G Protein-coupled pH-sensing Receptor *OGR1* Is a Regulator of Intestinal Inflammation

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Background: A novel family of proton-sensing G protein-coupled receptors, including *OGR1*, *GPR4*, and *TDAG8*, was identified to be important for physiological pH homeostasis and inflammation. Thus, we determined the function of proton-sensing OGR1 in the intestinal mucosa.

Mtehods: *OGR1* expression in colonic tissues was investigated in controls and patients with IBD. Expression of *OGR1* upon cell activation was studied in the Mono Mac 6 (MM6) cell line and primary human and murine monocytes by real-time PCR. *Ogr1* knockout mice were crossbred with *Il-10* deficient mice and studied for more than 200 days. Microarray profiling was performed using $Ogr1^{-/-}$ and $Ogr1^{+/+}$ (WT) residential peritoneal macrophages.

Results: Patients with IBD expressed higher levels of *OGR1* in the mucosa than non-IBD controls. Treatment of MM6 cells with TNF, led to significant upregulation of *OGR1* expression, which could be reversed by the presence of NF-κB inhibitors. Kaplan–Meier survival analysis showed a significantly delayed onset and progression of rectal prolapse in female $Ogr1^{-/-}/Il\text{-}10^{-/-}$ mice. These mice displayed significantly less rectal prolapses. Upregulation of gene expression, mediated by OGR1, in response to extracellular acidification in mouse macrophages was enriched for inflammation and immune response, actin cytoskeleton, and cell-adhesion gene pathways.

Conclusions: *OGR1* expression is induced in cells of human macrophage lineage and primary human monocytes by TNF. NF-κB inhibition reverses the induction of *OGR1* expression by TNF. *OGR1* deficiency protects from spontaneous inflammation in the *Il-10* knockout model. Our data indicate a pathophysiological role for pH-sensing receptor *OGR1* during the pathogenesis of mucosal inflammation.

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Key Words: GPCR, pH-sensing receptors, *OGR1*, IBD, microarrays, animal model

The mechanisms involved in the maintenance of mucosal homeostasis are important in our understanding of the pathophysiology of inflammatory bowel disease (IBD). Both forms of the disease, Crohn's disease (CD) and ulcerative colitis (UC), give rise to inflammation that is associated with extracellular acidification of mucosal tissue. Mucosal inflammation is interpreted as a local response to tissue damage and microbial invasion.

A number of studies suggest that an acidic environment affects the progression and resolution of inflammation.[1–3] Inflammation has been attributed to an increase in local proton concentration and lactate production[4] and subsequent proinflammatory cytokine production, such as tumur necrosis factor (TNF), interleukin-6 (IL-6), interferon gamma (IFN-γ), and interleukin-1-beta (IL-1β). TNF is one of the characterizing cytokines in IBD,[5,6] and anti-TNF targeted therapies are successful in both CD and UC.[7–10] Activated macrophages, which are key cellular mediators of acute and chronic inflammation, are primary producers of TNF.[11] TNF activates the nuclear transcription factor kappa B (NF-κB), one of the key regulators in chronic mucosal inflammation.[12,13]

G protein-coupled receptors (GPCRs), cell-surface molecules involved in signal transduction, are targeted by key inflammatory cytokines.[14] The ovarian cancer G protein-coupled receptor 1 (*OGR1*) family of receptors, which include *OGR1*, G protein-coupled receptor 4 *(GPR4*), and T-cell death associated

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gene *(TDAG8)*, sense extracellular protons through histidine residues located on the extracellular region of the receptors, resulting in the modification of a variety of cell functions.[15,16] Early signaling pathways of pH-sensing receptors triggered by acidification include phospholipase C activation, inositol trisphosphate formation, and subsequent Ca^{2+} release[15] or cyclic adenosine monophosphate production.[17,18] The increase of intracellular calcium influx and accumulation of cyclic adenosine monophosphate has been shown to regulate a vast range of cellular responses. Moreover, *OGR1* and *TDAG8* are alleged to act in opposition in a regulatory manner, either enhancing or inhibiting the production of proinflammatory cytokines respectively.[19]

TDAG8-mediated extracellular acidification inhibited lipopolysaccharide (LPS)-induced production of TNF and IL-6 in mouse peritoneal inflammatory macrophages.[2] Patients with CD demonstrate a defect in macrophage function resulting in an inadequate bacterial clearance from inflammatory sites.[20] In addition, macrophages from patients with CD showed impaired TNF-α secretion in response to bacterial challenge.[21] Furthermore, association results and in silico analysis have recently identified a locus within the *TDAG8* gene as one of the susceptibility loci associated with CD.[22] Onozawa et al[23] suggest that *TDAG8* is a negative regulator of inflammation, which is mediated through a G_s-coupled mechanism.[2] In contrast, *OGR1* is reported to act predominately through a G_q-coupled mechanism to stimulate proinflammatory cytokines production upon extracellular acidification.[19]

To date, few data on the role of *OGR1* in inflammation in IBD have been published. *OGR1* may play an important role in the regulation of the inflammatory pathways in IBD, and it may represent an interesting target for innovative therapies. Therefore, we investigated the role and function of *OGR1* in gut inflammation with a focus on myeloid cells. We used an immune-mediated inflammatory disease mouse model, namely interleukin-10 (*Il-10*) knockout (KO) mice, which spontaneously develop chronic colitis[24–26] and a human monocyte model. We show that *OGR1* expression is induced in monocytes by TNF and *OGR1* deficiency protects from spontaneous inflammation in the *Il-10* KO model.

MATERIALS AND METHODS

Details of reagents used and methods for gene expression are provided in the Supplementary Materials and Methods section (see Supplemental Digital Content 1, http://links.lww.com/IBD/A799).

pH Experiments

pH shift experiments were carried out in serum-free RPMI medium (1-41F24-I, Amimed), supplemented with 2 mM Glutamax (35050-038, Gibco), and 20 mM HEPES. The pH of all solutions was adjusted using a calibrated pH meter (Metrohm, Herisau, Switzerland) with NaOH or HCl, and the medium was equilibrated in a 5% CO_2 incubator for 36 hours. All data presented are referenced to pH measured at room temperature.

Culture of Cell Lines

The monocytic cell line MonoMac 6 (MM6, obtained from DMSZ) was cultured in RPMI (Sigma-Aldrich, Munich, Germany) supplemented with 10% fetal calf serum, 1% nonessential amino acids, and 1% oxalacetic acid–pyruvate–insulin medium supplement (Sigma-Aldrich), and maintained according to the American Type Culture Collection.

Patient Tissue Samples

Primary intact colonic epithelial cell crypts were isolated from normal human colonic tissue of patients undergoing bowel surgery as previously described.[27] Biopsies of human terminal ileum, colon, or rectum were taken from patients with CD or UC, or from control subjects undergoing colonoscopy for colon cancer screening. Biopsies from patients with colitis were taken endoscopically from inflamed areas. Written consent was obtained before specimen collection, and studies were approved by the local ethics committee.

Isolation of Human Peripheral Blood Monocytes

Normal human peripheral blood monocytes, obtained from the Swiss Red Cross Blood Service, were isolated from buffy coat samples, by density gradient centrifugation using Lymphoprep (Axis-Shield, Oslo, Norway). Purification was performed using EasySep Human Monocyte Enrichment Kit without CD16 Depletion and EasySep magnet (both from Stemcell, Vancouver, Canada) according to manufacturer's instructions. The purity of the monocytes was >85% as assessed by fluoroscein isothiocyanate–labeled anti-CD14 (557742, BD Biosciences, Allschwil, Switzerland) by flow cytometry (data not shown).

