Molecular Medicine

GPR84 and TREM-1 Signaling Contribute to the Pathogenesis of Reflux Esophagitis

Heba Abdel-Aziz,[1] Mathias Schneider,[2] Winfried Neuhuber,[3] Abdel Meguid Kassem,[4] Saleem Khailah,[4] Jürgen Müller,[5] Hadeel Gamal Eldeen,[4] Ahmed Khairy,[4] Mohamed T Khayyal,[6] Anastasiia Shcherbakova,[7] Thomas Efferth,[2] and Gudrun Ulrich-Merzenich[7]

[1]Department of Pharmacology, Institute of Pharmaceutical Chemistry, Westfalian Wilhelms University, Münster, Germany; [2]Department of Pharmaceutical Biology, Institute of Pharmacy and Biochemistry, Johannes Gutenberg University, Mainz, Germany; [3]Institute of Anatomy, University of Erlangen-Nürnberg, Erlangen, Germany; [4]Tropical Medicine Department and Gastrointestinal Endoscopy Unit, Faculty of Medicine, Cairo University, Cairo, Egypt; [5]Scientific Department, Steigerwald Arzneimittelwerk GmbH, Darmstadt, Germany; [6]Department of Pharmacology, Faculty of Pharmacy, Cairo University, Cairo, Egypt; and [7]Medical Clinic III, University Clinic Centre, Rheinische Friedrich-Wilhelms University, Bonn, Germany

Gastro-esophageal reflux disease (GERD) is one of the most common disorders in gastroenterology. Patients present with or without increased acid exposure indicating a nonuniform etiology. Thus, the common treatment with proton pump inhibitors (PPIs) fails to control symptoms in up to 40% of patients. To further elucidate the pathophysiology of the condition and explore new treatment targets, transcriptomics, proteomics and histological methods were applied to a surgically induced subchronic reflux esophagitis model in Wistar rats after treatment with either omeprazole (PPI) or STW5, a herbal preparation shown to ameliorate esophagitis without affecting refluxate pH. The normal human esophageal squamous cell line HET-1A and human endoscopic biopsies were used to confirm our findings to the G-protein–coupled receptor (GPR) 84 in human tissue. Both treatments reduced reflux-induced macroscopic and microscopic lesions of the esophagi as well as known proinflammatory cytokines. Proteomic and transcriptomic analyses identified CINC1–3, MIP-1/3α, MIG, RANTES and interleukin (IL)-1β as prominent mediators in GERD. Most regulated cyto-/chemokines are linked to the TREM-1 signaling pathway. The fatty acid receptor GPR84 was upregulated in esophagitis but significantly decreased in treated groups, a finding supported by Western blot and immunohistochemistry in both rat tissue and HET-1A cells. GPR84 was also found to be significantly upregulated in patients with grade B reflux esophagitis. The expression of GPR84 in esophageal tissue and its potential involvement in GERD are reported for the first time. IL-8 (CINC1–3) and the TREM-1 signaling pathway are proposed, besides GPR84, to play an important role in the pathogenesis of GERD.

Online address: http://www.molmed.org
doi: 10.2119/molmed.2015.00098

INTRODUCTION

Gastro-esophageal reflux disease (GERD) is one of the most common conditions confronting gastroenterologists, with an incidence exceeding 20% in the Western world (1). The disease strongly compromises the patients' quality of life and may lead to long-term implications, such as Barrett esophagus and esophageal adenocarcinoma.

Current therapy of GERD relies predominantly on the use of proton pump inhibitors (PPI). However, although PPIs are extremely effective in mucosal healing, they are not always effective in relieving heartburn. Up to 40% of GERD patients are refractory to PPI therapy and continue to experience symptoms while on PPIs (2). Accordingly, additional treatment options are needed and, for their development, a clearer understanding of the molecular mechanisms involved in GERD pathogenesis is required.

Traditionally, the caustic chemical injury inflicted by acidic gastric contents on the esophageal epithelium was seen as the primary culprit in the pathogenesis of GERD. More recent evidence suggests that immune-mediated mechanisms are involved, and, accordingly, acid suppression alone is not sufficient to treat the condition in all patients. Souza *et al.* (3) showed that reflux induces interleukin (IL)-8 secretion from the esophageal epithelium and that infiltration of inflammatory cells takes place before the appearance of any erosion on the surface of the esophageal epithelium. These and other findings (4–6) suggest that gastro-esophageal reflux may

Address correspondence to *Heba Abdel-Aziz, Scientific Department, Steigerwald Arzneimittelwerk GmbH, Havelstr. 5, D-64295 Darmstadt, Germany. Phone: +49-6151-3305-202; Fax: +49-6151-3305-471; E-mail: heba.abdelaziz@gmail.com.*
Submitted April 30, 2015; Accepted for publication November 23, 2015; Published Online (www.molmed.org) November 24, 2015.

activate inflammatory pathways first and then impair esophageal barrier function later. However, the mechanisms involved in such an inflammatory process require further elucidation.

In the present study, we established a subchronic surgical model of reflux esophagitis in rats. The effects of reflux induction were analyzed with a focus on the esophageal tissue using macroscopic and microscopic parameters, proteome- and RNA-microarray profiling, immunohistochemistry and Western blots (WBs). To further elucidate acid suppression–independent mechanisms in the treatment of GERD, we used omeprazole, a PPI used as standard treatment for GERD with additional acid-independent anti-inflammatory effects (7,8), and STW5, a herbal combination preparation with potent antiinflammatory activity, which has been shown to relieve heartburn and other concomitant reflux symptoms in patients with functional dyspepsia (9) and to be effective in a pharmacological model of acute reflux esophagitis without affecting the pH of the refluxate (10). The impact of inducing esophagitis in our rat model and the results of both treatments thereupon were assessed. Because both agents act through mechanisms independent of acid-suppressing activity, it is hoped that the study would lead to identifying novel interesting pathways that are involved in the pathogenesis of GERD. Normal human esophageal squamous cell line HET-1A and human endoscopic biopsies have also been examined in an attempt to correlate our experimental findings with the human situation.

MATERIALS AND METHODS

See the Data Supplement for detailed methodology and material sources.

Drugs

STW5 (Iberogast®; see the Data Supplement for constituents and standardization) was provided by Steigerwald Arzneimittelwerk as lyophilized powder. It was freshly reconstituted in water before use. Omeprazole was purchased from Sigma-Aldrich.

Antibodies

See the Data Supplement.

Induction of Reflux Esophagitis

Reflux esophagitis was induced according to Omura *et al.* (11) with few modifications (ligation with 2-0 silk thread; 3-mm-long piece of 18-Fr Nelaton catheter). The study was carried out according to The European Communities Council Directive of 1986 (86/609/EEC) and approved by the Ethical Committee for Animal Experimentation (NRW, Germany).

Experimental Design

Adult male Wistar rats were blindly allocated to five groups. Two groups received water, two received STW5 (0.5 or 2 mL/kg) and one received omeprazole (30 mg/kg) by oral gavage once daily starting 7 d before induction of esophagitis. One of the vehicle-treated groups was sham-operated. Esophagitis was induced in all other groups as described above. Animals were treated for a further 10 d and were then sacrificed under anesthesia. During anesthesia, blood for RNA analysis was withdrawn from the posterior vena cava. Finally, the lower 3 cm of the esophagus were excised, opened longitudinally, examined macroscopically and scored according to the severity of damage (0 = no visible damage to 5 = perforation).

Esophageal samples were weighed and cut into three parts: one fixed in 10% formalin and embedded in paraffin, one stored at –80°C in an RNAlater® tube for RNA array analysis and one used to prepare whole cell lysates and stored at –80°C.

Histopathological Examination

The 5-µm-thick paraffin sections were stained with hematoxylin-eosin and examined under a microscope. From each block, six sections were randomly chosen for examination by a histologist blinded to the treatment, and each was assigned a damage score, where 0 = no apparent damage, 1 = slight inflammation (edema, infiltration), 2 = moderate inflammation (epithelial damage) and 3 = severe inflammation (severe edema, extended ulceration, abscess).

Immunohistochemistry

Paraffin-embedded tissues were cut into 4-µm-thick sections and mounted on object slides with charged surface and stained with rabbit anti-GPR84 (G-protein–coupled receptor 84) or anti–IL-17 antibody. For determination of protein expression, the UltraVision polymer detection method was used as previously described (12).

Determination of Inflammatory Mediators

Whole cell lysates were used for the semiquantitative determination of 29 cytokines using a commercially available rat Proteome-Profiler cytokine array kits. Three membranes were used for each treatment group, with pooled samples from two to three animals per membrane. In addition, IL-1β and tumor necrosis factor (TNF)-α were quantified by rat-specific enzyme-linked immunosorbent assay (ELISA) (Quantikine, R&D Systems). Whole cell lysates were equalized to 200 µg protein and estimated according to the manufacturer's instructions.

