

Vanin-1 licenses inflammatory mediator production by gut epithelial cells and controls colitis by antagonizing peroxisome proliferator-activated receptor γ activity

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Colitis involves immune cell-mediated tissue injuries, but the contribution of epithelial cells remains largely unclear. Vanin-1 is an epithelial ectoenzyme with a pantetheinase activity that provides cysteamine/cystamine to tissue. Using the 2,4,6-trinitrobenzene sulfonic acid (TNBS)-colitis model we show here that Vanin-1 deficiency protects from colitis. This protection is reversible by administration of cystamine or bisphenol A diglycidyl ether, a peroxisome proliferator-activated receptor (PPAR) γ antagonist. We further demonstrate that Vanin-1, by antagonizing PPAR γ , licenses the production of inflammatory mediators by intestinal epithelial cells. We propose that Vanin-1 is an epithelial sensor of stress that exerts a dominant control over innate immune responses in tissue. Thus, the Vanin-1/pantetheinase activity might be a new target for therapeutic intervention in inflammatory bowel disease.

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Abbreviations used: ATRA, all trans-retinoic acid; BADGE, bisphenol A diglycidyl ether; COX, cyclooxygenase; 15-d-PGJ₂, 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂; GSH, glutathione; IBD, inflammatory bowel disease; IEC, intestinal epithelial cell; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; PPAR, peroxisome proliferator-activated receptor; SCID, severe combined immunodeficient; TNBS, 2,4,6-trinitrobenzene sulfonic acid.

Inflammatory bowel disease (IBD) is a chronic idiopathic inflammation of the gastrointestinal tract that manifests as ulcerative colitis and Crohn's disease in human. These pathologies exemplify the rupture of equilibrium between bacterial-driven inflammation and tolerogenic gut immunity (1–3). In healthy individuals, the intestinal mucosa is in a state of controlled inflammation and its homeostasis depends upon intimate contacts between innate immune and epithelial cells. Dendritic cells and macrophages participate in the early detection of inflammatory stress and trigger destructive responses associated with colitis. However, because of their frontline position, intestinal epithelial cells (IECs) likely play a key role in stress sensing. They are known to express Toll-like receptors

and MHC molecules and to secrete antibacterial peptides and chemokines upon activation. It is therefore important to evaluate the hierarchical contribution of epithelial cells to stress sensing and triggering of gut inflammation.

Vanin-1 is an ectoenzyme anchored at the surface of epithelial cells by a glycosylphosphatidyl inositol moiety (4, 5). Its pantetheinase activity is involved in the metabolic pathway of pantothenate (vitamin B5) and is a main provider of cysteamine to tissues (5). In Vanin-1-deficient mice, the lack of detectable tissue cysteamine is associated with an enhanced γ -glutamylcysteine synthetase activity leading to elevated endogenous glutathione (GSH) stores in tissues. This confers on Vanin-1^{-/-} animals a better resistance to oxidative stress exposure that can be abolished by administration of cystamine (the disulfide form of cysteamine) (6, 7). As a consequence, Vanin-1^{-/-} mice display a down-regulated tissue inflammation in response to systemic oxidative stress. In gut, oxidative

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stress can be generated by acute exposure to nonsteroidal anti-inflammatory drugs or chronic *Schistosoma mansoni* infection. In both situations, Vanin-1^{-/-} mice were shown to control the resulting inflammation and ensuing hemorrhages that normally provoke death (7). Collectively, these observations pointed to a proinflammatory role of Vanin-1, which may be involved in the development of IBDs. These pathologies result from an excessive immune response to gut-derived infectious stimuli in a context of unbalanced immune tolerance. Therefore, we investigated an experimental model of colitis after intrarectal administration of 2,4,6-trinitrobenzene sulfonic acid (TNBS) (8). This model has been widely used to dissect some of the molecules and the cellular mechanisms involved in acute or chronic colitis. Among them, the peroxisome proliferator-activated receptor (PPAR) γ was identified as a checkpoint in the control of antiinflammatory responses and gut homeostasis (9, 10). Indeed, PPAR γ ^{+/-} heterozygous mice exhibit increased susceptibility to experimental TNBS-colitis, and in human, ulcerative colitis patients display an impaired expression of PPAR γ in colonic epithelial cells (11, 12). In addition, ligand activation of PPAR γ was shown to reduce the severity of colitis in mouse models, and it was recently demonstrated that the positive clinical effect of 5-aminosalicylic acid in the treatment of IBD patients was linked to PPAR γ activation (13, 14). PPAR γ is a member of the steroid receptor family with various biological functions including differentiation, apoptosis, lipid metabolism, and antiinflammatory properties (15, 16). Ligands for PPAR γ include few identified natural compounds like poly-