Animal Models

All animal experiments were performed according to Swiss animal welfare laws and were approved by the Veterinary Authority of Basel-Stadt and the Veterinary Office of the Canton Zürich, Switzerland. *Ogr*1$^{-/-}$ (C57BL/6) mice, initially obtained from Deltagen, Inc., San Mateo, CA, were generated as described.[28] *Il-10* $^{-/-}$ mice (C57BL/6) mice and *Ogr*1$^{-/-}$ mice were crossed to generate *Ogr*1 $^{-/-}$/*Il-10* $^{-/-}$ colitis susceptible mice. Mice were observed until reaching either 200 days of age or suffering a prolapse. All mice were housed together in 1 room in a vivarium.

Genomic DNA Extraction and Genotyping

Genotyping was confirmed by PCR of tail genomic DNA. DNA extraction was performed according to standard NaOH methods. The PCR reactions used for *Ogr1* genotyping were performed as previously described,[28] oligonucleotides used are listed in the supplementary Material and Methods (see Supplemental Digital Content 1, http://links.lww.com/IBD/A799).

Murine Macrophage Isolation and Culture

Mature quiescent macrophages were isolated from the mouse peritoneal cavity without the aid of eliciting agents, as

described by Zhang et al.[29] Animals were killed by cervical dislocation to reduce influence on pH. Additional details are described in the supplementary Methods (see Supplemental Digital Content 1, http://links.lww.com/IBD/A799).

Evaluation of Inflammation in Murine Colitis

Typical inflammatory parameters were evaluated as previously described.[30,31]

Expression Profiling by Microarrays

Global whole-transcript analysis was performed using a GeneAtlas microarray system (Affymetrix) to compare response differences between *Ogr1*$^{+/+}$ and *Ogr1*$^{-/-}$ murine macrophages after 24 hours acidic pH shift. Mature murine quiescent peritoneal macrophages were isolated as described above, from age-matched female *Ogr1*$^{-/-}$ and *Ogr1*$^{+/+}$ mice (C57BL/6). Five replicates or mice per condition were used, and approximately 1×10^6 macrophages per mouse obtained. Cells were not pooled. Cells were treated with pH 6.7 equilibrated medium to activate *Ogr1*, and pH 7.7 to serve as negative controls. Cells were collected, and RNA and cDNA samples were prepared. Biotin-labeled cDNA samples were hybridized to GeneChip Mouse Gene 1.1ST Array Strip (Affymetrix, P/N 901628) after protocols provided by Affymetrix. Data were summarized on gene-level using RMA (Robust Multiarray Average). Data quality was assessed using the bioconductor/R package "arrayQualityMetrics,"[32] and reproducibility was assessed using Pearson's correlation for all the filtered expression values and hierarchical clustering. For all pairwise comparisons, differentially expressed genes were selected using ≥2.0-fold change, $P < 0.05$ significance, as determined using analysis of variance (as implemented by the R package, Linear Models for Microarray Data, "limma") and F-test for the complete experimental design. The results were analyzed by global ranked fold change and using Metacore software for pathway enrichment.

Statistical Analysis

For murine prolapse ratio comparison studies, statistical differences between genotypes were calculated by chi-square test with Fisher's exact test (exact significance, two-sided) and risk estimate test from contingency tables. The prolapse survival analysis was performed using Kaplan–Meier survival analysis (log rank Mantel–Cox test) and estimated median survival time. Groups of data were compared using nonparametric Mann–Whitney U test (mouse data) or Kruskal–Wallis one-way analysis of variance followed by Dunn's multiple comparison test (patient data). Data are presented as mean ± SEM for a series of n experiments. Probabilities (P, two-tailed) of $P < 0.05$ were considered statistically significant. Monocyte/macrophage expression data were analyzed using a one-way analysis of variance followed by the Tukey's post hoc test. Throughout this article, asterisks denote significant differences at $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

RESULTS

OGR1 mRNA Expression Is Increased in Patients with IBD

OGR1 mRNA expression in isolated crypts and terminal ileum, colon, or rectum specimens from patients with IBD and control subjects was confirmed by RT-qPCR. Ct values from the isolated crypts from 4 patients ranged from 27 to 30, indicating moderate expression of *OGR1* in colonic epithelium (data not shown). Compared with normal control subjects, *OGR1* expression increased 2.3-fold ($P < 0.05$) in patients with UC (n = 8) and 2.2-fold ($P < 0.01$) in patients with CD (n = 29) (Fig. 1).

OGR1 Expression Is Regulated by TNF in Myeloid Cells

A local decrease in pH usually occurs at inflammatory sites, and monocytes are rapidly recruited, followed by an increase in proinflammatory cytokines. MM6 cells were treated with IFN-γ, IL-1β, IL-6, TNF, or TGF-β, which are known to initiate immune and inflammatory responses in the mucosa. Stimulation by TNF resulted in significant upregulation of *OGR1* expression (≈4- to 5-fold; $P < 0.001$, Fig. 2A–B). No induction of *OGR1* occurred by IFN-γ, IL-1β, IL-6, or TGF-β at 6 hours (Fig. 2A–B) or at 1 hour, 5 hours, or 24 hours (data not shown). A concentration-dependent (0, 2.5, 10, 25, 50, and 100 ng/mL) induction of *OGR1* mRNA expression in MM6 cells by TNF was confirmed at 4 and 8 hours (Fig. 2C). Maximal OGR1 induction, after 8 hours of treatment was reached at TNF concentration 50 ng/mL (Fig. 2C). Induction of *OGR1* expression in MM6 cells by TNF returned to basal levels after 48 hours (Fig. 2D).

Treatment of MM6 cells with PMA, a known PKC activator but also commonly used to differentiate monocytes into macrophage-like cells,[33] led to increased *OGR1* expression (14-fold at 24 h, $P < 0.001$) (Fig. 2E).

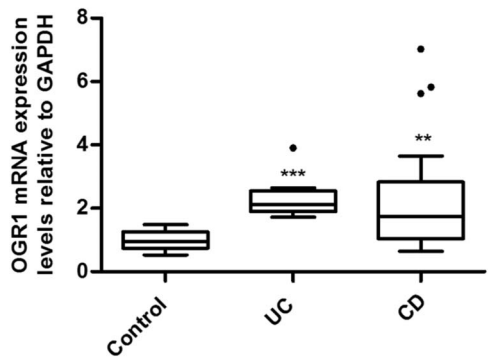

FIGURE 1. OGR1 expression in human intestinal mucosa; patients with IBD expressed higher levels of *OGR1* mRNA in the mucosa as compared with controls. Expression levels normalized to GAPDH. Biopsy specimens were taken from 29 patients with CD, 8 patients with UC, and 17 non-IBD control patients. Asterisks denote significant differences from the respective control ($**P < 0.01$, $***P < 0.001$).