Nuclear Factor (NF)-κB p65

NF-κB p65 DNA binding was measured in whole cell lysates by using a TransAM NF-κBp65 immunoassay-based kit.

Gene Microarrays

RNA was isolated from tissue and blood samples by a PaxGene tissue RNA kit and PaxGene blood RNA kit. The gene modulation was determined by gene microarrays in four animals per group as described previously (13). Full array data were submitted to GEO repository (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE63403).

Reverse Transcription and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

The following genes that were highly affected by the treatment and/or belonged to different biological pathways were selected for qRT-PCR: *IL-6*, *IL-17D*, *CCL4*, *CXCL1*, *GPR84* and *vascular*

adhesion molecule-1 (VCAM-1); 18sRNA served as endogenous control.

Western Blot Analysis

Western blots were performed by standard procedures as described earlier (14). A minimum of five independent samples per group were evaluated.

GPR84 Expression in HET-1A Cells

GPR84 expression was determined by WB in the capsaicin-stimulated normal human esophageal squamous cell line HET-1A with or without coincubation with STW5 (60 μL/mL) or omeprazole (30 μg/mL) for 18 h.

GPR84 Expression in Human Esophageal Samples

Tissue preparations were obtained from 34 patients who underwent routine upper gastrointestinal endoscopy and presented either with endoscopic normally appearing esophagus or reflux esophagitis. Patients with Barrett esophagus, esophageal polyps or moniliasis were not included. An informed consent was obtained from all patients, and all procedures were approved by the local ethics committee and were conducted according to the ethical guidelines of the Declaration of Helsinki (as revised in 2000).

A full medical history was taken from all patients, with special emphasis on indications for upper gastrointestinal endoscopy, symptoms and signs of upper and lower gastrointestinal tract (Table 1). For each patient, at least two endoscopic biopsies were obtained from the distal esophagus above the Z-line. On the basis of endoscopic findings, patients were graded according to the Los Angeles classification into the following: endoscopically normal esophagus, grade A esophagitis, grade B esophagitis or grade C esophagitis. Biopsies were taken by using biopsy forceps and immediately preserved in formalin (10% solution). They were embedded in paraffin within 24 h. GPR84 was assessed by immunohistochemical staining.

Samples were examined by an experienced pathologist blinded to the group assignment. GPR84 immunoreactivity was scored for each sample in six representative high-power fields. The staining intensity (SI) was semiquantitatively assessed by using the following score: 0 (no staining), 1 (weak), 2 (moderate) and 3 (strong).

Table 1. Patient demographic data and upper gastroendoscopy findings.

	Endoscopically normal esophageal mucosa (n = 18)	Grade A reflux esophagitis (n = 10)	Grade B reflux esophagitis (n = 6)
Sex (M/F)	13/5	7/3	3/3
Age (mean ± standard deviation)	46.1 ± 15.6	37.5 ± 15.1	45.8 ± 12.9
PPI use	8	4	3
Indication for upper gastroendoscopy			
Abdominal pain only	6	2	—
+ reflux	3	4	5
+ vomiting	3	2	1
+ weight loss	1	—	—
+ GI bleeding	1	—	—
Duodenal biopsy and aspiration	1	—	—
Severe heartburn	1	2	—
Dysphagia	1	—	—
Anemia	1	—	—
Assessment before reflux surgery	—	—	1
Additional upper gastroendoscopy findings[a]			
Hiatus hernia	3	—	3
Diffuse gastritis	8	8	—
Antral gastritis	1	2	1
Duodenitis	4	4	2
Pangastritis	—	—	2
Incompetent cardia	1	—	—
Gastric erosion	—	—	1
Gastric ulcer	2	—	—
Prepyloric ulcer	—	—	2
Pale gastric mucosa	1	—	—
None	2	—	—

[a]Patients may present with more than one upper gastroendoscopy finding.

Statistics

Microarray data of the esophagitis group express the fold changes in gene expression as compared with sham. Microarray data of the treatment groups express the fold changes as compared with the mean of the esophagitis group. All other results are expressed as mean ± standard error of the mean (SEM) or as box plots or scatterplots (histology). Differences between groups were compared by analysis of variance followed by appropriate multiple comparison testing.

To elucidate the internal structure of the inflammatory mediators assessed by proteome profiling, a factor analysis was calculated (15). See the Data Supplement for further details.

All supplementary materials are available online at www.molmed.org.

RESULTS

Reflux-Induced Macroscopic and Microscopic Lesions

Ten days after surgery, marked lesions had developed in the esophagi of vehicle-treated animals. Pretreatment with STW5 or omeprazole significantly reduced the number, size and depth of these lesions (Figures 1A, B).

Microscopic examination revealed massive mucosal damage and transmural

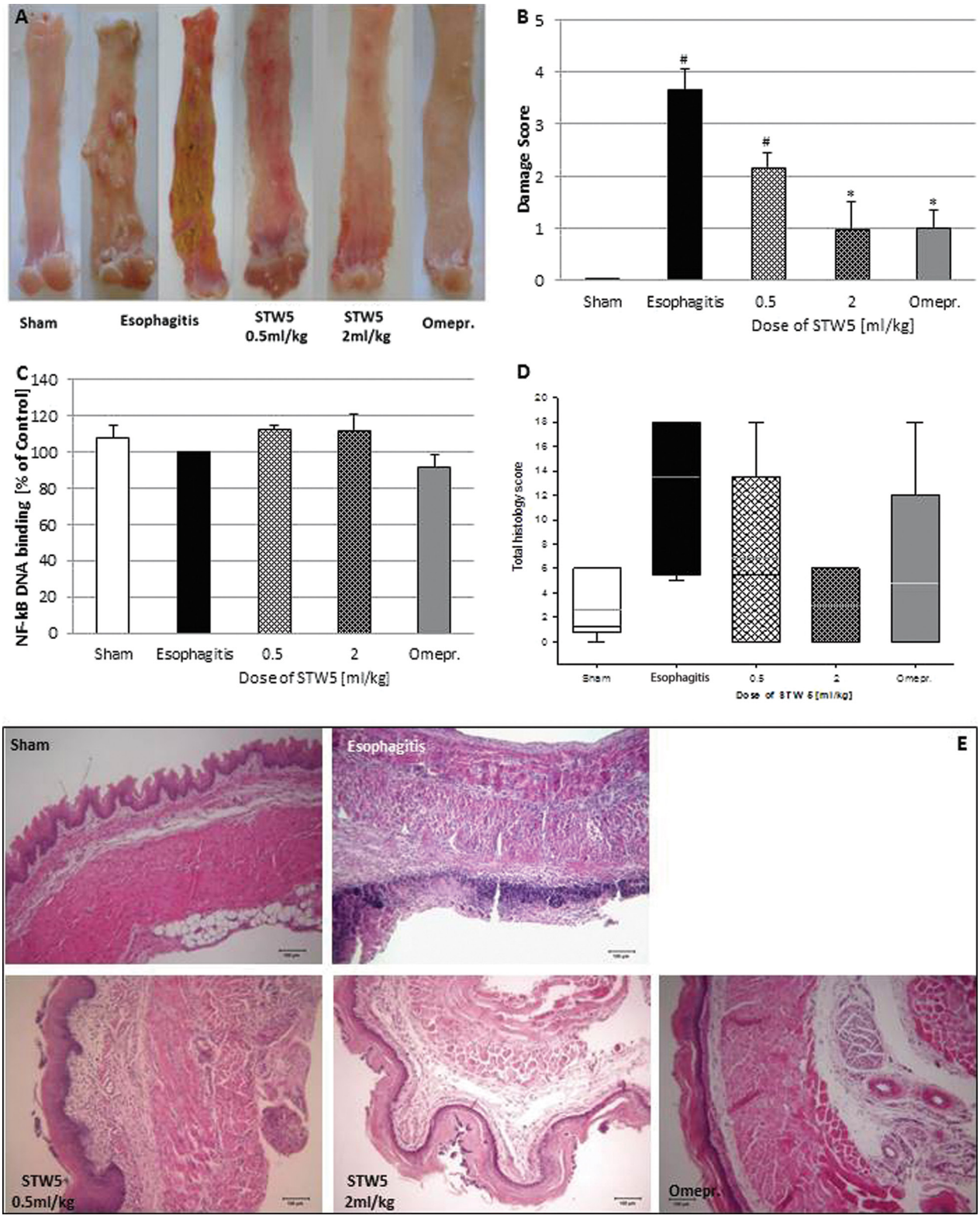

Figure 1. Subchronic reflux esophagitis induces macroscopic damage (A, B) and histological damage (D, E) in rats but does not affect NF-κB activity (C). Data are expressed as the mean ± SEM (B, C) or box plot (D) for six to nine animals. #Significant difference from sham-operated animals. *Significant difference from esophagitis group, $P < 0.05$.

infiltration with inflammatory cells in tissues from vehicle-treated esophagitis rats (Figures 1D–E). Esophagi from omeprazole-treated rats appeared almost unchanged compared with sham animals. Likewise, STW5 treatment significantly reduced infiltration and mucosal damage in a dose-dependent manner. Animals treated with 2 mL/kg STW5 had almost normal appearing esophagi.

