Research, Philipps University Marburg, Germany

*Both authors contributed equally to the work, M.O. and D.N. are considered equal senior authors

Summary. Background: The endocanabinoid system is involved in many inflammatory diseases, such as Crohn's disease (CD) and ulcerative colitis (UC). The distribution and expression of cannabinoid receptors 1 (CNR1) and 2 (CNR2) in combination with inflammatory cytokines and RAGE (receptor of advanced glycation end products), which is also overactive in these diseases, in dependency of the extent of inflammation and alteration of the colon barrier is still unclear and needs to be elucidated. Material and Methods: 10 specimens of CD patients who underwent colectomy and 14 colectomy specimens of patients suffering from UC were investigated histologically for inflammatory infiltrate, extent of fibrosis and for disturbance of the intestinal barrier. Immunohistochemistry was carried out to examine the distribution and localization of CNR1, CNR2 and RAGE. Additionally, qRT-PCR was performed to study the expression of CNR1, CNR2, RAGE and inflammatory cytokines (TNFα, TGFß, CTGF, IL12, IFNγ). 35 morphological and histological normal specimens of colectomy cases served as controls. Results: The expression level of CNR2 did not differ between the control group and the group of patients with IBD, while CNR1 displayed a significant up regulation, especially in cases of CD. A differential association between the expression of CNR1/CNR2 and RAGE with morphological changes and expression of molecular markers of inflammation could be established. Conclusion: We showed that cannabinoid receptors are expressed differentially in inflammatory bowel disease and that the expression seems to be influenced by the underlying disease and by localized inflammation.

Key words: CNR1, CNR2, Inflammatory bowel disease, Crohn's disease, Ulcerative colitis

Offprint requests to: Sebastian Stintzing, MD, Department of Medicine III, University Hospital Grosshadern, University of Munich, Marchinoninistrasse 15, 81377 Munich, Germany. e-mail: sebastian.stintzing@med.uni-muenchen.de

Introduction

The endocannabinoid system, with its possible immunomodulatory capability, has recently been shown to be altered in many chronic inflammatory diseases (Lu et al., 2006). Among them are atherosclerosis (Rajesh et al., 2007; Steffens and Mach, 2006), NASH (Mendez-Sanchez et al., 2007) and inflammatory bowel diseases (IBD), such as ulcerative colitis (UC) and Crohn's disease (CD) (Massa et al., 2004). These findings could explain former observations, where Cannabis preparations were used for many bowel disorders (Miligi et al., 2006) and anecdotic reports of patients experiencing relief after smoking marihuana. Two main receptors for cannabinoids (CNR1 and CNR2) have been characterized during the last years. Both are G-protein coupled receptors which are physiologically activated by lipid ligands like anandamide (AEA) and 2-arachidonoyl glycerol (2-AG). Noladin-ether was identified as a CNR1 specific ligand (Hanus et al., 2001). The highest expression of CNR1 receptors is found in the central and peripheral nervous system and is responsible for the psychotropic effects of tetrahydrocannabinol (THC). Further nonneural expression sites are the adipose tissue and endothelial cells (Cota et al., 2003; Samson et al.,

2003). The analysis of the physiological localisation inside the colonic tissue showed that CNR1 is expressed by colonic epithelium, smooth muscle and the submucosal myenteric plexus (Wright et al., 2005). CNR2 is mostly expressed on macrophages and plasma cells of the lamina propria and therefore seems to have a immunomodulatory function (Di Marzo and Izzo, 2006). Although many effects of cannabinoids in the gastrointestinal tract are not fully understood, the endocannabinoid system is involved in wound healing (Wright et al., 2005) and seems to have anti-inflammatory effects (Darmani and Johnson, 2004; Massa et al., 2004; Izzo, 2007). The use of anandamide (a CNR1 agonist) or the inhibition of the anandamide degrading enzyme fatty-acid-amidohydrolase (FAAH) to treat chemically induced colitis in mice showed reduced inflammation in comparison to the controls (Massa et al., 2004). Additionally, RAGE (receptor for advanced glycation end products) interactions were identified to be overactive in chronic intestinal inflammation (Foell et al., 2003). Foell et al. were able to demonstrate an up-regulation of the RAGE ligand S100A12 in biopsy specimens of patients suffering from CD or UC. Furthermore, in vitro experiments showed that S100A12 is secreted by activated neutrophils (Boussac and Garin, 2000) in a dose and time dependent manner in the presence of TNFα (Foell et al., 2003). TNFα is found in higher levels in all chronic inflammatory disorders, suggesting a possible way of mediating chronic inflammation in IBD through RAGE interaction. RAGE itself has proinflammatory features (Basta, 2008), whereas its soluble extracellular binding domain (s)RAGE has been shown to be able to suppress inflammation in mouse models of chronic colitis (Schmidt et al., 2001). Newer studies indicate a pivotal role of RAGE mediated activation of NF-κB signaling in the development of colitis associated cancer (Turovskaya et al., 2008).

Sharing the same inflammatory cytokines, both systems, the cannabinoid and RAGE system, mediate the immune response and therefore the severity of IBE.

To assess the severity of inflammation the expression of inflammatory cytokines can be investigated. Typical inflammatory cytokines playing a pivotal role in CD and UC are interferon γ, (IFNγ) (Hofmann et al., 1999; Strober et al., 2007), tumor necrosis factor α (TNFα) (Strober et al., 2007, Bouma and Strober, 2003) and interleukin 12 (IL12) (Mannon et al., 2006). Growth factors involved in the dysregulation of the cellular immunity seen in IBE are transforming growth factor beta (TGF-ß) (Becker et al., 2006) and connective tissue growth factor (cTGF) (Dammeier et al., 1998).

We here describe the expression levels of CNR1, CNR2 and RAGE in dependency of the inflammatory infiltrate and proinflammatory cytokines in patients suffering from IBD (CD or UC), and healthy controls to explore an association between two systems of chronic inflammation, the endocannabinoid and the RAGE system.

Materials and methods

All samples were obtained from the archives of the Department of Pathology of the University of Erlangen-Nuremberg or of the Institute of Pathology, Salzburger Landeskliniken, Paracelsus Private Medical University. All tissues were studied in an anonymous fashion, in accordance with the recommendations of the local ethics research committees. The study was performed according to the Austrian Gene Technology Act. Experiments were performed in accordance with the Helsinki declaration of 1975 (revised 1983) and the guidelines of the Salzburg State Ethics Research Committee (ethical agreement: AZ 209-11-E1/823-2006) since this was not a clinical drug trial or epidemiological investigation.

Patient characteristics

The control specimens derived from histologically normal mucosa samples from small and large intestines of 35 patients (median age of 63 years (range 17-86), 20 male and 15 female patients) with non-neoplastic (n=12, diagnosis: diverticulosis or intestinal incarceration) or neoplastic (n=23, colorectal cancer: TNM stage I (n=5), II, (n=9), III (n=8) and IV (n=1)) diseases. Samples from patients with colorectal cancer were taken at least 10 cm from the neoplastic lesions.