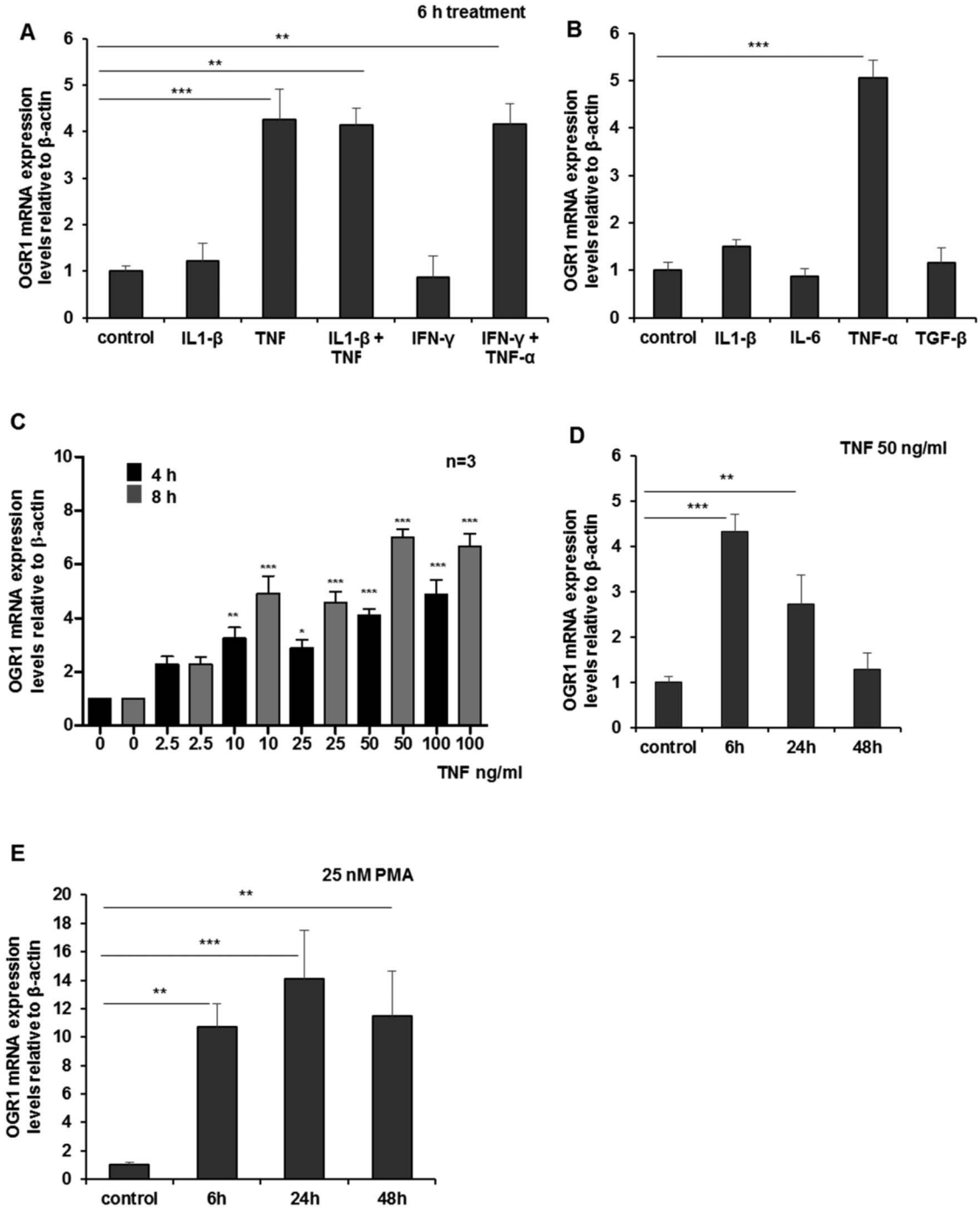

FIGURE 2. TNF and PMA induce *OGR1* expression in human monocytes; (A and B) MM6 cells were treated with cytokines for 6 hours. Treatment of MM6 cells with TNF led to significant upregulation of *OGR1*. No induction of *OGR1* occurred with other cytokines (IFN-γ, IL-1β, IL-6, and TGF-β) (50 ng/mL) tested. C, Concentration-dependent TNF (0, 2.5, 10, 25, 50, 100 ng/mL) induction of *OGR1* mRNA expression was confirmed at 4 and 8 hours. Maximal *OGR*1 induction was reached at TNF concentration 50 ng/mL at 8 hours. D, Induction of *OGR1* expression by TNF (50 ng/mL) returned to basal levels after 48 hours. E, Monocytic macrophagic differentiation of MM6 cells with PMA (25 nM), a specific activator of protein kinase C (PKC) and NF-κB, led to a significant increase in *OGR1* mRNA expression. Asterisks denote significant differences from the respective control (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Representative data of one of 3 qualitatively similar experiments shown unless indicated.

To confirm the relevance of our findings in MM6 cells, we tested OGR1-induction by TNF and PMA in primary human monocytes and mouse peritoneal macrophages. Concentration-dependent TNF and PMA induction of *OGR1* mRNA was confirmed in human monocytes (Fig. 3A–B). Similarly TNF-mediated OGR1 induction was observed in mouse macrophages (Fig. 4). The intensity of the response to TNF was comparable in MM6 cells and primary mouse macrophages (Fig. 2C–D [MM6]; Fig. 4). The induction time of primary human monocytes was considerably slower but of similar intensity. Similarly, primary human monocytes exhibited a slower induction and lower response to PMA compared with MM6 cells. No induction of the other pH-sensing GPCRs, *GPR4* and *TDAG8*, by any of the cytokines tested or by PMA was detected (data not shown).

TNF-, LPS-, or PMA-induced *OGR1* Expression Is Reversed by Akt, MAP, and PKC Kinase and NF-κB Inhibitors

To understand the pathways involved in TNF-, PMA-, or LPS-mediated induction of *OGR1* expression, we investigated the roles of Akt1/2 kinase, c-Jun N-terminal kinase (JNK), and PKC by using their specific inhibitors. Mitogen-activated protein kinases (MAPKs) play an important role in regulating the cellular response to various extracellular stimuli.[34] Signaling through PKC is known to activate MAPKs.[35] Activation occurs by sequential phosphorylation by JNK, extracellular signal-regulated kinase (ERK) 1/2, p38 MAPK, ERK5, and ERK3/4.[36] Activated MAPK kinase pathways may stimulate activator protein 1 (AP-1).[37,38] Exposure of monocytes and macrophages to TNF, LPS, and PMA results in activation of

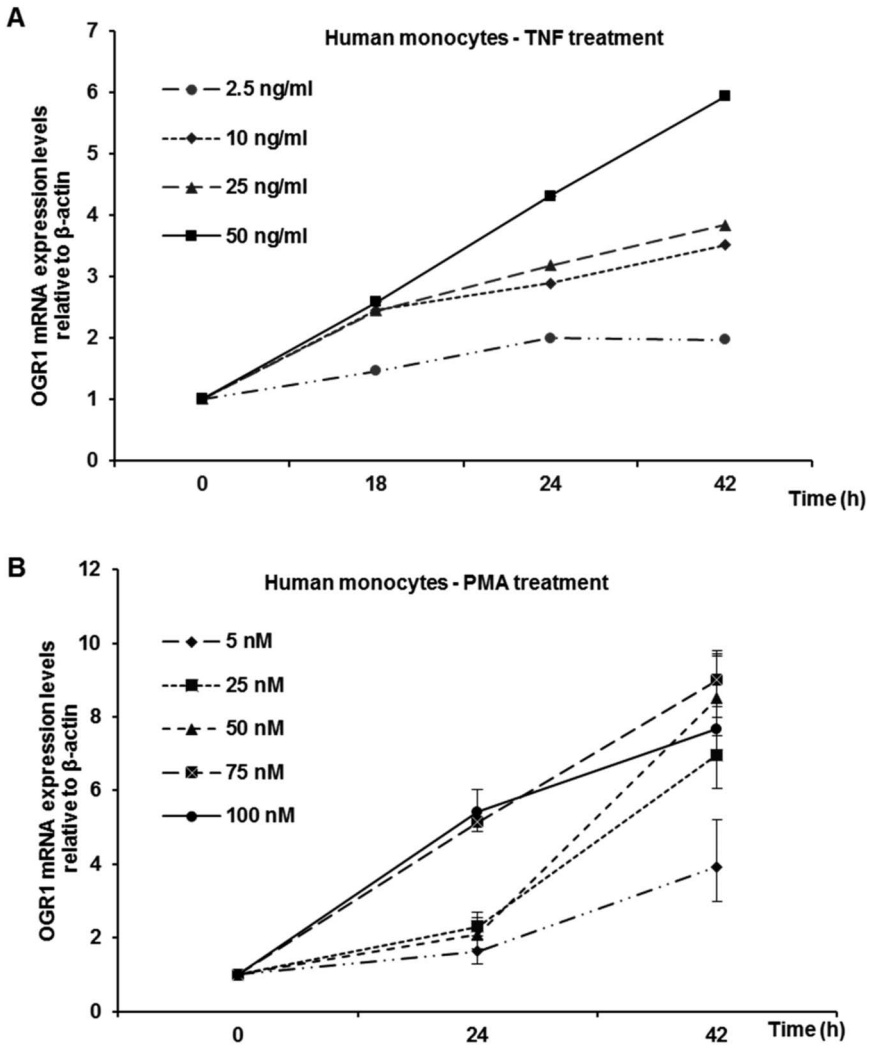

FIGURE 3. TNF- and PMA-dependent induction of OGR1 mRNA in primary human monocytes. A, Dose-dependence of TNF (0, 2.5, 10, 25, and 50 ng/mL) induction of *OGR1* mRNA was confirmed in primary human monocytes. B, PMA (0, 5, 25, 50, 75, and 100 ng/mL) induction of *OGR1* mRNA was confirmed in primary human monocytes. Representative data of one of 2 similar experiments shown.