Inflammatory Mediators and NF-κB Activity

In esophageal tissue, esophagitis led to the modulation of 1,708 genes, of which more than half were related to inflammation (inflammatory response: 30%; inflammatory disease: 23%). Other genes (Supplementary Figure S1) belonged to skeletal and muscular disorders (18%), connective tissue disorders (17%) and infectious diseases (12%). Major modulated transcripts related to proinflammatory cyto- and chemokines in the inflamed tissue are presented in Table 2 (detailed list: Data Supplement). Both treatments normalized proinflammatory gene expression. However, although STW5 and omeprazole showed a common modulation of 2,302 genes, there were differences in the way they suppressed the inflammatory response. For example, omeprazole did not reduce IL-17 expression, but like STW5, it did downregulate the IL-17 receptor (Supplementary Table S7). A further example is presented in a pathway analysis showing that even though both drugs downregulate *IL-6*, they activate different molecules in the cytokine signaling pathway. Whereas STW5 increased *TRAF6* and activated *mitogen-activated protein (MAP) kinases* of the TNF-α and IL-1 pathway, such as *MKK3/6* and *p38*, omeprazole signaled directly via downregulation of *JAK2* and the IL-6 pathway (Supplementary Figure S3). NF-κB was only weakly affected in esophagitis and thus its expression was less modulated by both treatments.

Table 2. Gene regulation of proinflammatory cyto- and chemokines as well as GPR84 and LOX-1 in esophageal tissue.

Genes	Esophagitis	PP	STW5 (0.5 mL/kg)	PP	STW5 (2 mL/kg)	PP	Omeprazole (30 mg/kg)	PP
IL-1A	1.4 (NS)	NE	–2.0*	NE	–1.6*	NE	–1.8**	NE
IL-1β	13.9$^{\#\#\#}$	↑	–2.2 (NS)	↓	–22.8***	↓	–5.9**	—
IL-1R	3.6$^{\#\#\#}$		–1.9**		–3.3**		–3.8***	
IL-6	11.5$^{\#}$; (0.1)	ND	–4.9*; (–3.9)	ND	–9.8***; (–1.8)	ND	–9.3***; (–7.4)	ND
IL-6R	2.6$^{\#\#\#}$		–1.5*		–2.5***		–2.3***	
IL-7R	12.7$^{\#\#\#}$		–2.6**		–9.3***		–6.7**	
IL-17D	–2.3$^{\#\#}$; (22.8)	ND	–2.0 (NS); (–11.3)	ND	–8.4***; (–33.9)	ND	2 (NS); (–7.4)	ND
IL-23A	2.1$^{\#}$	ND	–1.9*	ND	–2.8*	ND	–2.5**	ND
CCL1	1.5 (NS)	ND	–1.4 (NS)	ND	–1.1 (NS)	ND	–1.6 (NS)	ND
CCL2 (*MCP-1*)	11.1$^{\#\#}$	ND	–4.3**	ND	–10.8 (NS)	ND	–8.8***	ND
CCL3 (*MIP-1α*)	18.1$^{\#\#}$	↑$^{\#}$	–5.2**	↓*	–12.5***	↓*	–9.5***	↓
CCL4 (*MIP-1β*)	34.5$^{\#\#}$; (11.9)	↑$^{\#}$	–7.6 (NS); (–2.4)	↓	–13.0 (NS); (–81.0)	↓*	–30.5***; (–1,431.3)	↓
CCL5 (*RANTES*)	2.5 (NS)	↑$^{\#}$	–1.6 (NS)	↓$^{\#}$	–2.3*	↓*	–2.5*	↓*
CCL7	18.3$^{\#\#\#}$		–2.2 (NS)		–15.4***		–13.4***	
CXCL1 (*CINC1*)	17$^{\#\#}$; (0.001)	↑$^{\#}$	–2.3 (NS); (0.5)	↓*	–10.1**; (0.04)	↓*	–9.5***; (–5.6)	↓
CXCL2 (*CINC3*)	17.5$^{\#\#\#}$	↑$^{\#}$	–3.9*	↓	–12.0**	↓*	–15.5***	↓
CXCL3 (*CINC2*)	27.5$^{\#\#\#}$	↑$^{\#}$	–31.8**	↓*	–5.2**	↓*	–5.9**	↓
CXCL5 (*LIX*)	–1.4 (NS)	↑$^{\#}$	–1.7 (NS)	↓	–15.4 ***	↓	–2.3 (NS)	↓
CXCL9 (*MIG*)	2.7$^{\#}$	↑$^{\#}$	–1.6 (NS)	↓*	–3.0**	↓*	–2.7**	↓$^{\#}$*
GPR84	32.5$^{\#\#\#}$		-9.4***		-34.1***		-19.5***	
	14.6$^{\#\#\#}$		-8.2***		-16.9***		-14.6***	
	(18.3)		(1.2)		(–1.3)		(–8.8)	
LOX-1	11.1$^{\#\#\#}$		–4.5**		–27.8***		–19.5***	
TREM-1	8.4$^{\#\#}$		–2.8 (NS)		–16.0***		–7.2**	
TREM-3	8.7$^{\#\#\#}$		–5.2*		–34.5***		–5.4**	

Column 2 shows fold changes in the esophagitis group versus the sham-operated group. Columns 4, 6 and 8 show fold changes in the treatment groups compared with the esophagitis. Negative values indicate downregulation, positive values upregulation. $^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$ versus sham; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ versus esophagitis. Values between brackets are obtained by qRT-PCR. PP, proteome profiler (please see Figure 2 for detailed data): ↑, increase versus sham; ↓, decrease versus esophagitis; NE, no effect; ND, not detectable; NS, not significant.

To investigate whether this regulation of gene expression is also reflected in a modulation of protein expression, esophageal tissue homogenates were analyzed by using a proteome-profiler cytokine array. The esophagitis group showed a marked increase in the number and amount of proinflammatory cytokines (Figure 2A). This inflammatory response was prevented dose-dependently by treatment with STW5 and to a lesser extent by omeprazole. The observed inflammatory response was not accompanied by any change in NF-κB activity, as assessed by its DNA binding (Figure 1D) and the weak regulation of its gene expression. The lack of change in its expression was confirmed on protein level by WB (Supplementary Figure S4).

Factor Analysis Identified CINC1–3, MIP-1/3α, MIG, RANTES and IL-1β as Highly Relevant Mediators

Because inflammatory mediators, like most other molecules in a biological system, do not act in isolation, but rather as networks that affect one another, we used factor analysis to investigate the effects seen on the overall inflammatory response. It showed a four-component outcome that explains 85% of the total variance and therefore the internal structure of the mediators in a statistical connotation. The rotated component matrix (Supplementary Table S5) supports the determination of what the components 1–4 represent. Only the mediators with loadings (correlations) of ≥0.7 (bold) for each factor were used. The first component is most highly correlated with CINC1–3 and with MIP-1α, whereas IL-10 and IL-1β show a high correlation with component 1. Component 2 is highly correlated with CNTF and fractalkine. Component 3 is correlated to IL-1α. Component 4 is correlated to sICAM-1. Data suggest that at least components 3 and 4 are represented by IL-1α and sICAM-1, respectively, and one can narrow down the measurements for component 3 and 4 to IL-1α and sICAM-1, respectively. Component 1 is obviously not represented by a single molecule, but requires the measurement of several components (see the Data Supplement for further details).

TREM-1 Signaling

Pathway analysis is a further tool to investigate the correlation between different molecules in a biological system. Pathway analysis using the major modulated cyto- and chemokines in our model revealed that most were found to be part of the triggering receptor expressed on myeloid cells (TREM)-1 signaling pathway.

TREM-1 and *TREM-3* were upregulated during inflammation about eight-fold (Table 2). STW5 as well as omeprazole reversed this upregulation and subsequently upregulated/downregulated members of this signaling pathway (*SPIB, FOXO4, FOXO6 and PRDM1*), resulting in the decrease of the proinflammatory signature of cytokines in the treatment groups (Figure 3).