unsaturated fatty acids, some eicosanoids, the 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (15-d-PGJ₂), and certain drugs like the thiazolidinedione (17–20). After activation, PPAR γ forms heterodimer with the retinoic X receptor and the resulting complex binds PPAR-responsive elements (PPREs) within target gene promoters (15). Here we show that Vanin-1 modulates both the expression and the ligand-induced activation of PPAR γ in epithelial cells, therefore, regulating the expression of downstream proinflammatory target genes. Control of PPAR γ gene expression and function explains why Vanin-1 mutant mice better resist to colitis. These studies further show the importance of Vanin-1-dependent epithelium-derived signals in the triggering of colitis.

RESULTS

Vanin-1^{-/-} mice resist colitis-causing death: an epithelial-based mechanism

Mice were subjected to TNBS-induced colitis which provoked a rapid weight loss and death of 80% control BALB/c mice within 10 d (Fig. 1, A and B). In contrast, 70% of the Vanin-1^{-/-} mice survived during the same period and did not show considerable weight loss. Since Vanin-1 controls the production of cysteamine *in vivo*, we administered cysteamine (120 mg/kg) 2 d before TNBS injection and daily until death or sacrifice. This treatment completely reversed the protection conferred by the lack of Vanin-1 toward colitis since 90% of cysteamine-treated Vanin-1^{-/-} mice died within 5 d (Fig. 1 A). Thus, cysteamine restores the WT-like susceptibility to TNBS-induced colitis in Vanin-1^{-/-} mice.

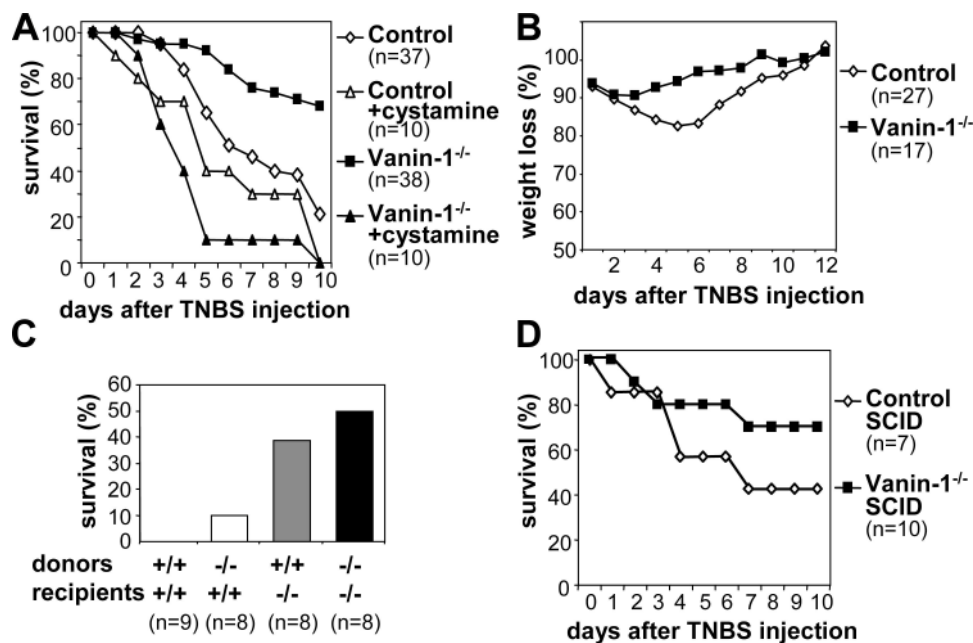


Figure 1. Protection of Vanin-1^{-/-} mice from colitis-causing death: an epithelial-based mechanism. (A) Survival rates of untreated or cysteamine-treated BALB/c control or Vanin-1^{-/-} mice after administration of TNBS. The number of mice is indicated. (B) Comparative weight loss

during TNBS-colitis between control and Vanin-1^{-/-} mice. (C) Survival at day 5 of bone marrow chimeric mice after induction of colitis. The genotype of donors and recipients is indicated. (D) Survival rates of immunodeficient BALB/c SCID and Vanin-1^{-/-} SCID mice to TNBS-induced colitis.