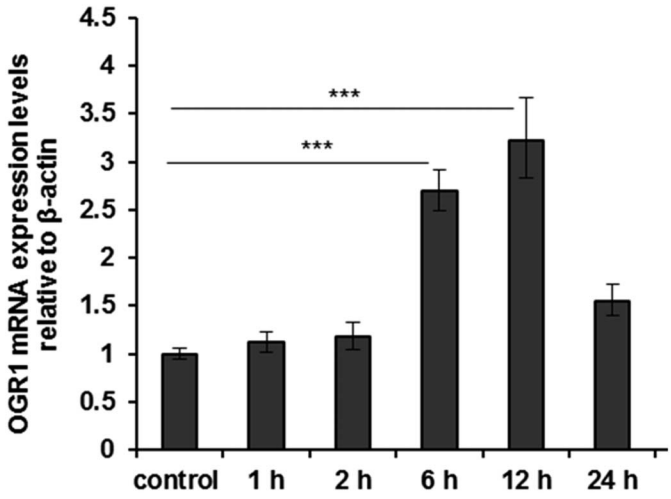

FIGURE 4. TNF induces OGR1 expression in murine macrophages. *OGR1* induction by TNF (25 ng/mL) was also confirmed in primary mouse residential peritoneal macrophages. Asterisks denote significant differences from the respective control (***$P < 0.001$). Representative data of one of 3 similar experiments shown.

the AP-1, NF-κB, caspase, and MAPK pathways.[39,40] Akt is a serine–threonine kinase and has been implicated in TNF-mediated activation of NF-κB.[41,42]

Based on our time course experiments (Fig. 2D), cells were stimulated with PMA, TNF, or LPS, in the presence of the appropriate kinase inhibitor, A6730 (9 μM), SP600125 (20 μM), and curcumin (25 μM), and harvested after 6 hours.

Exposure of MM6 cells to PMA, TNF, and LPS induced *OGR1* expression as 13.7-, 8.2-, and 10.3-fold, respectively. The Akt1/2 kinase inhibitor, A6730, significantly decreased TNF- and LPS-induced *OGR1* expression, by 5.6 (68%) and 8.2-fold (80%) respectively, but with less effect on PMA activation (4.8-fold decrease, 35% decrease) (n = 2, $P < 0.001$ or 0.01, Fig. 5A). SP600125, a JNK inhibitor, decreased *OGR1* induction by PMA 10.1-fold, (73%) TNF 4.0-fold (48%), and LPS 7.5-fold (80%). These results suggest that the JNK/AP1 pathway may be involved in *OGR1* regulation. Curcumin is a potent inhibitor of protein kinase C[43] and inhibits NF-κB activation through inhibition of IκB kinase and Akt activation.[44] Curcumin abolished the induction of all 3 activating agents (PMA, 12.7-fold [93%]; TNF, 7.2-fold [87%]; and LPS, 9.9-fold [96%] decrease). These preliminary kinase inhibitor studies suggest that Akt1/2, JNK, PKC, and IKK pathways play an important role in the induction of *OGR1* expression by PMA, TNF, and LPS.

Prompted by the results, we next tested a number of known NF-κB inhibitors. TNF-, PMA-, or LPS-mediated induction of *OGR1* was significantly reduced by simultaneous treatment of cells with NF-κB inhibitors: curcumin (25 μM), MG-132 (20 μM), AICAR (0.5 nM), BAY-11-7082 (20 μM), CAY10512 (0.3 μM), and SC-514 (25 μM) (Fig. 5A–C). In the presence of the inhibitor MG132, TNF induced *OGR1* expression decreased 3.2- and 5.7-fold, respectively (TNF, 95% decrease and PMA, 89% decrease) (n = 2, $P < 0.001$, Fig. 5B).

AICAR (5-aminoimidazole-4-carboxyamide) ribonucleoside blocks the expression of proinflammatory cytokines genes by a reduction in NF-κB DNA-binding activity.[45] BAY-11-7082 and SC-514 block NF-kB activation by inhibition of IκB kinase.[46,47] The resveratrol analog CAY10512 is a specific NF-κB inhibitor. Treatment with PMA, TNF, or LPS resulted in 10.2-, 3.0-, or 7.6-fold increase in *OGR1* expression, respectively, but in the presence of the NF-κB inhibitors induction of *OGR1* significantly decreased. *OGR1* expression decreased with inhibitor; AICAR 9.3-, 2.6-, and 7.3 -fold (91%, 88%, and 96%, respectively); BAY7082, 9.0-, 2.6-, and 6.1-fold (88%, 87%, and 81%, respectively); Cay10512, 5.6-, 1.8-, and 3.8-fold (56%, 62%, and 49%, respectively),; SC-514, 8.7-, 2.7-, and 7.4-fold (85%, 91%, and 97%, respectively) on PMA, TNF, and LPS stimulation, respectively (Fig. 5C). The results collectively suggest that NF-κB plays a key role in the regulation of *OGR1*.

In Silico Analysis of the *OGR1* Promoter

As the inhibitor studies suggested a strong role for AP-1[48] and NF-κB in the regulation of *OGR1* expression, we next performed an in silico promoter analysis of *OGR1*. Two alternative predicted promoter variants ≈9 kpb apart, exist for the *OGR1* gene on chromosome 14. In silico analysis using MatInspector software[49] (http://www.genomatix.de/matinspector.html) revealed several putative DNA-binding sites for AP-1, NF-κB, and HIF-1α within the proximal regions of the *OGR1* promoter variants. A schematic representation of these sites (TBSs) for *OGR1* variants 1 and 2 and binding sites are shown in Figures, Supplemental Digital Content 2 and 3, http://links.lww.com/IBD/A800 and http://links.lww.com/IBD/A801, respectively.

Cellular Responses Upon *OGR1* Activation by Extracellular Acidification in Murine Macrophages

We further investigated the effect of OGR1 deficiency on intestinal inflammation. We conducted a microarray study and compared the global gene expression of wild-type (WT) $Ogr1^{+/+}$ cells to $Ogr1^{-/-}$ cells in response to extracellular acidification. We selected the top 100 most differentially expressed genes by comparing the ranked fold change upon pH shift in WT compared with KO macrophages. Figure 6A shows a heat map summarizing gene expression across all samples in the 4 experimental conditions for these 100 genes.