LOX-1 Receptor and MAP Kinases

Transcript arrays showed marked upregulation of the *LOX-1* receptor (Table 2), which was largely prevented by both treatments. This result was reflected by the fact that LOX-1 protein was only detected in the esophagitis group in rats in WB (Figure 4A). All experimental groups with induced esophagitis showed an increase in total p38, a stress-induced MAP kinase, compared with the sham group. In addition, the phosphorylation of p38 increased. STW5 and omeprazole had no effect on the total expression of p38 but decreased the phosphorylation, albeit not to a significant extent, in the case of omeprazole (Figures 4C, D).

GPR84 Was Strongly Upregulated in Esophagitis

Interestingly, in our GERD-model *GPR84*, a former orphan receptor now regarded to play an important role in inflammation was among the most highly upregulated genes in esophageal tissue during esophagitis (Table 2) and was also upregulated in blood, albeit to a lower extent (2.6/4.4×; Supplementary Table S6). This upregulation was prevented by both drugs to similar extents. Gene upregulation was reflected in a marked increase in the amount of GPR84 protein detected in esophageal tissue homogenates by WB and its return to basal levels in the treatment groups (Figure 4A).

Distribution of GPR84 was investigated in rat esophageal tissue using immunohistochemical staining and was shown to be abundant mainly in the epithelial cells lining the esophagus, highly upregulated in the esophagitis group and decreased in the treatment groups (Figure 4F). To investigate the relevance of these findings in humans, we analyzed protein expression of the receptor in the esophageal epithelial cell line HET-1A. This experiment showed that GPR84 is not only expressed in rat, but also in human esophageal epithelial cells. Its expression was significantly upregulated upon incubation with capsaicin, which is known to induce an inflammatory state via TRPV1 activation (Figures 5A, B). This upregulation was prevented by coincubation with STW5 or omeprazole.

As a proof of concept for our theory of the involvement of GPR84 in esophagitis, we analyzed esophageal biopsies from a small cohort of patients by immunohistochemical staining. Samples were included from patients with either endoscopically normal or endoscopically inflamed (grade A or grade B reflux esophagitis) patients. Most patients expressed GPR84 to different extents in the squamous epithelium (Figures 5C–F); however, patients with more severe esophagitis (grade B) significantly expressed the receptor to a higher extent, as evidenced by the higher staining intensity.

DISCUSSION

Recent data suggest that immune-mediated mechanisms play a prominent role in the development of GERD and that IL-8 is one of the central cytokines (2,3). Indeed, in our model of subchronic GERD, the rat analogs to human IL-8 (the murine CINC1, -2 and -3) were elevated, supporting an IL-8 involvement in GERD. However, with our profiling approach,

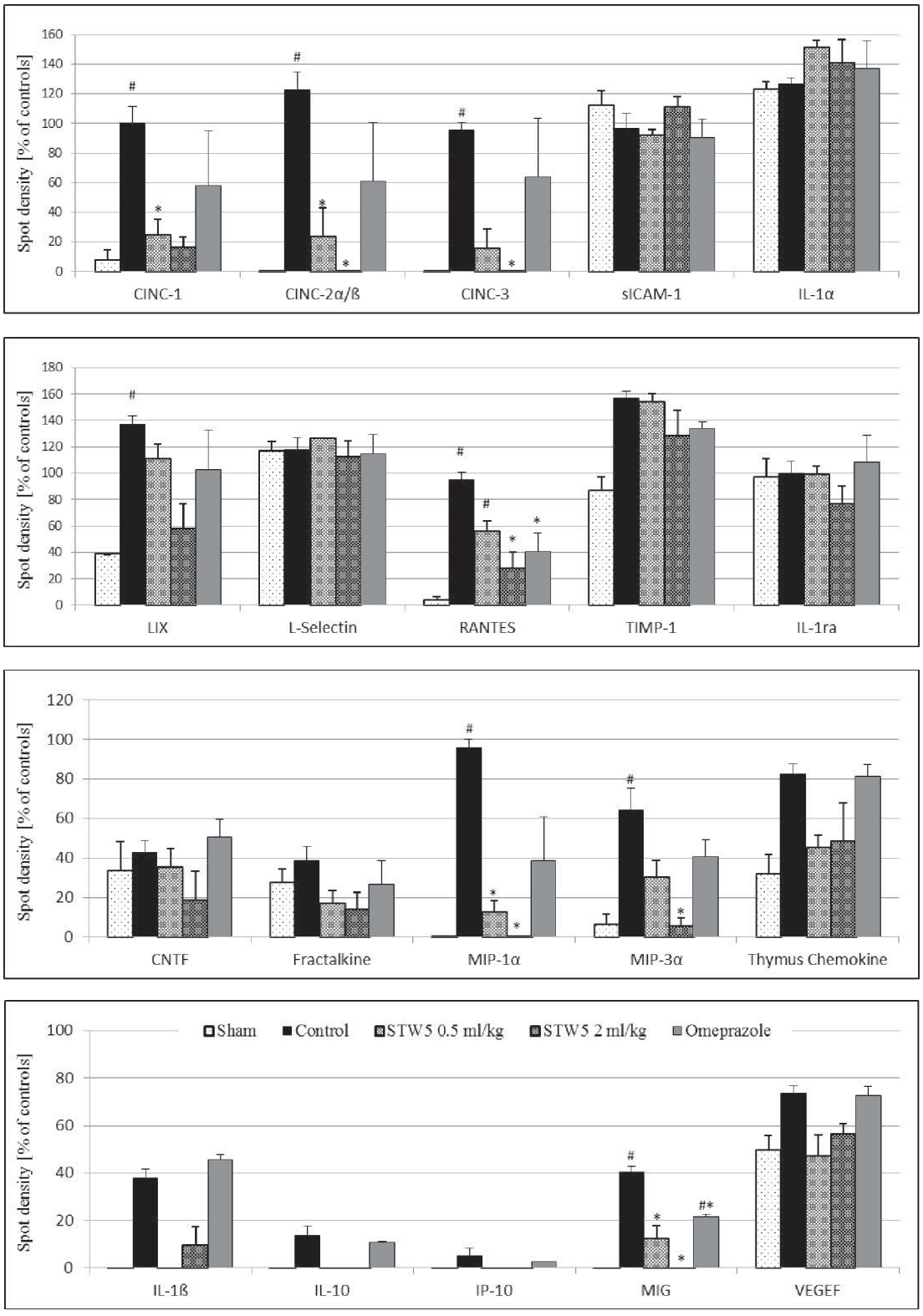

Figure 2. Subchronic reflux esophagitis increases proinflammatory cytokines in the esophageal tissue of rats with reflux esophagitis (proteome profiler). Data are expressed as the mean ± SEM for three arrays. #Significant difference from sham-operated animals. *Significant difference from esophagitis group, $P < 0.05$.