In our patient group suffering from Crohn's Disease (n=10) the median age was 48 years (range 39-63) with 2 female and 8 male participants. 14 patients suffered from ulcerative colitis (4 female and 10 male) with a median age of 61 years (range 45-63). CD patients underwent surgery because of persistent intestinal inflammation accompanied by symptomatic fibrostenosis. The UC patients were operated on because of treatment failure or long term intestinal inflammation accompanied by pseudopolyps. The diagnosis and extent of CD or UC were established by defined clinical, laboratory, endoscopic/radiological and histo-pathological criteria.

Tissue preparation

All specimens were reduced to small pieces immediately after arrival from the operating room and frozen (-20°C) until further processing. Some parts were routinely fixed with buffered formalin (5% (v/v)) instantly after removal, and embedded in paraffin. Five-μm-thick paraffin sections were cut and stored at room temperature until use. Parts of the frozen tissue were homogenized in RNAlater® using a freezer mill and stored at -80°C before the mRNA extraction was carried out.

RNA isolation and reverse transcription

For RNA analysis 700μl of the grounded material stored in RNAlater® was mixed with 400μl of RNAPure™ (Peg Lab Biotechnology Erlangen,

Germany). Further RNA isolation was done by peqGOLD RNAPureTM according to the manufacturer's recommendations. Total RNA concentration was evaluated photometrically and RNA integrity was confirmed on an agarose gel. RNA was frozen instantly and stored at -80°C until further processing. For reverse transcription we used 1 μg of the isolated RNA, 100 pmol oligo-dT, 50 pmol random primer and 100 U Superscript II (Invitrogen) following the recommendations of the supplier. The cDNA was stored at -20°C until use.

Quantitative real-time PCR

Real time RT-PCR was performed, as recently described (Neureiter et al., 2007). In short, the LightCycler FastStart DNA Master SYBR Green I kit (Roche) was used according to the manufacturer's instructions. The 20 μl probe contained 5 mM $MgCl_2$, 0.4 μM of each primer, 2 μl of the DNA template and 2 μl of the Master-Mix (including polymerase, dNTP). The PCR was performed as follows: (i) initial denaturation at 95°C for 10 min; (ii) 40 cycles of denaturation at 95°C for 10 s; annealing at 60°C for 5 s; and elongation at 72°C for 10s. (iii) A melting curve was arranged at 95, 60 and 95°C following the manufacturer's recommendations. Measurements were done 3-fold and standardized to GAPDH. Reactions without cDNA served as a negative control. The quantitative real-time PCR analyses were performed on a LightCycler real time PCR machine (Roche, Mannheim, Germany).

The sequences of primers are listed in table 1 and were synthesized at MWG Biotech AG (Ebersberg, Germany) or purchased from Qiagen (Hilden, Germany). Each PCR assay was repeated three times for each cDNA sample. To monitor amplification of possible contaminated DNA, distilled water served as a negative control.

Histochemical methods

Routine haematoxylin-eosin (H&E) staining was used to classify the extent of acute and chronic inflammation (including fibrosis), as well as the structural disturbance of the mucosa according to Shepherd et al. (1987) using a scoring system between 0 (no inflammation, no fibrosis, no structural changes) to 3 (severe inflammation, fibrosis, or substantial structural changes of the colon wall).

Immunohistochemical analysis

Immunohistochemical staining procedures were applied as described before (Quint et al., 2009) to analyse the tissue distribution of CNR1 and CNR2 in relation to RAGE, TNFα, CD45 and CD68 on consecutive slides. In short, 5 μm sections were deparaffinized using graded alcohols and antigen retrieval was carried out according to the primary antibody used (see table 2). Endogenous peroxidase blocking was carried out for 10 min with Peroxidase blocking reagent (Dako). Subsequently, primary

Table 1. Sequences and Primers used for real-time PCR experiments.

mRNA	Oligonucleotide	Sequence (5'-3')
GAPDH	GAPDH sense	CCA CAT CGC TCA GAC ACC AT
	GAPDH antisense	CCA GGC CAA TAC G
RAGE	RAGE sense	ACC AGG GAA CCT ACA GCT GTG T
	RAGE antisense	TAG AGT TCC CAG CCC TGA TCC T
CTGF	CTGF sense	AAC CGC AAG ATC GGC GT
	CTGF antisense	CCG TAC CAC CGA AGA TGC A
TGFß	TGFß sense	TGC GTC TGC TGA GGC TCA A
	TGFß antisense	TTG CTG AGG TAT CGC CAG GA
TNFα	TNFα sense	TCT CGA ACC CCG AGT GAC AA
	TNFα antisense	CGA CTG GAC CTG CCC CTC
IL12	IL12 sense	CTT GGA GCG AAT GGG CAT
	IL12 antisense	TCC ACT TTT CCT CCA AAT TTT CA
IFNγ	IFNγ sense	CTG TTA CTG CCA GGA CCC ATA TGT
	IFNγ antisense	CTC TGC ATT TTT CTG TCA CTC T
Target	Vendor	
CNR1	QuantiTect® Primer Assay (NM_001841) QT00012376, Qiagen Hilden Germany	
CNR2	QuantiTect® Primer Assay (NM_001841) QT00012376, Qiagen Hilden Germany	

Table 2. Antibodies used for immunohistochemistry and Western Blot analyses (pH 9: heat-induced epitope retrieval in pH=9 antigen retrieval buffer (Dako, Glostrup, Denmark), wb: waterbath).

Antibody	Species	Pretreatment	Dilution	Vendor
ß-actin	mouse monoclonal	None	1:5000	Sigma-Aldrich, USA
CNR1	rabbit polyclonal	wb/pH9	1:500	Abcam, Cambridge; UK
CNR2	rabbit polyclonal	wb/pH9	1:25	Abcam, Cambridge; UK
TNFα	rabbit polyclonal	wb/pH9	1:100	Abcam, Cambridge; UK
RAGE	mouse monoclonal	wb/pH6	1:50	Kindly provided by Dr. B Weigle (Dresden,Germany)
CD45	mouse monoclonal	wb/pH9	1:200	DAKO
CD68	mouse monoclonal	wb/pH9	1:200	DAKO

antibodies were applied for 30 min at RT. Primary rabbit and mouse antibodies were detected using the EnVision Detection System (Dako). Visualization was performed using Fast Red (Sigma, Germany or diaminobenzidine (Roche Molecular Biochemicals, Mannheim, Germany)) as the chromogen substrate according to the manufacturer's instructions. Nuclei were counterstained with hematoxylin. The stained slides were digitalized using the ImageAccess 9 Enterprise software (Imagic Bildverarbeitung, Glattbrugg, Switzerland). Images were evaluated using the particle analysis module with optimized binarisation method.