Acid-induced *OGR1*-mediated differentially upregulated genes in WT macrophages compared with *OGR1* KO macrophages include inflammatory response genes (*Tnfrsf13c, Ccl24, Cxcl13, C1qa, Nr4a1*) and immune response genes (*Iglv1, Cd79a, H2-Eb1, Tinagl1, Lst1, C1qa, C1qb Cd83, Ccl17*). Furthermore, genes associated with adhesion and ECM (*Sparc, Cyr61, Timp1, Aebp1, Ebp1, Siglec1, Cdh2, Mmp11, Serpine2, Tgm2*), and actin cytoskeleton (*Inhba, Fscn1, Sorbs2, Tuba1c, Map1b, Parva, Cnn3*)

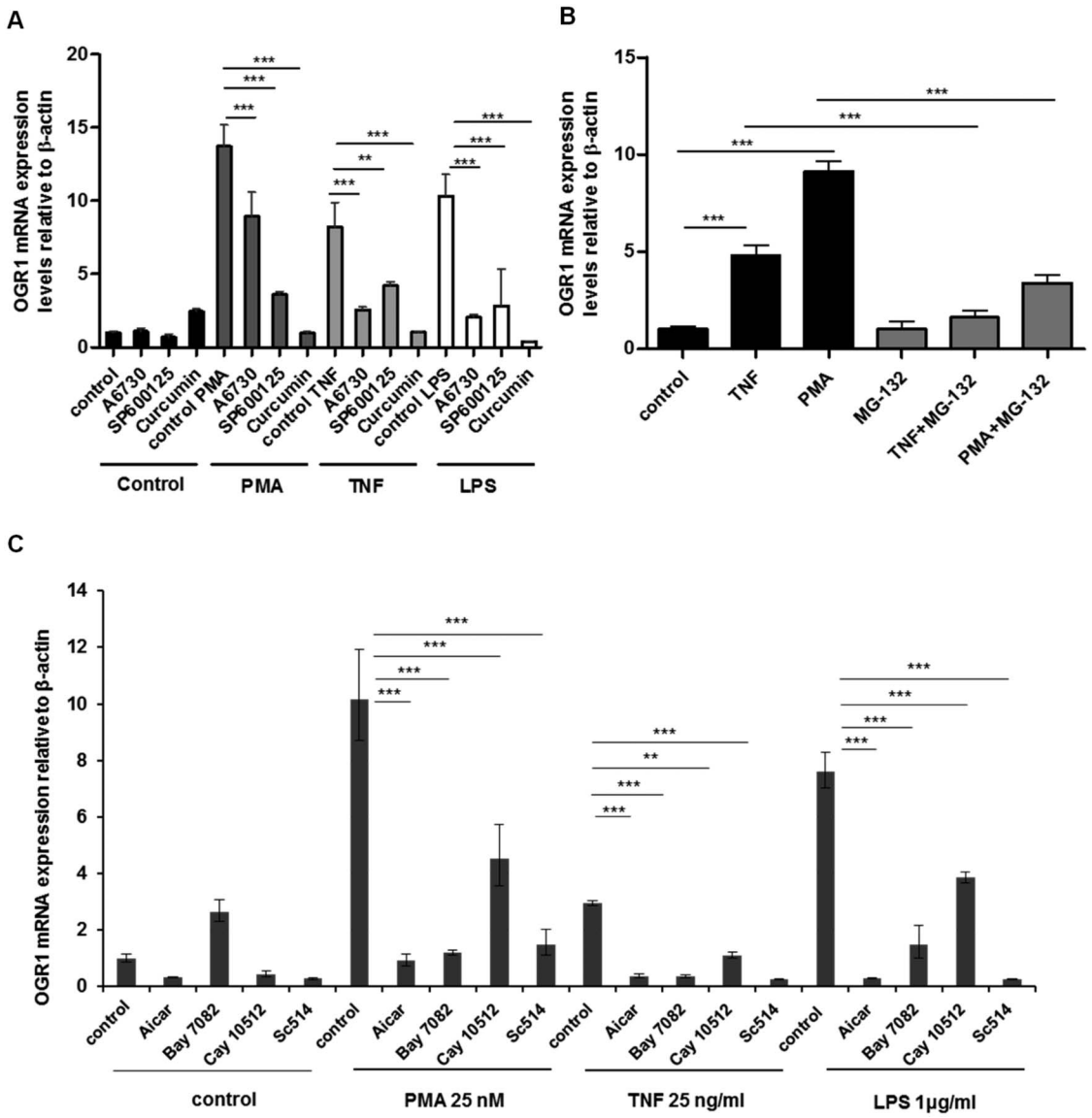

FIGURE 5. TNF-, PMA-, and LPS-mediated induction of OGR1 in MM6 cells was reversed by simultaneous treatment of cells with kinase and NF-kB inhibitors. A, Kinase inhibitors, A6730 (9 μM), SP600125 (20 μM), and curcumin (25 μM), reduced or abolished TNF- (25 ng/mL), PMA- (25 nM), or LPS-mediated (1 μg/mL) induction of *OGR1* in MM6 cells. B, Treatment with NF-kB inhibitor MG-132 (20 μM) significantly reduced TNF-mediated (50 ng/mL) or PMA-mediated (25 nM) induction of *OGR1* in MM6 cells. C, AICAR (0.5 nM), BAY-11-7082 (20 μM), CAY10512 (0.3 μM), and SC-514 (25 μM) also reduced TNF- (25 ng/mL), PMA- (25 nM), or LPS (1 μg/mL) mediated induction of *OGR1*. Asterisks denote significant differences from the respective control (**$P < 0.01$, ***$P < 0.001$). Representative data of one of 2 similar experiments shown.

were upregulated by acidic activation of *OGR1* in WT but not in *OGR1* KO macrophages. Interestingly, cholesterol homeostasis genes, (*Cyp11a1, Ephx2*), glucose response and insulin processing genes, (*Inhba, Cpe, Cma1, Igfbp7, Htra1, Sfrp4*), differentiation and bone development gene *Bmp-2* and transcription factor gene *Nrbf2* also increased in WT $Ogr1^{+/+}$ compared with $Ogr1^{-/-}$ KO cells at acidic pH.

The scatter plot represents the ratio fold change low to high pH *OGR1* KO/fold change low to high pH WT macrophages (Fig. 6B). The top 100 differentially expressed genes are shown in Table, Supplemental Digital Content 4, http://links.lww.com/IBD/A802. In addition, a list of genes and enrichment pathways, generated in GeneGo by comparison of pairs of WT low pH versus high pH to OGR1 KO low pH versus high pH, is shown in Table, Supplemental Digital Content 5, http://links.lww.com/IBD/A803 and Figure, Supplemental Digital Content 6, http://links.lww.com/IBD/A804, respectively. Results discussed in this article have been deposited in the NCBI Gene Expression Omnibus Accession number GSE60295 (http://www.ncbi.nlm.nih.gov/projects/geo).