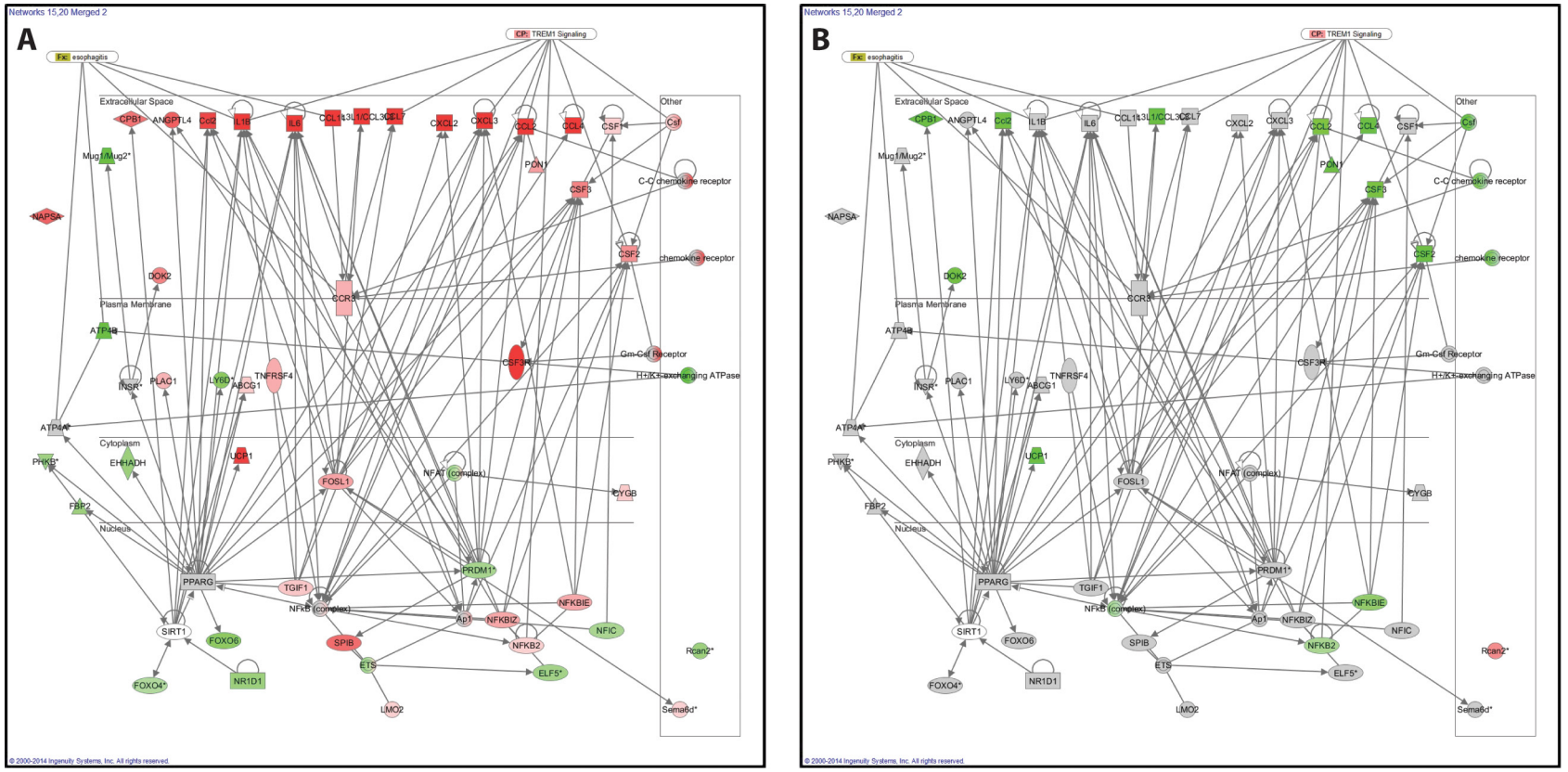

Figure 3. Pathway analysis reveals that most highly regulated inflammatory mediators are associated with the TREM-1 signaling pathway. The preprogrammed TREM-1 signaling pathway was merged with the preprogrammed genes known to be highly modulated during esophagitis (Ingenuity Systems 2014). This dataset was overlaid with the real gene expression array data obtained from the esophageal tissue of the different experimental groups to identify modulated genes. The red color indicates the upregulation of a particular gene expression, and the green color indicates downregulation. The intensity of the color signifies the magnitude of regulation. Grey color indicates no gene modulation. For a better overview, genes that were not modulated in any of the groups were removed from the pathway. The figure shows gene expression of the esophagitis group compared with the sham-operated group (A), the STW5- treated (0.5 and 2 mL/kg, respectively) groups in comparison to the esophagitis group (B, C) and the omeprazole-treated (30 mg/kg) group in comparison to the esophagitis group (D).

Continued on the next page

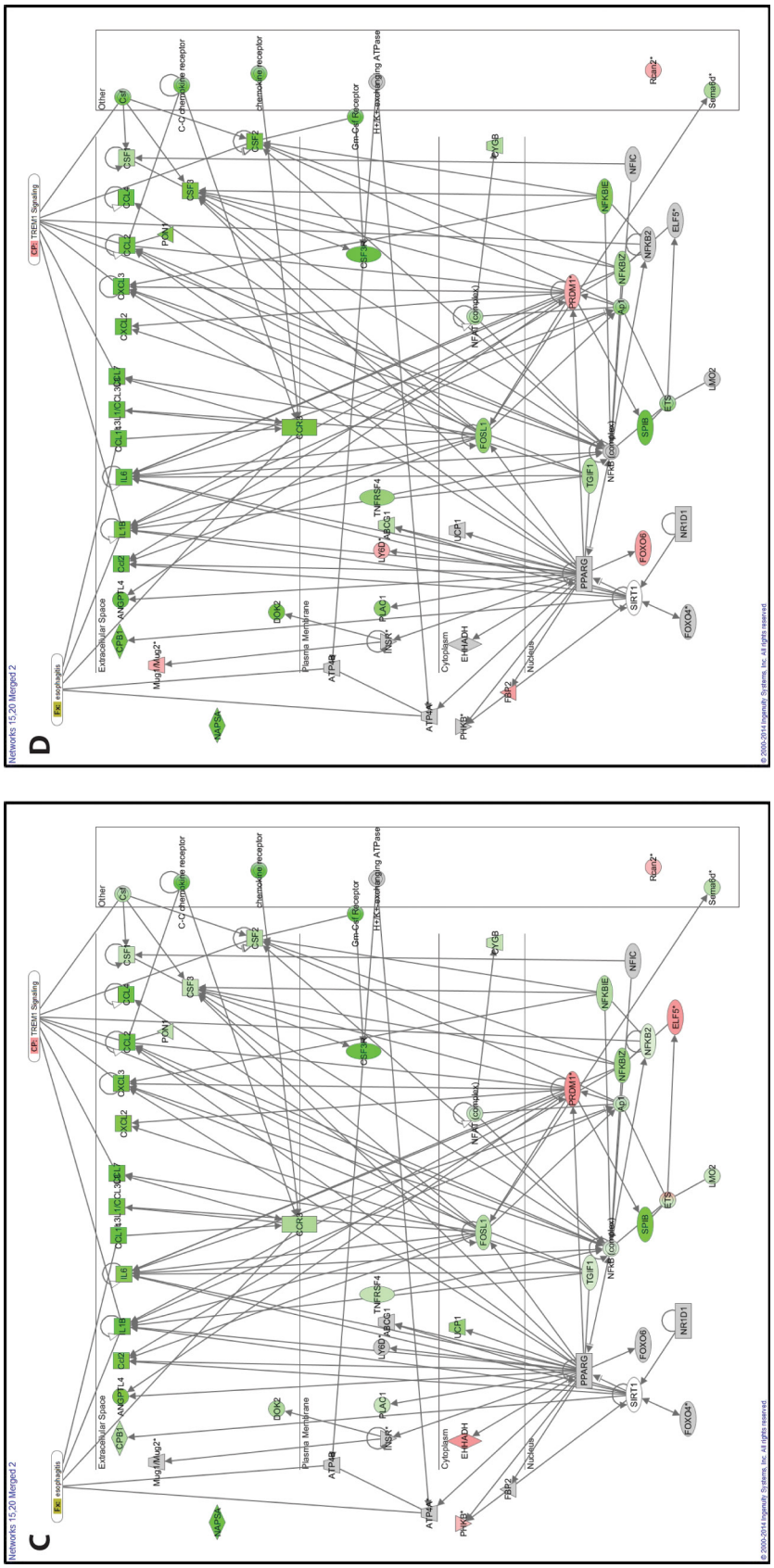

Figure 3. *Continued.*

we covered the whole transcriptome and can thereby further substantiate immune-mediated mechanisms, at least for the esophageal tissue in our model. The present data indicate the involvement of well-established proinflammatory mediators such as IL-6 and IL-1β, in addition to other mediators such as CCL3, CCL4 (MIP-1β) and RANTES, as yet unknown to be involved in esophagitis.

CCL4 functions as a potent chemoattractant for monocytes, T-lymphocytes, dendritic cells and natural killer cells. B-cells and professional APCs recruit regulatory T cells (Tregs) via CCL4. CXCL1 also called CINC-1 or Gro1 functions as a potent chemoattractant for neutrophils. It promotes growth of certain cancers and is part of a proinflammatory signature. Thus, besides the IL-8 analogs CINC1–3, chemokines capable of promoting the infiltration of inflammatory and/or immune-competent cells into the subepithelial space were upregulated.

Interestingly, transcripts of the NF-κB family were only weakly (one- to four-fold) upregulated, and its protein amount (WB) as well as its activity (as determined by ELISA) were not changed. In contrast, Fang *et al.* (5) recently reported a strong activation of the NF-κB pathway in duodenal and mixed models of GERD in mice. This discrepancy might be due to the differences between the models used. While duodenal reflux models are helpful to investigate certain aspects of the disease, they are not physiological and hence do not closely resemble the clinical situation.

Similar effects were observed for TNF-α, which was also undetectable in the proteome profiler and showed no difference between groups when measured with ELISA (Supplementary Figure S4). Because TNF-α was shown to be significantly elevated in a similar acute model of esophagitis (10), we hypothesized that during the development of chronicity, cytokine engagements may switch from TNF-α and NF-κB signaling to IL-6, IL-8 and chemokines such as CCL4. This phenomenon is worthy of further investigation.