Western blot analysis

Western blot analysis was done as described recently (Gahr et al., 2008). In short, proteins were isolated from fresh frozen tissue by adding 200μl 2x sample buffer (2 mM NEM, 2 mM PMSF, 4% SDS, 4% DTT, 20% glycerol, 0.01% bromophenol blue, 2 M urea, 0.01 M Na- EDTA and 0.15 M Tris-HCl) to a small tissue sample that was homogenized in a freezer mill. After centrifugation at 13,000 rpm for 10 min the supernatant was collected, placed in aliquots and stored at -20°C until use. After boiling at 95°C for 15 min, samples were centrifuged at 13,000 rpm for 10 min and then applied to 14% SDS-PAGE (precast gels; Invitrogen, Karlsruhe, Germany). After blocking in a buffer containing PBS, 0.1% Tween-20 and 4% low-fat milk powder overnight at 4°C, nitrocellulose membranes were incubated for 90 min with monoclonal mouse anti-human CNR1 (1:500, Abcam, Cambridge UK), monoclonal mouse anti-human CNR2 (1:500, Abcam, Cambridge UK), or ß-actin (1:5000, Sigma) antibodies. Membranes were washed twice for 10 min in a buffer containing PBS, 0.1% Tween-20 and 4% low-fat milk powder and incubated with anti-mouse IgG combined with peroxidase (1:1000, Sigma) for 1 h at room temperature. Reactive bands were detected with the ECL chemiluminescence reagent (Amersham Pharmacia-Biotech, Freiburg, Germany) using a Fluor-Chem 8900 digital image analyser (AlphaInnotech, San Leandro, CA). Human cerebral cortex normal tissue slides (ab4296) from Abcam (Cambridge, UK) were used as a positive control for the CNR1 and CNR2 antibodies. Quantification of Western blot bands was established by densitometry using the AIDA software program (German Resource Center for Genomics, Berlin, Germany).

Statistics

Statistical analysis was done using PASW Statistics 17.0 (SPSS GmbH Software, Munich, Germany). All data represent mean values ± SEM. Correlation coefficients were calculated according to Spearman's rank-order or Kendall's rank correlation analysis. Comparisons between different disease entities were assessed by univariate ANOVA using Bonferroni's post-hoc test to adjust for multiple comparisons. Statistical significance was determined at an alpha level of 0.05.

Results

Basic histological characterizations of the specimen

Basic histological characterizations of specimens: No fibrosis or structural disturbance of the colonic wall or any inflammation was observed in our morphological normal control group, whereas different grades of acute inflammation, as well as fibrotic remodeling, were detected in cases of CD and UC (ANOVA, $p<0.01$, see table 3). Additionally, the acute inflammation was significantly higher in CD than UC (ANOVA, $p=0.013$) and more structural changes were seen in UC than in CD (ANOVA, $p=0.008$).

mRNA and protein-expression of CNR1, CNR2 and RAGE

CNR1 mRNA was significantly elevated in cases of CD compared to controls and UC (descending order, ANOVA, $p<0.05$, Fig. 1a), which was confirmed by Western blot analysis of extracted proteins of controls and cases (see Fig. 1b). In contrast, CNR2 mRNA and protein levels were not differentially expressed between controls and cases as shown by qRT-PCR or Western blot (Fig. 1a,b). The RAGE mRNA level of control samples was intermediate between CNR1 and CNR2. Here, an increase in CD patients was also observed,

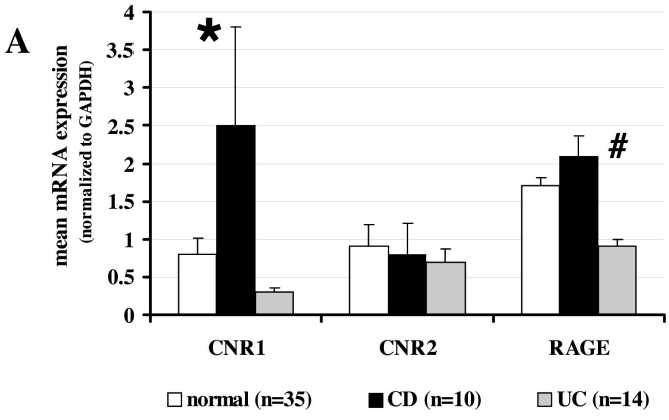
Table 3. Histological findings describing the grade of inflammation, structural changes of mucosal architecture and fibrosis of controls and cases of CD and UC.

	Controls (n=35)	Crohn's Disease (n=10)	Ulcerative Colitis (n=14)
Grade of acute inflammation[1,2]			
0	100	50	71
+	0	10	22
++	0	20	7
+++	0	20	0
Grade of chronic inflammation[1]			
0	100	10	7
+	0	40	63
++	0	50	33
+++	0	0	7
Structural changes[1,2]			
0	100	70	0
+	0	10	67
++	0	10	33
+++	0	10	0
Fibrosis[1]			
0	100	40	54
+	0	60	46

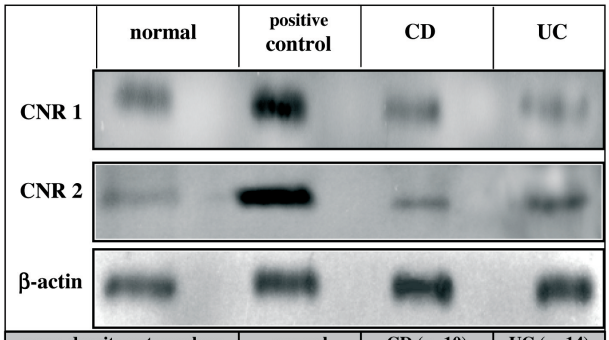
Values are given as percentage of whole groups. [1]: Significant difference between cases (CD/UC) and controls (ANOVA, $p<0.01$); [2]: Significant difference between CD and UC (ANOVA, $p<0.01$).

while a statistically significant suppression was found in UC samples (ANOVA, p=0.001).

The detailed immunohistochemical analysis of CNR1 distribution revealed descending expression levels in the submucosal myenteric plexus, smooth muscle cells and the mucosal epithelium, whereas immunohistochemical staining for CNR2 showed a less distinct pattern, as only the lamina propria in normal colon tissue and some cells of the inflammatory cell infiltrate in CD and UC displayed positivity (Fig. 2). Additionally, RAGE protein expression was mainly linked to inflammatory cells in CD and UC. This qualitative observation could be validated and characterized by quantitative image analysis (see Fig. 3a): The highest and most significant protein expression levels were found for CNR1 in CD, followed by UC and normal controls, and thus confirmed the mRNA expression data described above. Surprisingly, immunohistochemistry also revealed a significant increase of CNR2 expression in CD patients, which



B.



mean densitometry values		normal	CD (n=10)	UC (n=14)
CNR 1	mean/ß-actin	0.62	0.41	0.24
	normalized to normal	1	0.66	0.40
CNR 2	mean/ß-actin	0.05	0.04	0.04
	normalized to normal	1	0.85	0.84

Fig. 1. Expression of CNR1, CNR2 and RAGE in normal controls and cases of CD and UC. **A.** Mean mRNA expression, normalized to GAPDH values of each patient ± SEM for 3 independent experiments in triplicate. * p<0.05 vs. normal and DC cases. # p=0.001 vs. normal and UC cases. **B.** Representative Western blot analysis.