Under normal conditions, Vanin-1 is predominantly expressed by epithelial but not hematopoietic cells. To definitively establish the major contribution of epithelial cells in the control of colitis, we generated chimeras by reconstituting BALB/c control or Vanin-1^{-/-} irradiated mice with WT or Vanin-1^{-/-} Thy1-depleted bone marrows. These chimeric animals were subsequently submitted to TNBS-induced colitis and their survival was checked over 5 d. As shown in Fig. 1 C, Vanin-1^{-/-} recipients displayed a considerably reduced mortality rate independently of the genotype of the grafted bone marrow. Indeed, ~40–50% Vanin-1^{-/-} versus 0–10% control mice survived. Because the differential sensitivity to colitis might reflect the impact of the Vanin-1 mutation on the development of gut lymphocytes, we introduced the Vanin-1 mutation on the severe combined immunodeficient (SCID) mouse background. As shown in Fig. 1 D, although the lack of lymphocytes was associated with a reduced mortality, Vanin-1-deficient SCID BALB/c mice better resisted TNBS-colitis compared with Vanin-1^{+/+} SCID controls.

Thus, the protection conferred by the absence of Vanin-1 against lethal TNBS-colitis depends on a primordial epithelial-based response. These results document the preponderant role played by epithelial cells in the control of the severity of the colitis.

Lack of Vanin-1 is associated with mild tissue damage after colitis induction

The colon of mice was examined 2 d after TNBS injection, and the severity of clinical signs of colitis was evaluated macroscopically using the Wallace score. As shown in Fig. 2 A, control mice had severe macroscopic lesions characterized by numerous hemorrhagic and necrotic zones widely distributed

along the whole colon and extending into the cecum corresponding to a Wallace score of 8 (Fig. 2 B). In contrast, Vanin-1^{-/-} colons remained relatively healthy (Wallace score ~ 4).

Histologically, TNBS-treated WT colons were characterized by a severely disrupted mucosa with large areas of ulceration and extensive necrosis sparing few glands, subepithelial edema, and dense inflammatory infiltrate (Fig. 2 C). In contrast, the improved survival of Vanin-1^{-/-} mice was correlated to considerably less alterations of the colon with preserved mucosa integrity, reduced submucosal edema, and poorer leukocyte infiltration. This was quantified on several TNBS-treated animals using the Ameho score and represented in Fig. 2 D. As expected, cystamine-treated Vanin-1^{-/-} mice showed similar alterations to that observed in control animals (Fig. 2). These results demonstrate that Vanin-1 deficiency in the epithelial compartment prevents massive tissue infiltration by inflammatory cells and consequently the development of colonic lesions.

Vanin-1 controls the inflammatory response in injured colon

To further document the impact of Vanin-1 deficiency on colitis we scored the production of inflammatory effectors in colon of mice killed 2 d after TNBS injection. Using a cytokine array (Fig. 3, A and B), we observed the induction of a particular set of cytokines and chemokines in the colons of WT mice compatible with a major inflammatory reaction. This set includes the proinflammatory cytokine IL-6 tissue inhibitor of metalloproteinase-1, involved in tissue remodeling and wound healing, the neutrophil chemoattractant macrophage inflammatory protein (MIP)-2 and keratinocyte chemoattractant, and MIP-1 α and monocyte chemoattractant