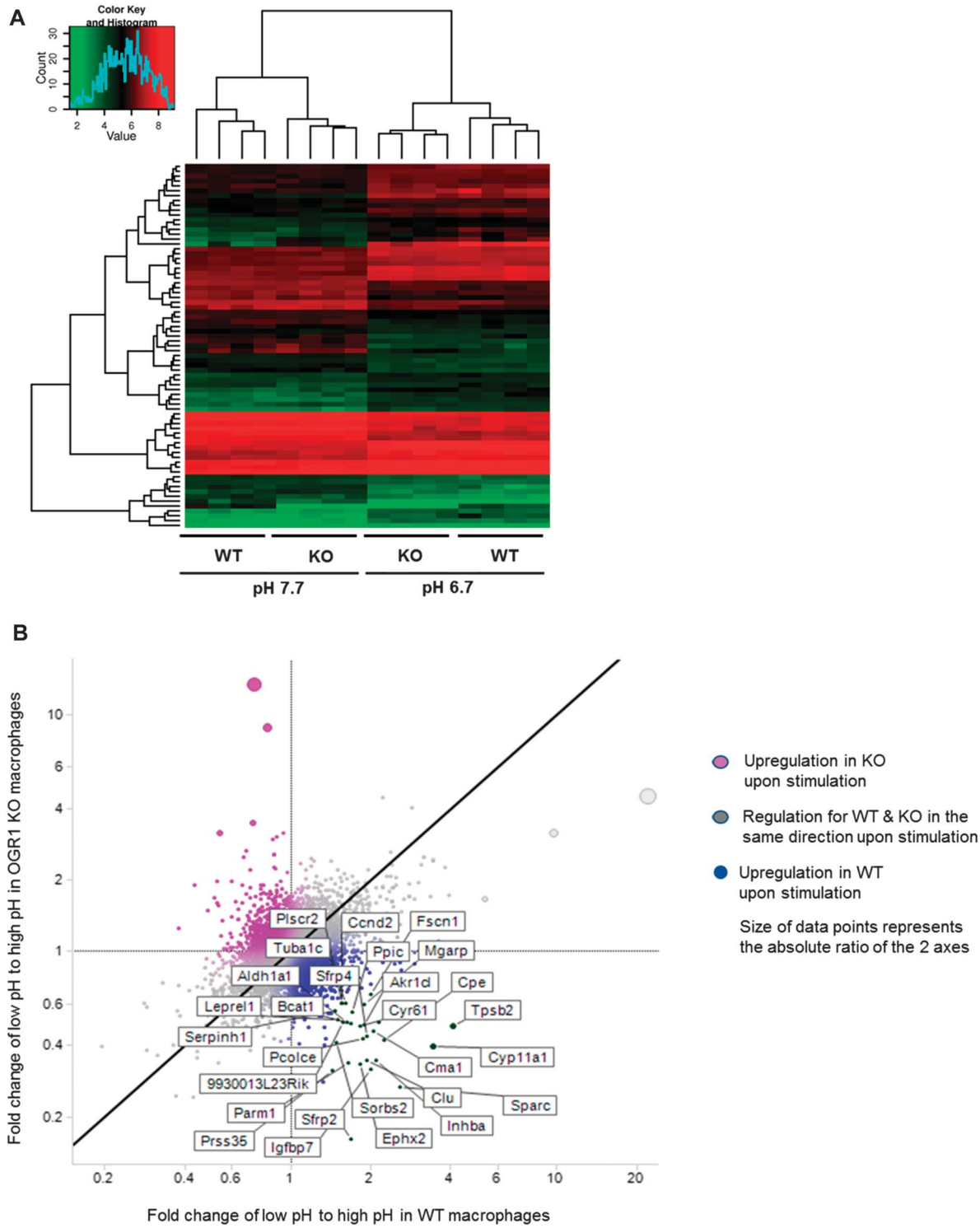

FIGURE 6. Global gene expression of acid response of mouse macrophages. A, Top 100 genes from the whole-transcript microarray analysis of acid response (pH 6.7) of WT and Ogr1 KO murine macrophages. Control condition, pH 7.7 is shown on the left. Changes in gene expression within each comparison are represented as $\log_2$-transformed fold changes (≥2.0-absolute fold-change, $P < 0.05$ significance). B, Differentially expressed genes for acid response in WT and *OGR1* KO macrophages. Fold changes in low to high pH shift of *OGR1* KO macrophages are depicted on y-axis and fold changes low to high pH WT macrophages are shown on the x-axis. The highest ranking differentially expressed genes in the acid response of WT mouse macrophages are shown in the lower right quadrant of the scatter plot, and the upper left quadrant shows the highest ranking differentially expressed genes in the acid response of *OGR1* KO mouse macrophages.

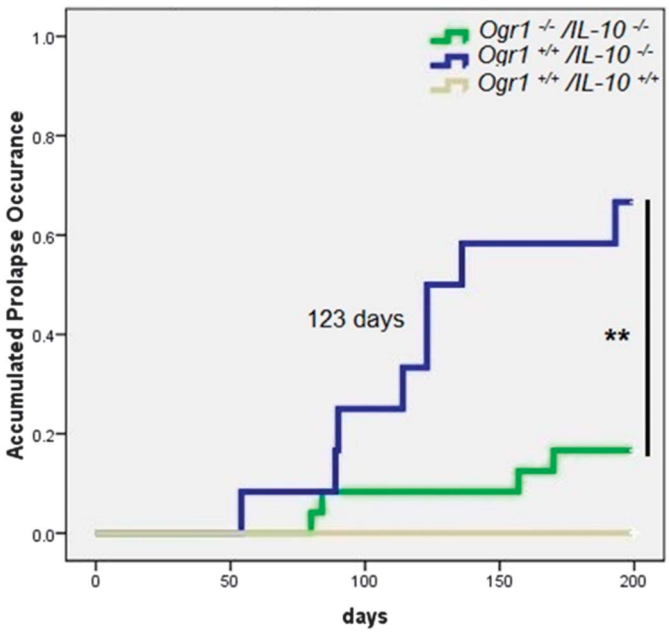

FIGURE 7. *OGR1*-deficient mice show delayed onset and severity of prolapse in a spontaneous *IL-10* KO mouse model. Kaplan–Meier survival analysis showed a significantly delayed onset and progression of rectal prolapse in female $Ogr1^{-/-}/Il\text{-}10^{-/-}$ *mice* (estimated median survival time: >200 days versus 123 days, $^{**}P = 0.002$, log rank [Mantel–Cox] test). Green solid lines, $Ogr1^{-/-}/Il\text{-}10^{-/-}$ mice (16.7% prolapses, n = 24); blue solid line, $Ogr1^{+/+}/Il\text{-}10^{-/-}$ mice (66.7% prolapses, n = 12); gray solid lines, $Ogr1^{+/+}/Il\text{-}10^{+/+}$ mice (0% prolapses, n = 31). No rectal prolapses were detected in any of the $Ogr1^{+/+}/Il\text{-}10^{+/+}$ mice in the breeding colony in the study, 200 days.

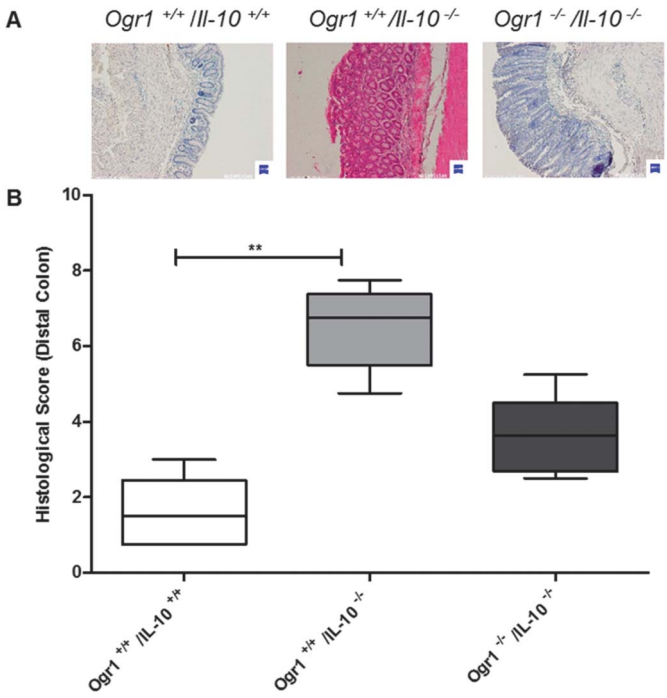

FIGURE 8. *OGR1*-deficient mice exhibit a trend to less inflammation in a spontaneous *IL-10* KO mouse model. A, Microscopic analysis of terminal colon sections from $Ogr1^{-/-}/Il\text{-}10^{-/-}$, $Ogr1^{+/+}/Il\text{-}10^{-/-}$, and $Ogr1^{+/+}/Il\text{-}10^{+/+}$ 80-day-old mice, staining by Hematoxylin and eosin. Representative images are shown. B, Histological score, based on evaluation of morphological changes of epithelium and immune cell infiltration, of distal colon from $Ogr1^{-/-}/Il\text{-}10^{-/-}$, $Ogr1^{+/+}/Il\text{-}10^{-/-}$ and $Ogr1^{+/+}/Il\text{-}10^{+/+}$ 80-day-old mice. Data presented as mean ± SEM; n ≥5 per group; Asterisks denote significant differences from the respective control ($^{**}P < 0.01$). All mice were female.