Table 4. Overall correlation analysis of CNR1, CNR2 and RAGE mRNA and protein expression levels with morphological changes and different inflammatory markers.

	CNR1		CNR2		RAGE	
	mRNA	Protein	mRNA	Protein	mRNA	Protein
Inter-correlations						
CNR1 mRNA/Protein				0.702**		
CNR2 mRNA/Protein	0.743**	0.702**				
RAGE mRNA/Protein	0.395*	0.815**				0.815**
Morphology						
Grade of acute inflammation	0.441**	0.490*				0.353*
Grade of chronic inflammation		0.609**		0.392*		0.535**
Fibrosis		0.485*		0.386*		0.334*
Structural changes						
Markers of inflammation						
CTGF[a]	0.338*	0.606**		0.612**		0.341*
TGFß[a]	0.662**	0.528*		0.716**	0.567**	
TNFα[a/b]	0.602**	0.667**		0.693**/0.701**	0.411**	
IL12[a]	0.766**	0.476*		0.676**	0.285*	
IFNγ[a]	0.401*	0.583**		0.450*		0.352*
CD68[b]		0.765**		0.696**		0.651**
CD45[b]		0.816**		0.637**		0.605**

[a]: mRNA analysis, [b]: protein analysis. *: p<0.05, **: p<0.01 (using Spearman's rank-order or Kendall's rank correlation analysis).

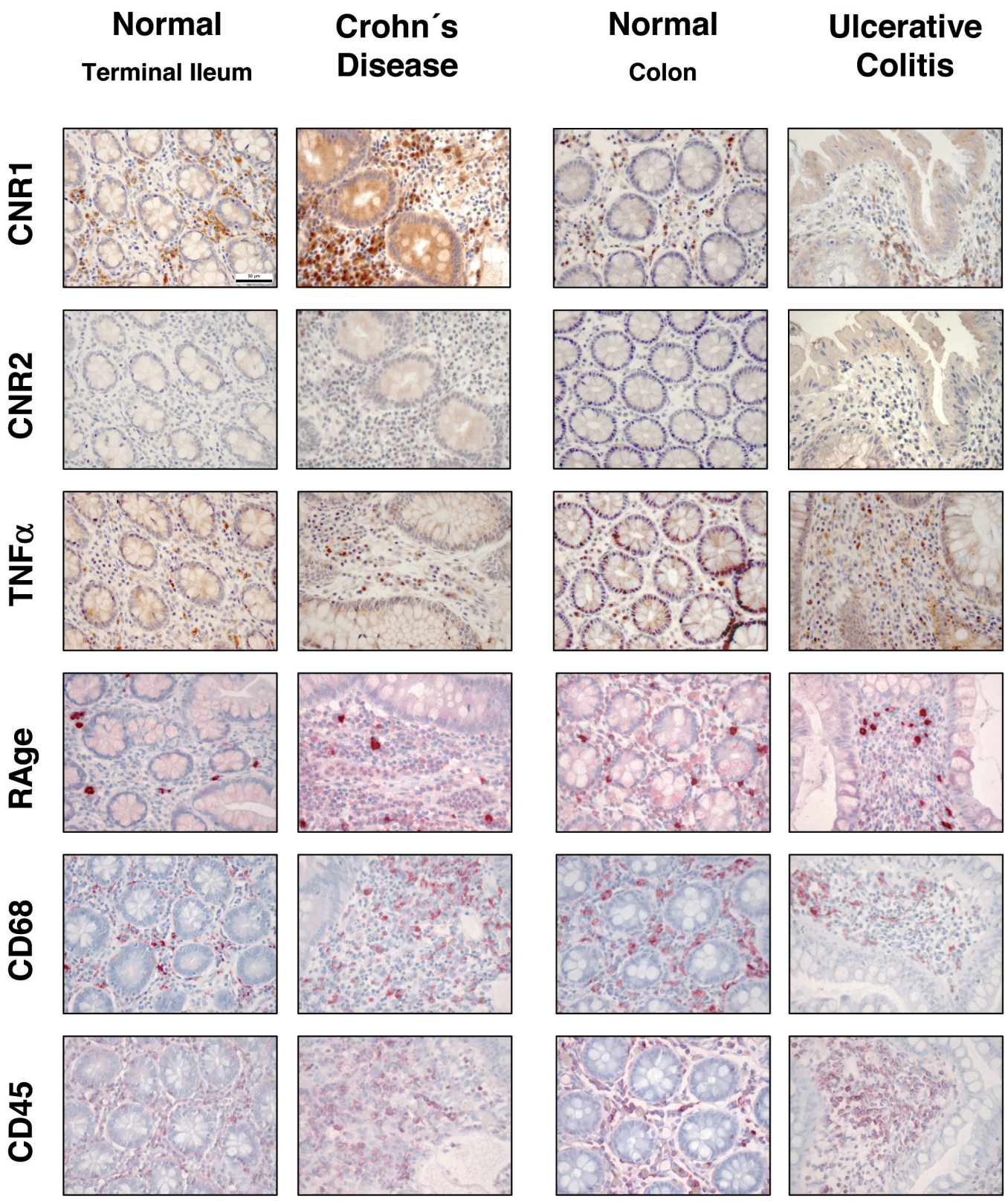


Fig. 2. Immunohistochemical analysis of CNR1, CNR2, TNFα, RAGE, CD68 and CD45. Representative cases show an upregulation of the investigated markers compared to the respective control tissues from the same patient. CNR1 is more expressed in epithelial cells in CD than in UC, while CNR2 shows a stronger expression in UC than CD. Bar: 50 μm.

contrasts with the mRNA and protein levels in these patients (Fig. 1a,b). The lowest protein expression levels were observed for RAGE, again with higher levels in cases than controls. When specifying the protein expression for epithelial and non-epithelial cells, CNR2 was significantly more detected in epithelial than non-epithelial cells, in contrast to CNR1 and RAGE (Fig. 3b, ANOVA, $p<0.01$).

Expression of inflammatory cytokines

In CD patients, mRNA expression of the pro-inflammatory cytokines TNFα and IL12 was strongly upregulated compared to normal controls, while these parameters only showed a weak increase in UC cases (Fig. 4a). The matrix modulating factor CTGF and the pro-inflammatory IFNγ were increased in both patient groups, while the strong pro-fibrogenic TGFß was increased in CD but decreased in UC cases.

These findings are paralleled by protein analysis via immunohistochemistry (Figs. 2, 4b). The amount of CD68 and CD45 expressing inflammatory cells was significantly higher in CD and UC than in controls. Surprisingly, the TNFα protein was detected in large amounts in both CD and UC samples, in contrast to mRNA findings.