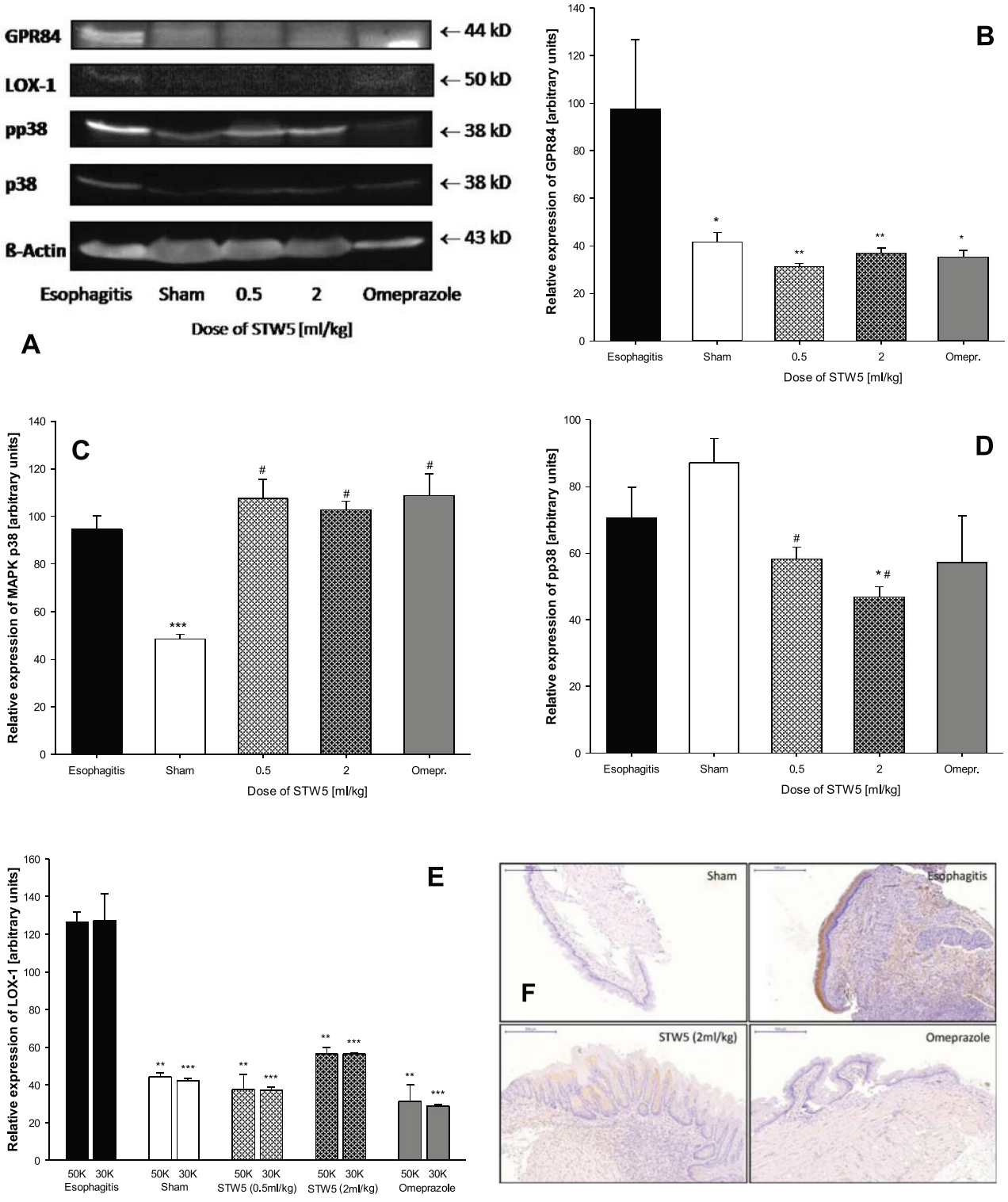

Figure 4. Modulation of GPR84, LOX-1 and mitogen-activated protein kinases in rats with RE. (A) Representative Western blots of GPR84, p38 and LOX-1. (B, C) Quantification of GPR84 or LOX-1 normalized each with β-actin. (D) Quantification of pp38 normalized with p38. (E) Quantification of p38 normalized with β-actin. Data represent the analysis of a minimum of five tissue samples from five animals per group. $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$ versus esophagitis group. $^{\#}P \leq 0.05$ versus sham. (F) Immunohistochemical staining of GPR84 (representative pictures).

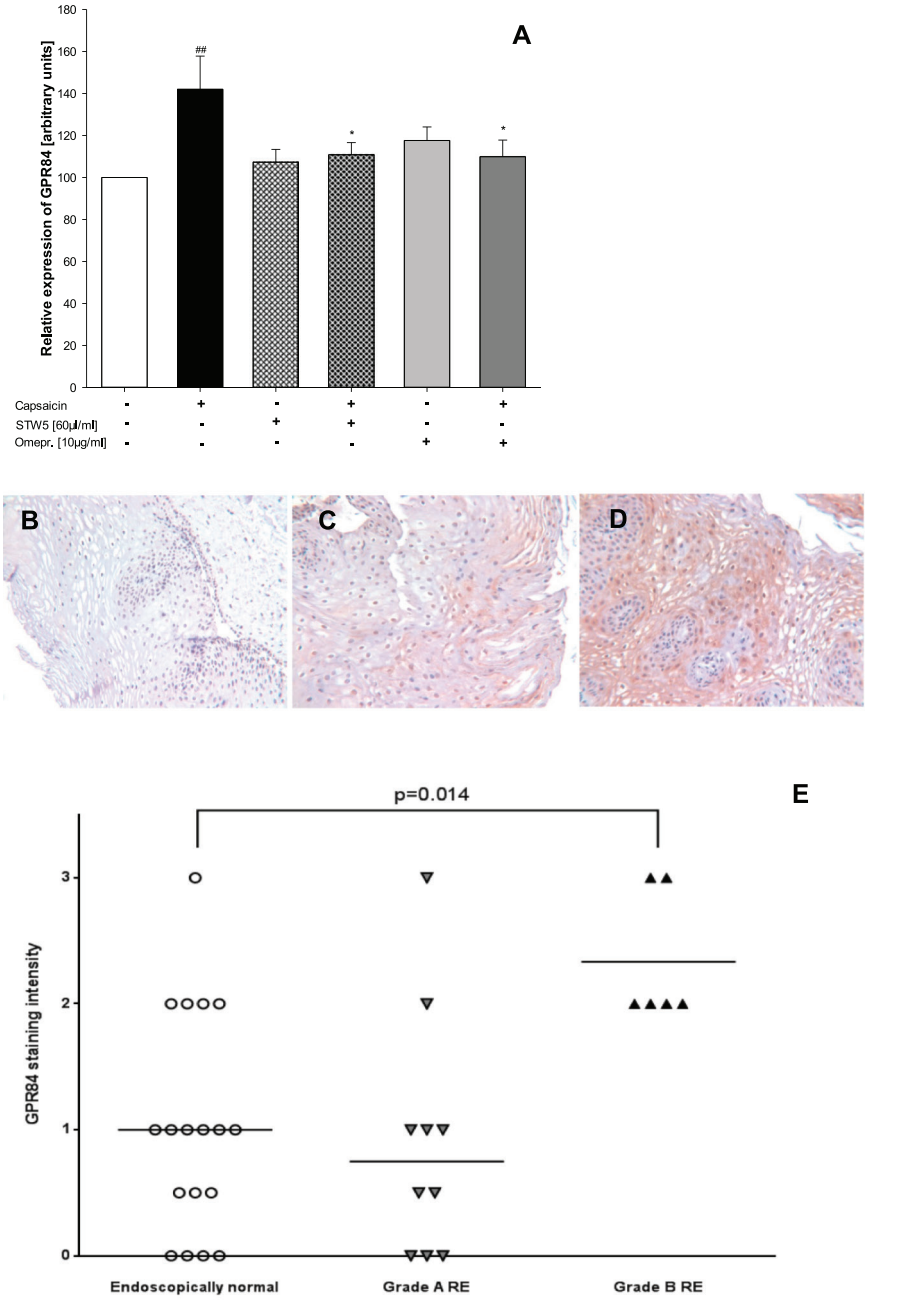

Figure 5. Modulation of GPR84 expression in HET-1A cells and human esophageal biopsies. (A) Quantification of GPR84 normalized with β-actin. (B–D) Immunohistochemical staining of GPR84 (representative pictures) showing no staining (B), moderate staining (C) and strong staining (D). (E) Quantification of GPR84 expression in human esophageal biopsies. $^{*}P < 0.05$ versus capsaicin; $^{\#\#}P < 0.01$ versus untreated cells.

To identify potential therapeutic targets, we focused on those genes/molecules modified by the disease and normalized by both treatments. Because STW5 did not show any acid-suppressing activity (10) and omeprazole was shown to possess additional antiinflammatory activity (8,16,17), this comparison should identify targets not related to acid suppression.