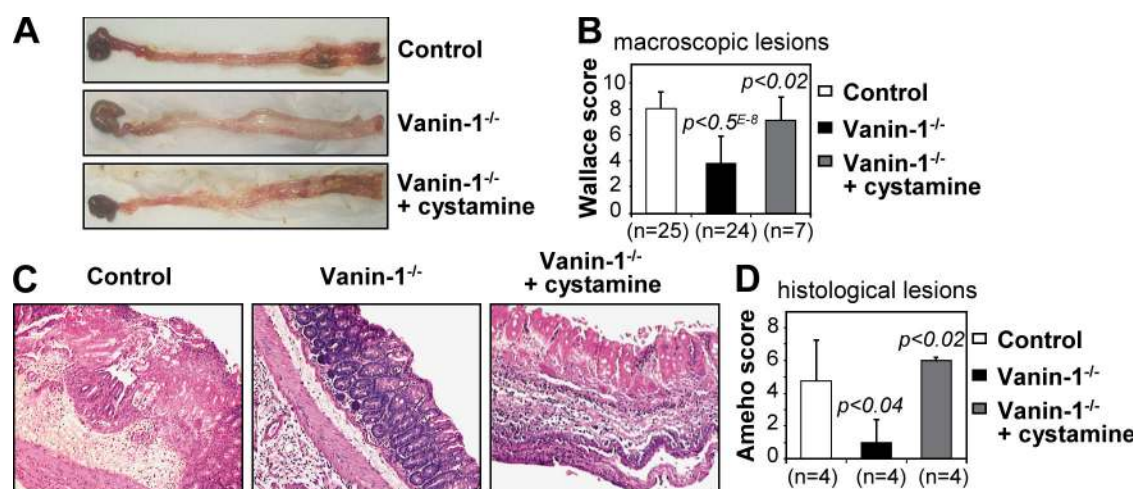


Figure 2. Reduced tissue damage in Vanin-1^{-/-} colons after colitis induction: cystamine restores the susceptibility to TNBS-driven colitis in Vanin-1^{-/-} mice. (A) Morphologic examination of colons of TNBS-treated BALB/c control mice and Vanin-1^{-/-} animals receiving or not receiving a treatment with cystamine. (B) The severity of the colon injury

was evaluated using the macroscopic Wallace score. (C) Representative histological sections of colon tissues of TNBS-treated control, Vanin-1^{-/-}, and cystamine-treated Vanin-1^{-/-} mice. (D) The severity of the lesions was evaluated using the histological Ameho score. The number of mice is indicated. All of these analyses were performed 2 d after TNBS administration.

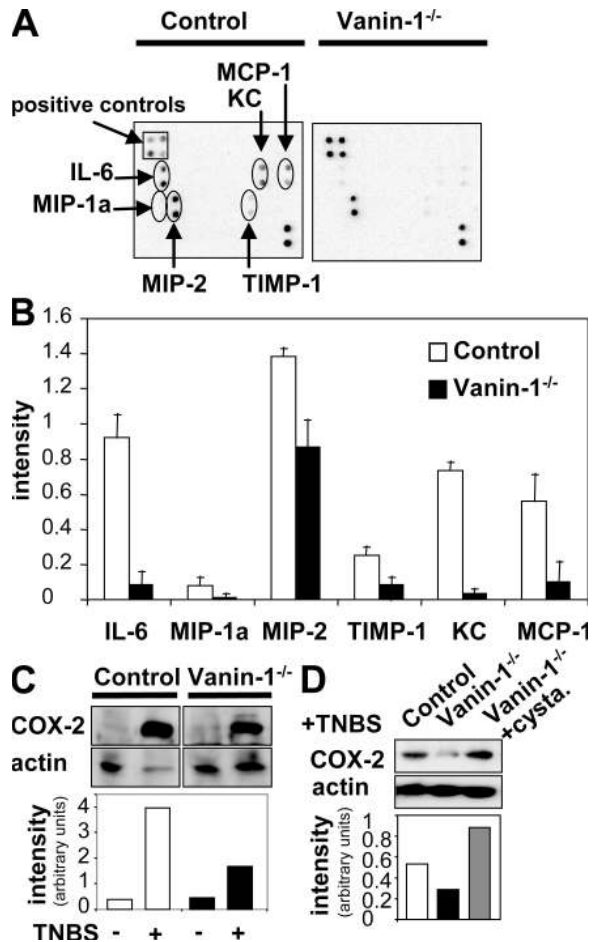


Figure 3. Vanin-1 controls the inflammatory response in injured colon. (A) ELISA-based cytokine arrays were hybridized with total colon tissue lysates derived from control or Vanin-1^{-/-} mice 2 d after colitis induction. Relevant signals are indicated. (B) Quantification of signal intensities resulting from the cytokine array experiment. (C) Comparative Western blot analysis of the expression of COX-2 in total colon extracts from control and Vanin-1^{-/-} mice before and 2 d after TNBS administration. Quantification of the results is represented in the bottom panel. (D) Similar experiment including total colon extracts from Vanin-1^{-/-} mice submitted to a treatment with cystamine. Quantification is shown in the bottom panel.