Correlation analysis between CNR1-2, Rage and morphology, as well as markers of inflammation

The expression of mRNA and protein of CNR1, CNR2 and RAGE were correlated to morphological and molecular findings (Table 4). The expression of CNR1 showed a significantly positive linkage to expression of CNR2 and RAGE on mRNA and protein levels. The morphological characterization of the specimen showed a correlation of CNR1/2 and RAGE protein, but not mRNA expression, with inflammation (especially chronic inflammation and fibrosis) but not with structural changes. Strong correlations could be established between CNR1 and RAGE with markers of inflammation on the protein and mRNA level. The inflammatory markers only correlated with CNR2 protein, but not mRNA levels.

Discussion

The functions of the endocannabinoid (receptor)

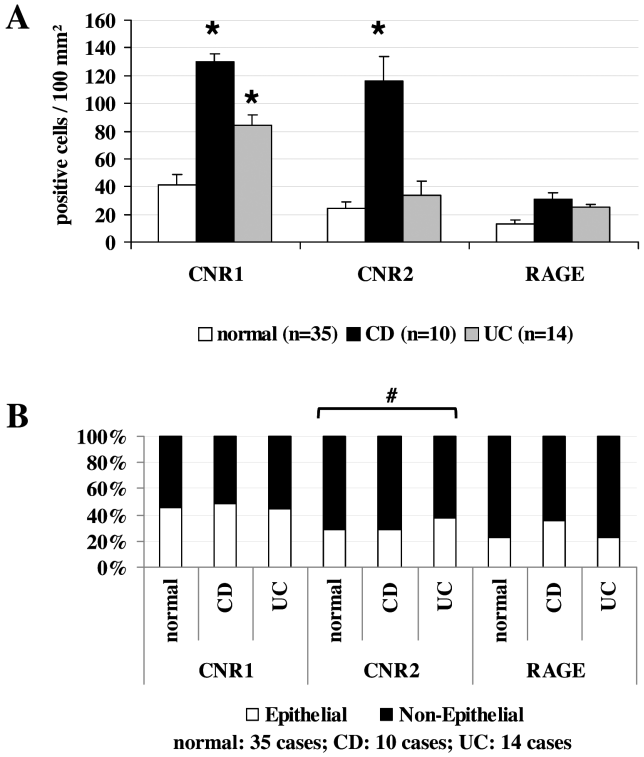


Fig. 3. Quantification of protein expression levels of CNR1, CNR2 and RAGE. Values were determined by immunohistochemistry in normal controls and cases of CD and UC. **A.** Mean ± SEM of overall expression. *: $p<0.05$ vs. normal controls. **B.** Detailed expression analysis for epithelial and non-epithelial cells. #: $p<0.01$ vs. CNR1 and RAGE expression pattern.

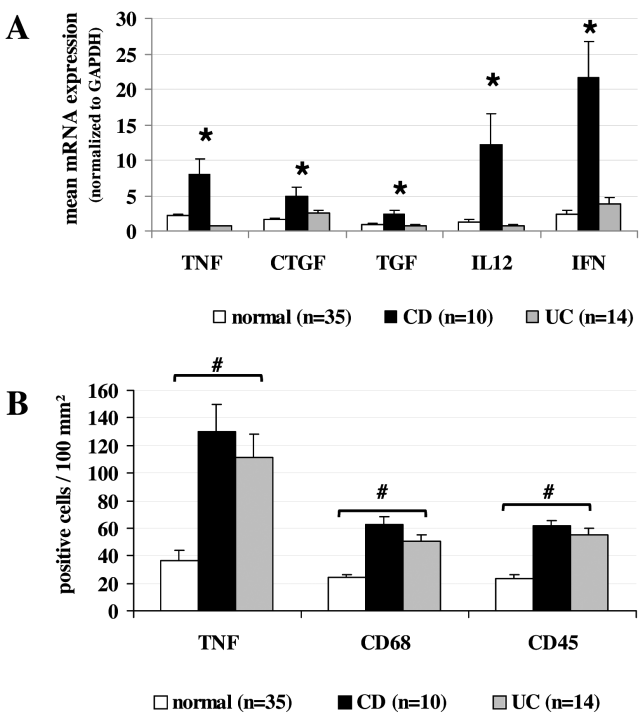


Fig. 4. Expression of inflammatory cytokines and markers. **A.** Mean mRNA expression of TNFα, CTGF, TGFß, IL12 and IFNγ ± SEM for 3 independent experiments in triplicate. Expression was normalized to GAPDH values of individual samples. **B.** Mean number of immunohistochemically positive cells/100 mm² for protein expression of TNF· and the macrophage markers CD68 and CD45. Values are mean ± SEM, * $p<0.05$ vs. normal/CU and CD, # normal vs. CD/CU.

system in intestinal homeostasis and inflammation still remain unclear to a large extent. The receptors for endogenous and exogenous cannabinoids are CNR1 and CNR2, and show a distinct distribution pattern in the gut under normal physiologic and under pathophysiologic conditions. We here found expression of CNR1 in the submucosal myenteric plexus and also to a lesser extent in smooth muscle and mucosal epithelial cells. Our results thus confirm previous reports which state a similar distribution pattern for CNR1 in the gut (Kulkarni-Narla and Brown, 2000; Casu et al., 2003; Izzo and Camilleri, 2008). We also confirmed a low expression of CNR2 in normal colonic epithelial, with signs of increased expression under inflammatory conditions and in the inflammatory cell infiltrate, especially in CD as demonstrated by immunohistochemistry in our patient cohort (Sanger, 2007; Duncan et al., 2008). Comparing in detail the expression levels of CNR1 and 2 in epithelial and non-epithelial cells, a shift towards an upregulation especially of CNR2 in epithelial cells inside CD and CU could be observed, in line with published data and reflecting the association between epithelial and mucosal immune cells, as well as disease severity (Wright et al., 2005; Marquez et al., 2009). Although the functional relevance of this observation is not known, the overexpression of CNR2 in epithelial cells of human adenomatous polyps and carcinomas is a promising target for therapeutical interventions (Izzo and Camilleri, 2009; Patel et al. 2010).