Factor analysis identified CINC1–3 as well as MIP-1/3, MIP-1/3α, MIG, RANTES and IL-1β as highly relevant. The analysis further highlights that the variance between the experimental groups cannot be explained by the measurement of single cytokines. Thus, for example, the most important factor 1 is not represented by a single cytokine but rather in the form of a group measurement of chemokines of the CC family. Thus, factor analysis further supports the hypothesis that immune responses mediated by chemokines play a role in GERD. The relevance of CINC1–3, MIP-3 and IL-1β was also supported by gene expression analysis.

During inflammation, the phosphorylation and total amount of p38 increased. P38 belongs to the stress-induced MAP kinases. Four members of the p38 MAP kinase family have been identified (p38α, p38β, p38γ, p38δ). The importance of the different kinases appears to be cell type–dependent and context-specific (18). The isoforms p38α/β regulate, for example, TNF-α expression (18). In our model, TNF-α or NF-κB (tissue mRNA, proteome profiler/ELISA and WB) were not clearly involved. However, the release of IL-6 and IL-8 is known to be linked to p38 signaling (19,20) and has so far not been reported for reflux esophagitis.

The strong upregulation of total p38 in the esophagitis groups is likely to mask changes in the phosphorylation status induced by the treatments. STW5 and omeprazole did not influence the total amount of p38. Thus, changes in pp38 due to treatment need to be considered with caution. Although, we did not measure isoforms of p38, we clearly demonstrate an involvement of p38 in our reflux model.

Because inflammatory mediators, like most other molecules in a biological system, do not act in isolation, but rather in orchestra, affecting one another, pathway analysis was used to further characterize the inflammatory response. Most of the chemokines, which were found to be modulated in the present study, can activate members of the transmembrane receptor family TREM.

So far, at least five members of the TREM family have been identified (TREM-1, -2, -3, -4 and -5), with TREM-1 being expressed on monocytes, macrophages, neutrophils and hepatic vascular endothelium (21,22) and functioning as an amplifier of acute and chronic inflammatory responses (23,24). Ultimately, the activation of the TREM-1 signaling pathway results in downstream production of a variety of proinflammatory cytokines, including TNF, MCP/CCL2, MIP-1α and IL-8, amplifying innate and adaptive immune responses (25,26). Our data not only supports an important role for TREM-1 in the release of well-known proinflammatory cytokines (MCP/CCL2, MIP-1α, IL-1, IL-6 and IL-8), but extends it to members of the CCL/CXCL chemokines, at least for subchronic experimental esophagitis. So far, TREM-1 has been associated with acute and chronic inflammatory bowel disease (IBD). Soluble TREM-1 was shown to be elevated in serum and expressed on $CD68^+$ macrophages in the intestinal lamina propria in patients with acute and chronic IBD (25,27). Recently, isolated PBMCs from primary specimens of patients with rheumatoid arthritis showed an increased expression of TREM-1, with a subsequent increased activation of ERK and MAP kinases, secretion of IL-8 and RANTES (28). This result further supports the relevance of our findings, which link TREM-1 to MAP kinase signaling, IL-8 and RANTES in "subchronic" inflammatory conditions. An association of this signaling complex with GERD is novel.

We can further characterize transcription factors, other than NF-κB, linked to the activation of TREM-1. The transcription factors *FOXO4* and *FOXO6* were downregulated, whereas *SPIB* was upregulated. While SPIB is reported to be crucial for the development of M-cells (specialized epithelial cells that initiate mucosal immune responses by sampling luminal antigens) in the gut (29), the FOXO family represents a group of transcription factors that is required for a number of stress-related transcriptional programs. It comprises four FOXO proteins: FOXO1, FOXO3, FOXO4 and FOXO6. Whereas FOXO6 is largely specific to neurons, the other three factors are widely distributed in most tissues (30). The simultaneous downregulation of the gene expression of *TREM-1* and *TREM-3* and the reversal of the upregulation and downregulation of *SPIB*, *FOXO4* and *FOXO6* after treatment with STW5 and omeprazole support a role of these transcription factors in inflammatory signaling. Recently, mice deficient in TREM-1 were generated, resulting in attenuated diethylnitrosamine-induced hepatocellular carcinoma (31), and TREM-1 blockade was shown to improve the clinical course of a murine model of IBD (25). Taken together, these data support a central role of TREM-1 in gastrointestinal (GI) inflammation and cancer progression. Thus, TREM-1 represents a highly interesting potential novel target and might be relevant to the treatment of GERD.

Physiologically, chemokines are expressed to recruit and direct cell trafficking/migration (32). They control the movement of certain transmembrane G protein–coupled receptor (GPCR)-expressing cells. Gene expression of one of these GPCRs, GPR84, was highly increased in the esophagitis model. This receptor has only recently been identified (33,34), and so far no close homologs were detected. Its ligands are medium-chain free fatty acids (FFAs), and it functions obviously as a lipid/FFA sensor (35). It was suggested that GPR84 acts as a proinflammatory receptor linking fatty acid metabolism to immunological responses (34–36). Other fatty acids such as arachidonic acid and saturated fatty acids have been demonstrated to increase proinflammatory IL-8 along with numerous other inflammatory factors, including IL-6, TNF-α, IL-1β and CXCL1 (37). Agonists of GPR84 were shown to amplify IL-8 production by PMNs, and GPR84 agonists work as chemo-attractants to both PMNs and macrophages (38). We found a simultaneous upregulation of GPR84 and of the rat analogs of IL-8 (CINCs), indicating that GPR84 is involved in proinflammatory pathways regulating the secretion of cytokines, thereby forming a link to the immune system. Furthermore, our data support a link of GPR84 to inflammation, since it is strongly upregulated in the esophagitis group and downregulated upon antiinflammatory treatments.

Because GPR84 is expressed in blood leukocytes (38), its upregulation, as seen in the RNA microarray and WB, could have been due to neutrophilic infiltration into the inflamed tissue. However, immunohistochemical staining of rat tissue showed clearly that the majority of GPR84 was localized in the epithelial lining of the esophageal mucosa. To investigate the presence of this receptor also in human esophageal mucosa and to test the possible relevance of these findings in human GERD, we analyzed protein expression of the receptor in the normal human squamous cell line HET-1A via WB. Indeed, we were able to detect the receptor in the cell line and to cause an upregulation of its expression by incubation with capsaicin as a TRPV1 agonist known to cause an inflammatory state in esophageal mucosal cells (39). This upregulation was prevented by coincubation with STW5 or omeprazole, confirming our *in vivo* findings.

As a proof of concept for our theory of the involvement of GPR84 in esophagitis, we analyzed esophageal biopsies from a small cohort of patients by immunohistochemical staining. This study had several limitations. It included only a small number of patients (34 in total), and the patient population was very heterogeneous. Most patients, even those with endoscopic normally appearing mucosa had reflux symptoms or other upper GI inflammatory conditions, indicating that these patients also might have had some low-grade inflammation and more than one-third was already on PPI therapy. Therefore, most patients were expressing GPR84 to different extents, and the lack of a healthy control group made it impossible to differentiate

between endoscopically normal and grade A esophagitis. Nevertheless, the more severely inflamed patients (grade B esophagitis) showed a significant up-regulation of GPR84 expression, which lends further evidence, at least to the presence of a correlation between the receptor and esophagitis.

To the best of our knowledge, we report here for the first time the discovery of GPR84 in esophageal tissue and of the GPR84 protein in tissue of the alimentary tract in general (thus far, mRNA was detected in zebra fish and human intestines [33,40]) and its possible implications in esophagitis.

CONCLUSION

By using a subchronic experimental model of esophagitis, the findings substantiate the concept that immunological processes linked to chemotaxis of immune-competent cells to esophageal tissue play a crucial role in the pathophysiology of GERD. Esophageal cells release an array of proinflammatory cytokines/chemokines that activate receptors and signal cascades, of which CINC1–3 (IL-8), TREM-1 and GPR84 appear to be relevant for the regulation of the inflammatory process. These receptors and/or signal cascades may prove to be promising targets for developing new therapeutic measures for better management of GERD.

ACKNOWLEDGMENTS

We would like to thank EJ Verspohl (Münster, Germany) for hosting this work in his laboratory and for his support in planning and execution of the study and A Hegazy (Cairo, Egypt) for helping in establishing the animal model, as well as D Rohr (Mainz, Germany) and F Hartbrod (Bonn, Germany) for their technical assistance. We also thank the German Academic Exchange Service (DAAD) and the Egyptian Ministry of Higher Education for supporting this study with a short-term research scholarship and Steigerwald Arzneimittelwerk GmbH (Darmstadt, Germany) for financial support.

DISCLOSURE

J Müller is employed by Steigerwald Arzneimittelwerk GmbH. H Abdel-Aziz was not affiliated with that company at the time of study, but joined employment later.