OGR1 Deficiency Protects from Development of Spontaneous Colitis in Mice

To investigate whether the *OGR1*-dependent changes upon acidification have functional consequences during IBD, we applied a mouse model of spontaneous colitis. Analysis of the occurrence of prolapse in the colon over the course of 200 days showed that only 16.7% of female $Ogr1^{-/-}/Il\text{-}10^{-/-}$ mice (n = 24) developed rectal prolapse. The incidence was significantly lower than that of female $Ogr1^{+/+}/Il\text{-}10^{-/-}$ littermate mice (66.7%, n = 12) maintained in the same vivarium room during the same time period ($^{**}P = 0.007$; odds ratio for female mice $Ogr1^{-/-}/Ogr1^{+/+}$ = 0.100 [95% confidence limit, 0.020–0.500], chi-square test with Fisher's exact test with 2 sides). Kaplan–Meier survival analysis showed a significantly delayed onset and progression of rectal prolapse in female $Ogr1^{-/-}/Il\text{-}10^{-/-}$ mice (estimated median survival time: >200 days versus 123 days for female $Ogr1^{+/+}/Il\text{-}10^{-/-}$ mice, $^{**}P = 0.002$, log rank [Mantel–Cox] test) (Fig. 7). No prolapses were observed in control $Ogr1^{+/+}/Il\text{-}10^{+/+}$ mice (n > 100 animals).

Histologically, consistent with the prolapse ratios, $Ogr1^{-/-}/Il\text{-}10^{-/-}$ mice showed less inflammation (score 3.7 ± 1.03) (Fig. 8A); however, this difference did not reach statistical significance from $Ogr1^{+/+}/Il\text{-}10^{-/-}$ female mice (6.5 ± 1.12) ($P > 0.05$, Fig. 8B). The same trend in MPO levels of female $Ogr1^{-/-}/Il10^{-/-}$ mice was observed (0.12 ± 0.034 versus 0.41 ± 0.072, $P > 0.05$, Fig. 9A). There were no differences in colon length, relative spleen weight (Fig. 9B–C), and in cytokines mRNA expression levels (Fig. 10).

DISCUSSION

Our article provides evidence for a role of the pH-sensing receptor *OGR1*, in inflammatory processes such as intestinal inflammation. We show that *OGR1* mRNA expression is upregulated ≈2-fold during intestinal inflammation in patients with IBD. To what extent this translates into upregulated protein expression cannot currently be assessed because of a lack of suitable antibodies. We further show that the proinflammatory cytokine TNF, a major mediator in IBD-associated inflammation, induces *OGR1* expression in human and murine myeloid cells. TNF upregulates *OGR1* expression for short periods (6–12 h); however, the effect is not sustained for longer periods, after 24 to 48 hours *OGR1* expression returns to basal levels.

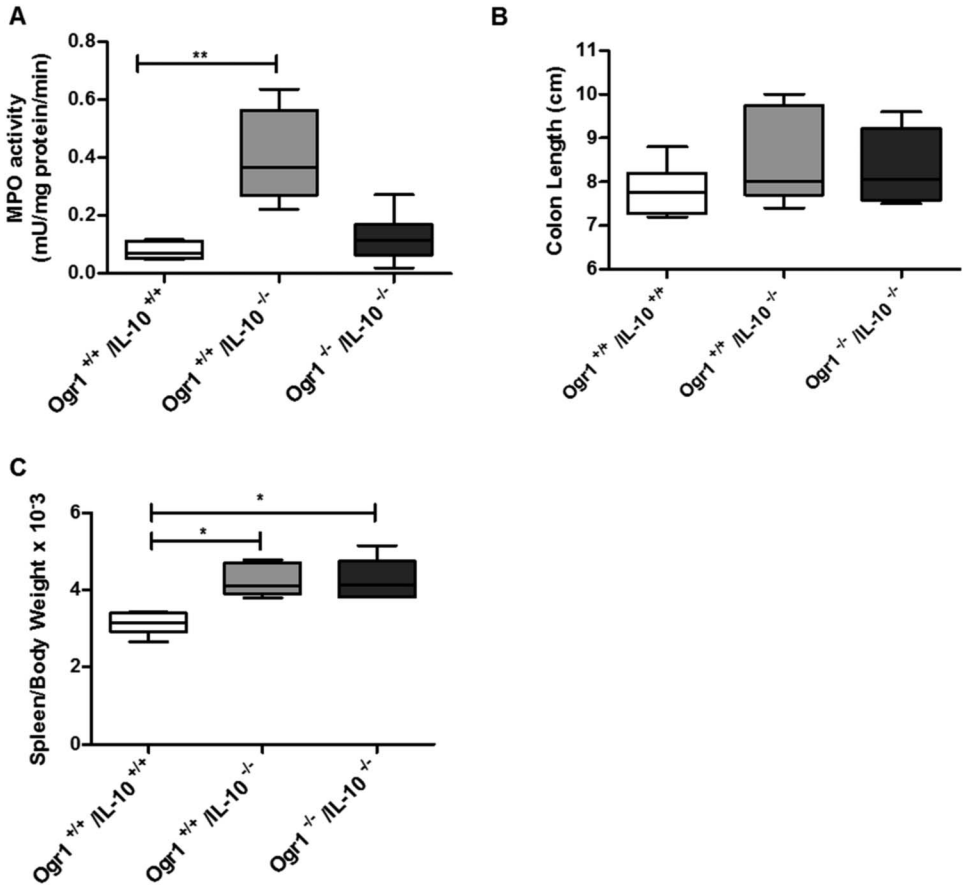

FIGURE 9. The development of IBD and progression of prolapse between *Ogr1*$^{-/-}$/*IL-10*$^{-/-}$and *Ogr1*$^{+/+}$/*Il-10*$^{-/-}$ *female* mice. A, Comparison of MPO activity in colon tissue (B). Assessment of colon length. C, Relative spleen weight. Asterisks denote significant differences from the respective control (*$P < 0.05$, **$P < 0.01$).

Similar to our findings, Lum et al[50] reported that expression of *GPR4*, a related proton-sensing GPCR, is upregulated several-fold by TNF and H_2O_2 in human brain microvascular endothelial cells. TNF-mediated induction of *GPR4* occurred after 2 hours and was sustained for 24 hours However, in contrast to OGR1, we did not observe induction of *GPR4* and *TDAG8* expression upon treatment with TNF, PMA, or LPS in MM6 cells. *OGR1* expression induced by TNF, PMA, or LPS was prevented by treatment with PI-3 (Akt1/2), MAP, and PKC inhibitors and with NF-κB inhibitors AICAR, BAY-11-7082, CAY10512, and SC-514. LPS stimulates production of TNF in MM6 cells.[51,52]

We further show that genetic deletion of *OGR1* ameliorates inflammation at least in female mice. Acidification and signaling through *OGR1* induced a multitude of cellular responses in the microarray analysis. In murine macrophages, acid-induced *OGR1*-mediated enriched upregulated genes are involved in inflammatory responses, further supporting our finding that *OGR1* signaling upon pH changes may play an important role in mucosal inflammation. Notably, upregulation of nuclear receptor subfamily 4 group A member 1 (NR4A1, also known as NUR77) was detected (see Table, Supplemental Digital Content 5, http://links.lww.com/IBD/A803). NR4A1 functions as an immediate early-response gene and plays a key role in mediating inflammatory responses in macrophages.[53] Additionally affected pathways are actin cytoskeleton modulation and cell adhesion. This may also be relevant as antiadhesion strategies for the treatment of IBD have been recently successful and vedolizumab as an antibody against α4β7 integrin recently has been approved for therapy of Crohn's disease by the FDA and EMA. In an *OGR1*-overexpressing Caco2 model, we also observed enrichment of inflammatory response, including NR4A1, actin cytoskeleton, and adhesion and ECM genes upon acidification (de Vallière, Solange Vidal, Ieuan Clay, et al, unpublished data, 2015). In this study, the genes Inhba and Nr4a1, which are linked to the myocardin-related transcription factor pathway,[54] were also found to be strongly regulated by pH change.