protein (MCP)-1, known to control macrophage recruitment. In contrast, Vanin-1^{-/-} tissue extracts presented only low levels of these inflammatory mediators (Fig. 3, A and B). Similarly, we found a lower induction of cyclooxygenase (COX)-2 expression in TNBS-treated Vanin-1^{-/-} than WT colons (Fig. 3, C and D). COX-2 is an inducible enzyme that catalyzes prostaglandin synthesis acting as potent lipid inflammatory mediators. Accordingly, as shown by Western blot analysis, cystamine administration boosted COX-2 expression in TNBS-treated Vanin-1^{-/-} colons (Fig. 3 D).

These findings demonstrate that Vanin-1 regulates the production of inflammatory signals in an injured colon. This explains the modest leukocyte recruitment in TNBS-treated

Vanin-1^{-/-} colons which was further confirmed by flow cytometry analysis of Mac1^{high}-F4/80⁺ macrophages and Mac-1^{low}Gr1⁺ granulocytes obtained from dissociated tissues (unpublished data).

Bisphenol A diglycidyl ether, a PPAR γ antagonist, abrogates Vanin-1^{-/-} protection against colitis

PPAR γ activation by agonists exerts antiinflammatory effects and attenuates TNBS-colitis (11, 21). This incited us to determine whether PPAR γ contributed to the down-regulation of the inflammatory processes observed in TNBS-treated Vanin-1^{-/-} mice. This was investigated by daily intraperitoneal administration of the bisphenol A diglycidyl ether (BADGE) PPAR γ antagonist (30 mg/kg), starting 2 d before TNBS injection (22, 23). All BADGE-treated mice developed a severe colitis to a level comparable to that of TNBS-exposed BALB/c mice. In Vanin-1^{-/-} mice, BADGE administration restored the sensitivity to TNBS-colitis recapitulating typical colon lesions with high inflammatory cell infiltrates and necrotic lesions (Fig. 4, A–C). This was not caused by an intrinsic toxicity of BADGE itself because mice submitted to an injection of BADGE alone, without intrarectal TNBS administration, did not die or present sign of colonic damage over the 15 d of experimentation (unpublished data). These findings suggested that the protective phenotype associated with the Vanin-1 deficiency was fully abrogated by PPAR γ inactivation.

Vanin-1 regulates the expression of PPAR γ in epithelial cells

Because BADGE suppresses the resistance of the Vanin-1^{-/-} mice to TNBS-induced inflammation, we explored whether the Vanin-1 molecule directly regulated PPAR γ expression and/or activation. First, despite high variability between animals we did not find a considerable difference in PPAR γ protein expression between control and Vanin-1^{-/-} mice by Western blot analysis on whole colon tissue extracts (Fig. 4 D). After TNBS administration, a comparable increase in PPAR γ expression was observed in WT and Vanin-1^{-/-} colons (Fig. 4 D). In Vanin-1^{-/-} mice, cystamine administration, which abrogates protection against colitis only slightly, reduced PPAR γ levels, whereas BADGE administration had no impact. Thus, on whole colon extracts, PPAR γ expression did not correlate with the clinical outcome. Furthermore, BADGE treatment, despite its antagonistic effect, did not seem to modulate PPAR γ expression. A major difficulty in this type of analysis is caused by the largely distributed PPAR γ expression among numerous cell types in colon, whereas Vanin-1 expression is mostly restricted to epithelial cells. Therefore, we performed the same analysis on protein lysates obtained from purified resting IECs, which show considerably higher PPAR γ expression levels in Vanin-1^{-/-} compared with control IECs (Fig. 4 E). Because of the toxicity of TNBS administration, we were unable to obtain enough viable purified IECs from TNBS-treated mice to be able to perform the same analysis.