There is growing evidence suggesting that endocannabinoids and CNR receptors, especially CNR1, are up-regulated during colon inflammation, although most of the data arise from animal models of colitis, such as the dinitrobenzene sulphonic acid (DNBS) or croton oil mouse models (Di Marzo and Izzo, 2006; Massa and Monory, 2006; Storr et al., 2008). In these models it was shown that higher levels of the endocannabinoid anandamide lead to minor changes of experimentally induced colitis (Izzo and Camilleri, 2008). In humans, higher levels of anandamide and 2-arachydonoylglycerol (2-AG) were detected in celiac disease, colorectal cancer or adenomatous polyps, while 2-AG was decreased in patients with diverticulitis (Ligresti et al., 2003; D'Argenio et al., 2006; Guagnini et al., 2006). Interestingly, the expression of CNR1 and CNR2 was not changed under these conditions, while levels of anandamide, but not 2-AG, increased. CNR1 and CNR2 were observed in inflammatory bowel diseases, especially in the acute phase (Wright et al., 2005; D'Argenio et al., 2006; Marquez et al., 2009). Here we show in a small patient group that the amount of CNR1, as measured by immunohistochemistry, is significantly increased in colitis samples, irrespective of the underlying aetiology. This is, in part, in contrast to the work of Marquez et al. (2009) which was able to show the same pattern in CNR2 immunoreactivity but was also able to show a decrease in CNR1 immunoreactivity in UC cases in the quiescent status in the epithelium. Looking closer to the distribution of CNR1 immunoreactivity (Fig. 3b), the epithelial proportion did not change at all, but the overall immunoreactivity did (see Fig. 3a). This may be due to the fact that Marquez et al. (2009) investigated samples from newly diagnosed patients, whereas our UC group consisted of patients with a longer medical history of the disease. The accumulation of CNR1 positive cells might be a sign for chronic changes inside the colonic epithelium and submucosal space, and not so much associated with changes that are due to acute inflammation which may drive the CNR2 expression. The upregulation of CNR1 has been proposed to exert a protective effect by limiting intestinal inflammation, promoting epithelial wound healing and regulating intestinal motility (Izzo et al., 2001; Massa et al., 2004; Wright et al., 2005). In our specimens, we detected a difference between CNR mRNA expression and the immunoreactivity detected by immunohistochemistry. This could be attributed to posttranscriptional or posttranslational modifications of mRNA and protein precursors, e.g. by miRNA-dependent RNA interference pathways or by differential regulation of receptor stabilization or internalization and trafficking. Recently, a regulatory role for various miRNAs has been described in IBD (Iborra et al.) and the regulation of inflammatory processes in general (Sonkoly and Pivarcsi, 2009), although it is so far unclear if components of the endocannabinoid system are also under the control of this pathway. In addition, it has been described that the proteasomal degradation pathway is deregulated in patients with IBD, which can lead to enhanced stability of pro-inflammatory molecules and mediators, e.g. Smad7 (Monteleone et al., 2005). Recently, the pharmacologic inhibition of proteasomal activities has been shown to ameliorate experimental IBD in mice by interfering with NFκB signaling (Schmidt et al., 2001), which is also involved in the here described pathways of CNR and RAGE. Interestingly, CNR2 protein expression was significantly increased only in CD patients when compared to our normal group, which is in line with the observed increased severity of acute and chronic inflammation in these patients compared to UC and controls. These findings support previous results of CNR2 expression on lymphocytes and the involvement in dendritic cell maturation and other inflammatory processes (Wacnik et al., 2008; Wright et al., 2008).

The patients were randomly and prospectively assigned to our study and were planned for elective surgical procedures. However, immunohistochemistry and other experiments revealed a considerable degree of acute and chronic inflammation in these patients which was not reflected by the disease activity indices. This could be attributed to a possible enhanced activity of CNR2 (as observed by immunohistochemistry in CD patients) or higher levels of its ligands, which were not analysed here, as CNR2 activation leads to reduced secretion of proinflammatory cytokines (Klein, 2005; Lunn et al., 2006).

RAGE, a member of the immunoglobuline superfamily of cell surface receptors, has recently been linked to several inflammatory diseases (e.g. diabetes, liver fibrosis or inflammatory bowel disease) by mediators of the S100/calgranulin family (Foell et al., 2003; Zen et al., 2007; Lohwasser et al., 2009). Activation of RAGE and its ligands has been linked to TNFα and NFκB signalling, which are both key mediators of inflammation in inflammatory bowel diseases (Reinecker et al., 1993; Breese et al., 1994; Rogler et al., 1998). While other reports showed that the expression of the RAGE ligand S100A12 is especially involved in IBD pathogenesis and maintenance, only limited data is available on RAGE expression under these conditions (Foell et al., 2003; Zen et al., 2007). Interestingly, the expression of RAGE has not been investigated in a larger patient cohort so far and we show here that expression of RAGE is not affected by IBD in comparison to control patients. We therefore speculate that RAGE ligands, but not RAGE itself, are crucial for IBD pathogenesis. Although both pathways, cannabinoid and RAGE signalling, are involved in chronic inflammatory processes in the GI tract, we could only establish a role for CNR1 and CNR2 in IBD.

The statistical analysis of mRNA and/or protein levels of CNR1, CNR2 and RAGE with markers of inflammation revealed medium to strong positive correlations at statistically significant levels. Interestingly, the correlation between the cannabinoid receptors and inflammatory markers is stronger than for RAGE, which confirms the observations from histology specimens. It was previously shown in other fibrotic or autoimmune diseases that the endocannabinoid receptor system is involved in the pathogenesis of tissue remodelling and inflammatory response (Marquart et al., Izzo and Camilleri, 2008) and our findings also show a strong correlation between CNR1 and CNR2 protein with CTGF and TGFß expression. Furthermore, we also confirmed a correlation with known markers of inflammatory disease activity in IBD, e.g. TNFα, IL12, IFNγ or expression of CD68 and CD45 proteins. These findings strongly indicate a regulatory role, especially of CNR1 and CNR2, for the investigated receptors in IBD and could present novel targets for future interventions (Storr et al., 2008, 2009; Izzo and Camilleri, 2009; Marquez et al., 2009).

In conclusion, the expression of CNR1, and to a lesser extend CNR2, is increased in inflammatory bowel diseases and correlates with the degree of inflammation, structural changes and the expression of related mediators. We also confirmed a distinct morphological expression pattern of these receptors depending of the underlying disease condition. RAGE does not seem to support the observed inflammatory processes in our patients, although further examination and randomized clinical trials with cannabinoids or inhibitors of degradation enzymes of endocannabinoids should be carried out to enlighten the clinical role of these pathways in Crohn's disease and ulcerative colitis.

Acknowledgements. The excellent technical assistance of A. Hartl, S. Leitner and A. Taut is gratefully acknowledged. The authors declare no conflict of interest.

References

Basta G. (2008). Receptor for advanced glycation endproducts and atherosclerosis: From basic mechanisms to clinical implications. Atherosclerosis 196, 9-21.

Becker C., Fantini M.C. and Neurath M.F. (2006). TGF-beta as a T cell regulator in colitis and colon cancer. Cytokine Growth Factor Rev. 17, 97-106.

Bouma G. and Strober W. (2003). The immunological and genetic basis of inflammatory bowel disease. Nat. Rev. Immunol. 3, 521-533.

Boussac M. and Garin J. (2000). Calcium-dependent secretion in human neutrophils: a proteomic approach. Electrophoresis 21, 665-672.

Breese E.J., Michie C.A., Nicholls S.W., Murch S.H., Williams C.B., Domizio P., Walker-Smith J.A. and MacDonald T.T. (1994). Tumor necrosis factor alpha-producing cells in the intestinal mucosa of children with inflammatory bowel disease. Gastroenterology 106, 1455-1466.