Another strongly regulated gene was activin. Activin A is released early in the cascade of circulatory cytokines during systemic inflammatory episodes, roughly coincident with TNF and before IL-6 and follistatin are elicited. Activin A protein is also elevated in patients with IBD and in experimental colitis.[55] Recently, activin A was identified to regulate macrophage switch between polarization states.[56] This skew towards

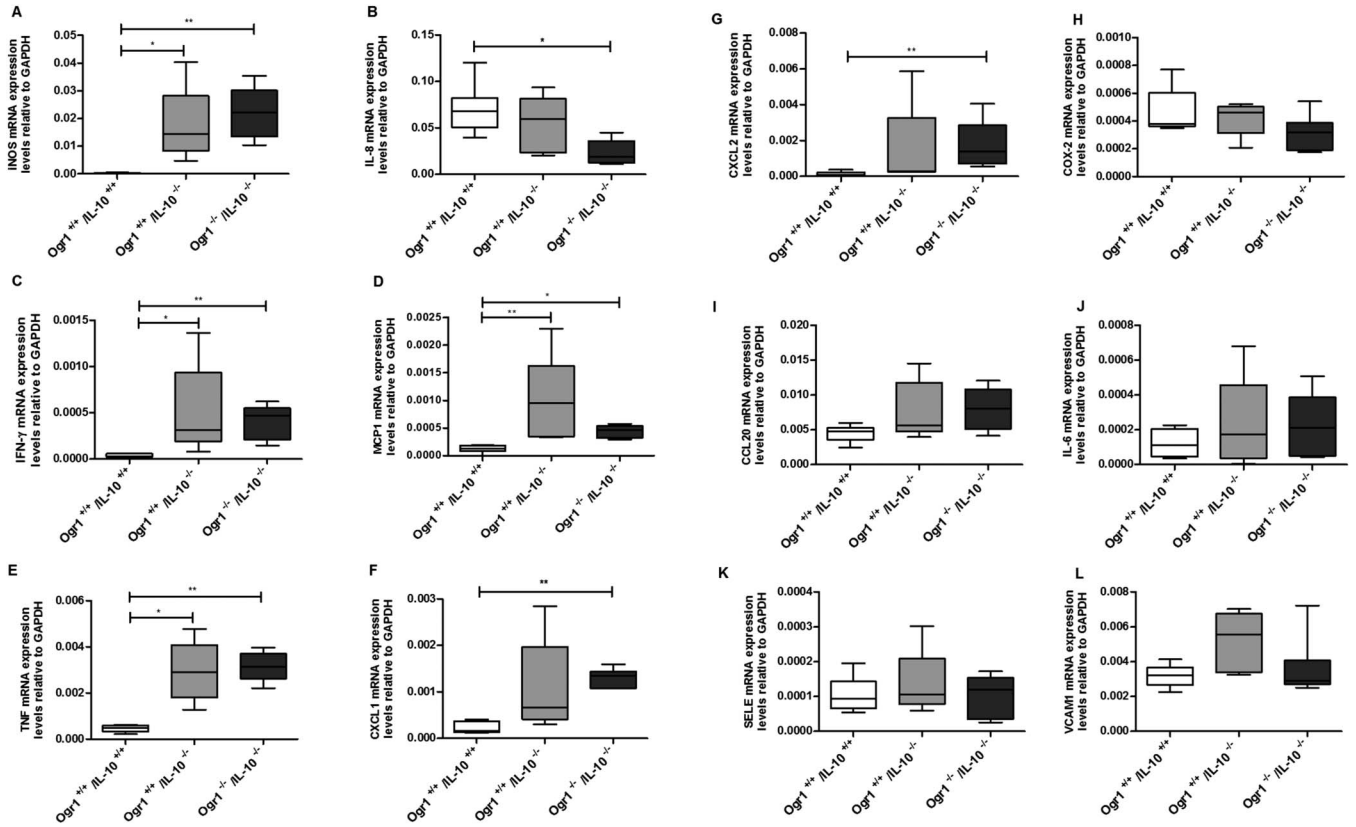

FIGURE 10. Expression levels of cytokines in colons of female *Ogr1*$^{-/-}$/*Il-10*$^{-/-}$, *Ogr1*$^{+/+}$/*Il-10*$^{-/-}$, and *Ogr1*$^{+/+}$/*Il-10*$^{+/+}$ (WT control) mice were determined by real-time PCR and normalized to GAPDH. (n = 6–9 mice per group). The homogenate of each mouse colon sample was tested in triplicate. Data presented as mean ± SEM; Asterisks denote significant differences from the respective control (*$P < 0.05$, **$P < 0.01$). No statistical difference between colon and mesenteric lymph nodes of female *Ogr1*$^{-/-}$/*Il-10*$^{-/-}$ mice and female *Ogr1*$^{+/+}$/*Il-10*$^{-/-}$ mice was observed ($P > 0.05$, Kruskal–Wallis one-way analysis of variance followed by Dunn's multiple comparison test).

a proinflammatory phenotype occurs by promoting the expression of M1 (GM-CSF) markers, and impairing the acquisition of M2 (M-CSF) markers, while downregulating the production of *Il-10*.[56] Furthermore, *SPARC* (secreted protein acidic and rich in cysteine) was found to be strongly regulated by *OGR1*. *SPARC* is a gene whose methylation has been related to IBD.[57,58] *SPARC* exacerbates colonic inflammatory symptoms in DSS-induced murine colitis. Compared with WT, *SPARC* KO mice had less inflammation with fewer inflammatory cells and more regulatory T cells.[59]

Why would pH-sensing be so important during intestinal inflammation? First, pH homeostasis is important for the maintenance of normal cell function. pH is normally tightly controlled within a narrow range. Normal pH of blood and tissue is controlled at ≈pH 7.2 to 7.4. Maintaining homeostasis requires cells to sense their external environment, communicate with each other, and respond rapidly to extracellular signals. This can be achieved by hydrophobic molecules, ion channels, catalytic receptor, and G protein-coupled receptors.[60]

Under physiological conditions, there are also counterplayers of *OGR1* expressed in the mucosal tissue. *TDAG8* mediates extracellular proton-induced inhibition of proinflammatory cytokine production in mouse macrophages.[2] Onozawa et al[23] showed that *TDAG8*-deficient mice exhibit enhanced arthritic symptoms compared with WT animals; suggesting that *TDAG8* attenuates inflammation by negatively regulating the function of the macrophages, T cells, and B cells. In search for genetic components and causal genetic variants of IBD, genome-wide association studies have identified numerous susceptibility regions that are marked by single nucleotide polymorphisms.[22,61,62] Association results and in silico analysis identified a locus within the *TDAG8* gene as susceptibility locus in CD,[22] supporting that *TDAG8* acts as a negative regulator of inflammation. Khor et al[63] propose that the presence of *TDAG8* as IBD-risk loci is necessary to maintain intestinal homeostasis due to its immune modulatory effect.

To summarize, ORG1 expression is induced in human and murine myeloid cells by TNF, PMA, and LPS, whereby simultaneous treatment with NF-κB inhibitors caused a reversal of this effect. Upregulated genes induced by extracellular low pH by proton-sensing OGR1 in murine macrophages were enriched for inflammatory and immune response, actin cytoskeleton, and cell-adhesion gene sets. The deficiency of pH-sensing receptor

OGR1 protects from spontaneous inflammation in the *Il-10* KO model. Thus, pH sensors may be interesting new targets for pharmacological intervention in intestinal inflammation.

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Author contributions: C. de Vallière performed experiments, analyzed the data and wrote the first draft of the manuscript; Y. Wang, S. Vidal, I. Clay, M. R. Spalinger, I. Tcymbarevich, A. Terhalle, M.-G. Ludwig, T. Suply, performed experiments and were involved in data analysis, M. Fried, G. A. Kullak-Ublick, I. Frew-Wagner, M. Scharl, J. J. Eloranta, K. Seuwen, C. A. Wagner, G. Rogler conceived, designed and supervised the study and respective experiments. All authors wrote, corrected and approved the manuscript.

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