REFERENCES

1. Ronkainen J, Agreus L. (2013) Epidemiology of reflux symptoms and GORD. *Best Pract. Res. Clin. Gastroenterol.* 27:325–37.
2. Souza RF. (2010) Bringing GERD management up to PAR-2. *Am. J. Gastroenterol.* 105:1944–6.
3. Souza RF, *et al.* (2009) Gastroesophageal reflux might cause esophagitis through a cytokine-mediated mechanism rather than caustic acid injury. *Gastroenterology.* 137:1776–84.
4. Huo X, Souza RF. (2013) Acid burn or cytokine sizzle in the pathogenesis of heartburn? *J. Gastroenterol. Hepatol.* 28:385–7.
5. Fang Y, *et al.* (2013) Gastroesophageal reflux activates the NF-kappaB pathway and impairs esophageal barrier function in mice. *Am. J. Physiol. Gastrointest. Liver Physiol.* 305:G58–65.
6. Yamaguchi T, *et al.* (2005) Cytokine-induced neutrophil accumulation in the pathogenesis of acute reflux esophagitis in rats. *Int. J. Mol. Med.* 16:71–7.
7. Park JY, *et al.* (2014) Proton pump inhibitors decrease eotaxin-3 expression in the proximal esophagus of children with esophageal eosinophilia. *PLoS One.* 9:e101391.
8. Huo X, *et al.* (2014) In oesophageal squamous cells exposed to acidic bile salt medium, omeprazole inhibits IL-8 expression through effects on nuclear factor-kappaB and activator protein-1. *Gut.* 63:1042–52.
9. Madisch A, Mueller MH, Vinson BR, Weiser D. (2008) M1803 herbal preparation STW 5 significantly improved concomitant reflux symptoms in patients with functional dyspepsia: a subgroup analysis of placebo-controlled trials. *Gastroenterology.* 134:A-422.
10. Abdel-Aziz H, *et al.* (2010) Effect of an herbal preparation, STW 5, in an acute model of reflux oesophagitis in rats. *J. Pharmacol. Sci.* 113:134–42.
11. Omura N, *et al.* (1999) Establishment of surgically induced chronic acid reflux esophagitis in rats. *Scand. J. Gastroenterol.* 34:948–53.
12. Krishna S, *et al.* (2015) A randomised, double blind, placebo-controlled pilot study of oral artesunate therapy for colorectal cancer. *EBioMedicine.* 2:82–90.
13. Ulrich-Merzenich G, *et al.* (2012) Novel neurological and immunological targets for salicylate-based phytopharmaceuticals and for the anti-depressant imipramine. *Phytomedicine.* 19:930–9.
14. Ulrich-Merzenich G, Zeitler H, Panek D, Bokemeyer D, Vetter H. (2007) Vitamin C promotes human endothelial cell growth via the ERK-signaling pathway. *Eur. J. Nutr.* 46:87–94.
15. Do K-A, Qin ZS, Vannucci M (eds.). (2013) *Advances in Statistical Bioinformatics. Models and Integrative Inference for High-Throughput Data*. New York: Cambridge University Press. 514 pp.
16. Kobayashi T, Ohta Y, Inui K, Yoshino J, Nakazawa S. (2002) Protective effect of omeprazole against acute gastric mucosal lesions induced by compound 48/80, a mast cell degranulator, in rats. *Pharmacol. Res.* 46:75–84.
17. El-Nezhawy AO, Biuomy AR, Hassan FS, Ismaiel AK, Omar HA. (2013) Design, synthesis and pharmacological evaluation of omeprazole-like agents with anti-inflammatory activity. *Bioorg. Med. Chem.* 21:1661–70.
18. Sabio G, Davis RJ. (2014) TNF and MAP kinase signalling pathways. *Semin. Immunol.* 26:237–45.
19. Lee KE, *et al.* (2013) Helicobacter pylori and interleukin-8 in gastric cancer. *World J. Gastroenterol.* 19:8192–202.
20. Feng W, Song Y, Chen C, Lu ZZ, Zhang Y. (2010) Stimulation of adenosine A(2B) receptors induces interleukin-6 secretion in cardiac fibroblasts via the PKC-delta-P38 signalling pathway. *Br. J. Pharmacol.* 159:1598–607.
21. Chen LC, Laskin JD, Gordon MK, Laskin DL. (2008) Regulation of TREM expression in hepatic macrophages and endothelial cells during acute endotoxemia. *Exp. Mol. Pathol.* 84:145–55.
22. Bouchon A, Dietrich J, Colonna M. (2000) Cutting edge: inflammatory responses can be triggered by TREM-1, a novel receptor expressed on neutrophils and monocytes. *J. Immunol.* 164:4991–5.
23. Amatngalim GD, Nijnik A, Hiemstra PS, Hancock RE. (2011) Cathelicidin peptide LL-37 modulates TREM-1 expression and inflammatory responses to microbial compounds. *Inflammation.* 34:412–25.
24. Ford JW, McVicar DW. (2009) TREM and TREM-like receptors in inflammation and disease. *Curr. Opin. Immunol.* 21:38–46.
25. Schenk M, Bouchon A, Seibold F, Mueller C. (2007) TREM-1: expressing intestinal macrophages crucially amplify chronic inflammation in experimental colitis and inflammatory bowel diseases. *J. Clin. Invest.* 117:3097–106.
26. Bouchon A, Cella M, Grierson HL, Cohen JI, Colonna M. (2001) Activation of NK cell-mediated cytotoxicity by a SAP-independent receptor of the CD2 family. *J. Immunol.* 167:5517–21.
27. Park JJ, *et al.* (2009) Correlation of serum-soluble triggering receptor expressed on myeloid cells-1 with clinical disease activity in inflammatory bowel disease. *Dig. Dis. Sci.* 54:1525–31.
28. Chen X, *et al.* (2015) Inactivation of DAP12 in PMN inhibits TREM1-mediated activation in rheumatoid arthritis. *PLoS One.* 10:e0115116.
29. de Lau W, *et al.* (2012) Peyer's patch M cells derived from Lgr5(+) stem cells require SpiB and are induced by RankL in cultured "miniguts." *Mol. Cell. Biol.* 32:3639–47.
30. Tikhanovich I, Cox J, Weinman SA. (2013) Forkhead box class O transcription factors in liver function and disease. *J. Gastroenterol. Hepatol* 28 Suppl 1:125–31.

31. Wu J, *et al.* (2012) The proinflammatory myeloid cell receptor TREM-1 controls Kupffer cell activation and development of hepatocellular carcinoma. *Cancer Res.* 72:3977–86.
32. Ansari AW, Heiken H, Meyer-Olson D, Schmidt RE. (2011) CCL2: a potential prognostic marker and target of anti-inflammatory strategy in HIV/AIDS pathogenesis. *Eur. J. Immunol.* 41:3412–8.
33. Wittenberger T, Schaller HC, Hellebrand S. (2001) An expressed sequence tag (EST) data mining strategy succeeding in the discovery of new G-protein coupled receptors. *J. Mol. Biol.* 307:799–813.
34. Bouchard C, Page J, Bedard A, Tremblay P, Vallieres L. (2007) G protein-coupled receptor 84, a microglia-associated protein expressed in neuroinflammatory conditions. *Glia.* 55:790–800.
35. Wang J, Wu X, Simonavicius N, Tian H, Ling L. (2006) Medium-chain fatty acids as ligands for orphan G protein-coupled receptor GPR84. *J. Biol. Chem.* 281:34457–64.
36. Nagasaki H, *et al.* (2012) Inflammatory changes in adipose tissue enhance expression of GPR84, a medium-chain fatty acid receptor: TNFalpha enhances GPR84 expression in adipocytes. *FEBS Lett.* 586:368–72.
37. Munoz A, Costa M. (2013) Nutritionally mediated oxidative stress and inflammation. *Oxid. Med. Cell Longev.* 2013:610950.
38. Suzuki M, *et al.* (2013) Medium-chain fatty acid-sensing receptor, GPR84, is a proinflammatory receptor. *J. Biol. Chem.* 288:10684–91.
39. Ma J, Harnett KM, Behar J, Biancani P, Cao W. (2010) Signaling in TRPV1-induced platelet activating factor (PAF) in human esophageal epithelial cells. *Am. J. Physiol. Gastrointest. Liver Physiol.* 298:G233–40.
40. Huang Q, *et al.* (2014) A medium-chain fatty acid receptor Gpr84 in zebrafish: expression pattern and roles in immune regulation. *Dev. Comp. Immunol.* 45:252–8.

Cite this article as: Abdel-Aziz H, *et al*. (2015) GPR84 and TREM-1 signaling contribute to the pathogenesis of reflux esophagitis. *Mol. Med*. 21:1011–24.