Casu M.A., Porcella A., Ruiu S., Saba P., Marchese G., Carai M.A., Reali R., Gessa G.L. and Pani L. (2003). Differential distribution of functional cannabinoid CB1 receptors in the mouse gastroenteric tract. Eur. J. Pharmacol 459, 97-105.

Cota D., Marsicano G., Tschop M., Grubler Y., Flachskamm C., Schubert M., Auer D., Yassouridis A., Thone-Reineke C., Ortmann S., Tomassoni F., Cervino C., Nisoli E., Linthorst A.C., Pasquali R., Lutz B., Stalla G.K. and Pagotto U. (2003). The endogenous cannabinoid system affects energy balance via central orexigenic drive and peripheral lipogenesis. J. Clin. Invest. 112, 423-431.

D'Argenio G., Valenti M., Scaglione G., Cosenza V., Sorrentini I. and Di Marzo V. (2006). Up-regulation of anandamide levels as an endogenous mechanism and a pharmacological strategy to limit colon inflammation. FASEB J. 20, 568-570.

Dammeier J., Brauchle M., Falk W., Grotendorst G.R. and Werner S. (1998). Connective tissue growth factor: a novel regulator of mucosal repair and fibrosis in inflammatory bowel disease? Int. J. Biochem. Cell Biol. 30, 909-922.

Darmani N.A. and Johnson J.C. (2004). Central and peripheral mechanisms contribute to the antiemetic actions of delta-9-tetrahydrocannabinol against 5-hydroxytryptophan-induced emesis. Eur. J. Pharmacol. 488, 201-212.

Di Marzo V. and Izzo A.A. (2006). Endocannabinoid overactivity and intestinal inflammation. Gut 55, 1373-1376.

Duncan M., Mouihate A., Mackie K., Keenan C.M., Buckley N.E., Davison J.S., Patel K.D., Pittman Q.J. and Sharkey K.A. (2008). Cannabinoid CB2 receptors in the enteric nervous system modulate gastrointestinal contractility in lipopolysaccharide-treated rats. Am. J. Physiol. Gastrointest Liver Physiol. 295, G78-G87.

Foell D., Kucharzik T., Kraft M., Vogl T., Sorg C., Domschke W. and Roth J. (2003). Neutrophil derived human S100A12 (EN-RAGE) is strongly expressed during chronic active inflammatory bowel disease. Gut 52, 847-853.

Gahr S., Peter G., Wissniowski T.T., Hahn E.G., Herold C. and Ocker M. (2008). The histone-deacetylase inhibitor MS-275 and the CDK-

inhibitor CYC-202 promote anti-tumor effects in hepatoma cell lines. Oncol. Rep. 20, 1249-1256.

Guagnini F., Valenti M., Mukenge S., Matias I., Bianchetti A., Di Palo S., Ferla G., Di Marzo V. and Croci T. (2006). Neural contractions in colonic strips from patients with diverticular disease: role of endocannabinoids and substance P. Gut 55, 946-953.

Hanus L., Abu-Lafi S., Fride E., Breuer A., Vogel Z., Shalev D.E., Kustanovich I. and Mechoulam R. (2001). 2-arachidonyl glyceryl ether, an endogenous agonist of the cannabinoid CB1 receptor. Proc. Natl. Acad. Sci. USA 98, 3662-3665.

Hofmann M.A., Drury S., Fu C., Qu W., Taguchi A., Lu Y., Avila C., Kambham N., Bierhaus A., Nawroth P., Neurath M.F., Slattery T., Beach D., McClary J., Nagashima M., Morser J., Stern D. and Schmidt A.M. (1999). RAGE mediates a novel proinflammatory axis: a central cell surface receptor for S100/calgranulin polypeptides. Cell 97, 889-901.

Iborra M., Bernuzzi F., Invernizzi P. and Danese S. (2010). MicroRNAs in autoimmunity and inflammatory bowel disease: Crucial regulators in immune response. Autoimmun Rev. (in press).

Izzo A.A. (2007). The cannabinoid CB(2) receptor: a good friend in the gut. Neurogastroenterol Motil 19, 704-708.

Izzo A.A. and Camilleri M. (2008). Emerging role of cannabinoids in gastrointestinal and liver diseases: basic and clinical aspects. Gut 57, 1140-1155.

Izzo A.A. and Camilleri M. (2009). Cannabinoids in intestinal inflammation and cancer. Pharmacol Res 60, 117-125.

Izzo A.A., Fezza F., Capasso R., Bisogno T., Pinto L., Iuvone T., Esposito G., Mascolo N., Di Marzo V. and Capasso F. (2001). Cannabinoid CB1-receptor mediated regulation of gastrointestinal motility in mice in a model of intestinal inflammation. Br. J. Pharmacol. 134, 563-570.

Klein T.W. (2005). Cannabinoid-based drugs as anti-inflammatory therapeutics. Nat. Rev. Immunol. 5, 400-411.

Kulkarni-Narla A. and Brown D.R. (2000). Localization of CB1-cannabinoid receptor immunoreactivity in the porcine enteric nervous system. Cell Tissue Res. 302, 73-80.

Ligresti A., Bisogno T., Matias I., De Petrocellis L., Cascio M.G., Cosenza V., D'Argenio G., Scaglione G., Bifulco M., Sorrentini I. and Di Marzo V. (2003). Possible endocannabinoid control of colorectal cancer growth. Gastroenterology 125, 677-687.

Lohwasser C., Neureiter D., Popov Y., Bauer M. and Schuppan D. (2009). Role of the receptor for advanced glycation end products in hepatic fibrosis. World J. Gastroenterol. 15, 5789-5798.

Lu D., Vemuri V.K., Duclos R.I. Jr and Makriyannis A. (2006). The cannabinergic system as a target for anti-inflammatory therapies. Curr. Top. Med. Chem. 6, 1401-1426.

Lunn C.A., Reich E.P. and Bober L. (2006). Targeting the CB2 receptor for immune modulation. Expert. Opin. Ther. Targets 10, 653-663.

Mannon P.J., Fuss I.J., Dill S., Friend J., Groden C., Hornung R., Yang Z., Yi C., Quezado M., Brown M. and Strober W. (2006). Excess IL-12 but not IL-23 accompanies the inflammatory bowel disease associated with common variable immunodeficiency. Gastroenterology 131, 748-756.

Marquart S., Zerr P., Akhmetshina A., Palumbo K., Reich N., Tomcik M., Horn A., Dees C., Engel M., Zwerina J., Distler O., Schett G. and Distler J.H. (2010). Inactivation of the cannabinoid receptor CB1 prevents leukocyte infiltration and experimental fibrosis. Arthritis Rheum. 62, 3467-3476.

Marquez L., Suarez J., Iglesias M., Bermudez-Silva F.J., Rodriguez de Fonseca F. and Andreu M. (2009). Ulcerative colitis induces changes on the expression of the endocannabinoid system in the human colonic tissue. PLoS One 4, e6893.

Massa F. and Monory K. (2006). Endocannabinoids and the gastrointestinal tract. J. Endocrinol. Invest. 29, 47-57.

Massa F., Marsicano G., Hermann H., Cannich A., Monory K., Cravatt B.F., Ferri G.L., Sibaev A., Storr M. and Lutz B. (2004). The endogenous cannabinoid system protects against colonic inflammation. J. Clin. Invest. 113, 1202-1209.

Mendez-Sanchez N., Zamora-Valdes D., Pichardo-Bahena R., Barredo-Prieto B., Ponciano-Rodriguez G., Bermejo-Martinez L., Chavez-Tapia N.C., Baptista-Gonzalez H.A. and Uribe M. (2007). Endocannabinoid receptor CB2 in nonalcoholic fatty liver disease. Liver Int. 27, 215-219.

Miligi L., Costantini A.S., Benvenuti A., Kriebel D., Bolejack V., Tumino R., Ramazzotti V., Rodella S., Stagnaro E., Crosignani P., Amadori D., Mirabelli D., Sommani L., Belletti I., Troschel L., Romeo L., Miceli G., Tozzi G.A., Mendico I. and Vineis P. (2006). Occupational exposure to solvents and the risk of lymphomas. Epidemiology 17, 552-561.

Monteleone G., Del Vecchio Blanco G., Monteleone I., Fina D., Caruso R., Gioia V., Ballerini S., Federici G., Bernardini S., Pallone F. and MacDonald T.T. (2005). Post-transcriptional regulation of Smad7 in the gut of patients with inflammatory bowel disease. Gastroenterology 129, 1420-1429.

Neureiter D., Zopf S., Leu T., Dietze O., Hauser-Kronberger C., Hahn E.G., Herold C. and Ocker M. (2007). Apoptosis, proliferation and differentiation patterns are influenced by Zebularine and SAHA in pancreatic cancer models. Scand. J. Gastroenterol. 42, 103-116.

Patel K.D., Davison J.S., Pittman Q.J. and Sharkey K.A. (2010) Cannabinoid CB(2) receptors in health and disease. Curr. Med. Chem. 17, 1393-1410.

Quint K., Stintzing S., Alinger B., Hauser-Kronberger C., Dietze O., Gahr S., Hahn E.G., Ocker M. and Neureiter D. (2009). The expression pattern of PDX-1, SHH, Patched and Gli-1 is associated with pathological and clinical features in human pancreatic cancer. Pancreatology 9, 116-126.

Rajesh M., Mukhopadhyay P., Batkai S., Hasko G., Liaudet L., Huffman J.W., Csiszar A., Ungvari Z., Mackie K., Chatterjee S. and Pacher P. (2007). CB2-receptor stimulation attenuates TNF-alpha-induced human endothelial cell activation, transendothelial migration of monocytes, and monocyte-endothelial adhesion. Am. J. Physiol. Heart Circ. Physiol. 293, H2210-2218.

Reinecker H.C., Steffen M., Witthoeft T., Pflueger I., Schreiber S., MacDermott R.P. and Raedler A. (1993). Enhanced secretion of tumour necrosis factor-alpha, IL-6, and IL-1 beta by isolated lamina propria mononuclear cells from patients with ulcerative colitis and Crohn's disease. Clin. Exp. Immunol. 94, 174-181.

Rogler G., Brand K., Vogl D., Page S., Hofmeister R., Andus T., Knuechel R., Baeuerle P.A., Scholmerich J. and Gross V. (1998). Nuclear factor kappaB is activated in macrophages and epithelial cells of inflamed intestinal mucosa. Gastroenterology 115, 357-369.

Samson M.T., Small-Howard A., Shimoda L.M., Koblan-Huberson M., Stokes A.J. and Turner H. (2003). Differential roles of CB1 and CB2 cannabinoid receptors in mast cells. J. Immunol. 170, 4953-4962.

Sanger G.J. (2007). Endocannabinoids and the gastrointestinal tract: what are the key questions? Br. J. Pharmacol. 152, 663-670.

Schmidt A.M., Yan S.D., Yan S.F. and Stern D.M. (2001). The multiligand receptor RAGE as a progression factor amplifying

immune and inflammatory responses. J. Clin. Invest. 108, 949-955.

Shepherd N.A., Jass J.R., Duval I., Moskowitz R.L., Nicholls R.J. and Morson B.C. (1987). Restorative proctocolectomy with ileal reservoir: pathological and histochemical study of mucosal biopsy specimens. J. Clin. Pathol. 40, 601-607.

Sonkoly E. and Pivarcsi A. (2009). Advances in microRNAs: implications for immunity and inflammatory diseases. J. Cell Mol. Med. 13, 24-38.

Steffens S. and Mach F. (2006). Cannabinoid receptors in atherosclerosis. Curr. Opin. Lipidol. 17, 519-526.

Storr M.A., Keenan C.M., Emmerdinger D., Zhang H., Yuce B., Sibaev A., Massa F., Buckley N.E., Lutz B., Goke B., Brand S., Patel K.D. and Sharkey K.A. (2008). Targeting endocannabinoid degradation protects against experimental colitis in mice: involvement of CB1 and CB2 receptors. J. Mol. Med. 86, 925-936.

Storr M.A., Keenan C.M., Zhang H., Patel K.D., Makriyannis A. and Sharkey K.A. (2009). Activation of the cannabinoid 2 receptor (CB2) protects against experimental colitis. Inflamm. Bowel Dis. 15, 1678-1685.

Strober W., Fuss I. and Mannon P. (2007). The fundamental basis of inflammatory bowel disease. J. Clin. Invest. 117, 514-521.

Turovskaya O., Foell D., Sinha P., Vogl T., Newlin R., Nayak J., Nguyen M., Olsson A., Nawroth P.P., Bierhaus A., Varki N., Kronenberg M., Freeze H.H. and Srikrishna G. (2008). RAGE, carboxylated glycans and S100A8/A9 play essential roles in colitis-associated carcinogenesis. Carcinogenesis 29, 2035-2043.

Wacnik P.W., Luhr K.M., Hill R.H., Ljunggren H.G., Kristensson K. and Svensson M. (2008). Cannabinoids affect dendritic cell (DC) potassium channel function and modulate DC T cell stimulatory capacity. J. Immunol. 181, 3057-3066.

Wright K., Rooney N., Feeney M., Tate J., Robertson D., Welham M. and Ward S. (2005). Differential expression of cannabinoid receptors in the human colon: cannabinoids promote epithelial wound healing. Gastroenterology 129, 437-453.

Wright K.L., Duncan M. and Sharkey K.A. (2008). Cannabinoid CB2 receptors in the gastrointestinal tract: a regulatory system in states of inflammation. Br. J. Pharmacol. 153, 263-270.

Zen K., Chen C.X., Chen Y.T., Wilton R. and Liu Y. (2007). Receptor for advanced glycation endproducts mediates neutrophil migration across intestinal epithelium. J. Immunol. 178, 2483-2490.

Accepeted December 29, 2010