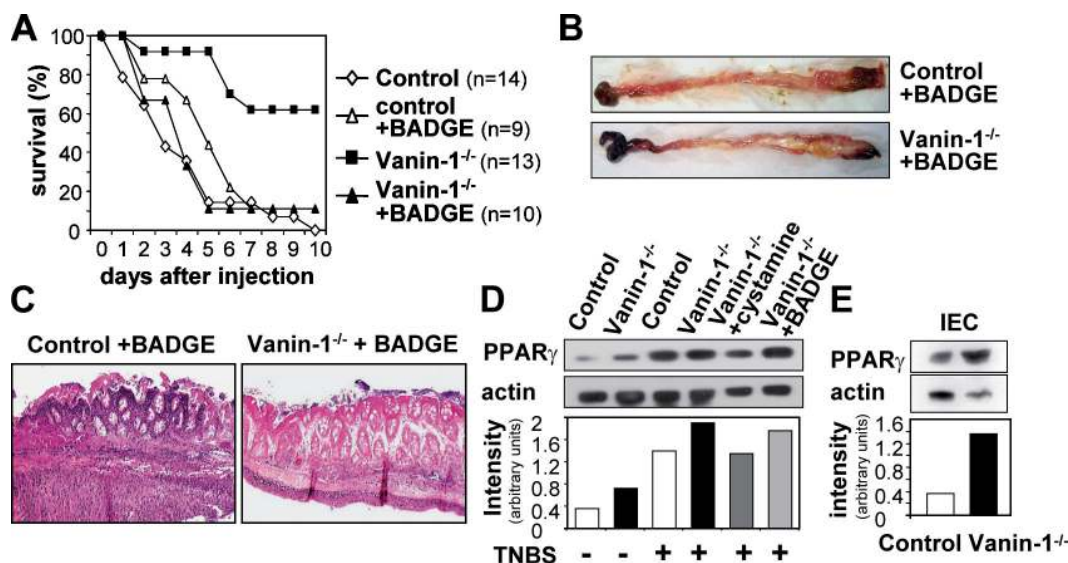


Figure 4. BADGE abrogates the protection against colitis associated with Vanin-1 deficiency. (A) Comparative survival rates of untreated and BADGE-treated control and Vanin-1^{-/-} mice during TNBS-colitis. (B) Macroscopic appearance of colons from BADGE-treated control and Vanin-1^{-/-} mice 2 d after TNBS induction. (C) Representative histological colon sections 2 d after TNBS administration. (D) Western blot

analysis of the PPAR γ expression in total colon extracts from control, Vanin-1^{-/-} mice, and Vanin-1^{-/-} animals treated with cystamine or BADGE. Samples were prepared 2 d after TNBS induction. The bottom panel represents a quantification of the results. (E) Western blot analysis of the expression of the PPAR γ protein by IEC purified from control or Vanin-1^{-/-} colons. Quantification is shown in the bottom panel.

We then turned to similar analyses on two distinct cell lines. The mouse MTE4.14 thymic epithelial cell line naturally expresses Vanin-1. Using a lentiviral-based RNA interference strategy, we derived clones showing an 80% reduction of Vanin-1 cell surface expression (named Vanin-1RNAi MTE4.14 cells). In parallel, the immature mouse intestinal cell line mIC, which lacks Vanin-1 expression, was transfected with Vanin-1 cDNA to generate stable Vanin-1(+) mIC transfectants. As shown in Fig. 5, the absence or reduced level of Vanin-1 was systematically associated with an up-regulation of the PPAR γ mRNA, quantified by real-time PCR. Indeed, levels of PPAR γ mRNA were, respectively eight- and fivefold higher in the Vanin-1RNAi MTE4.14 and the Vanin-1(-) mIC cells than in their Vanin-1(+) counterparts (Fig. 5 A). This was correlated with changes in PPAR γ protein level and measured by Western blot analysis (Fig. 5 B). The augmented PPAR γ mRNA expression could be reversed by incubating Vanin-1RNAi MTE4.14 cells with 1 nM cystamine for 12 h (Fig. 5 C). The prostaglandine 15-d-PGJ₂ has been identified as a potent endogenous ligand of PPAR γ that also increases PPAR γ mRNA expression in cell lines (24, 25). We found using real-time PCR that 15-d-PGJ₂ (5 μ M) boosted PPAR γ mRNA expression in Vanin-1(+) MTE4.14 cells by sixfold but did not further augment that found in Vanin-1RNAi MTE4.14 where the level of PPAR γ expression was already high (Fig. 5 D). In conclusion, we document a reverse relationship between Vanin-1 and PPAR γ transcript levels and an impact of cystamine on PPAR γ transcription.

The activation of PPAR γ is modulated by Vanin-1 in epithelial cells

Activation of PPAR γ results in the translocation of the protein into the nucleus and its subsequent binding to specific DNA elements called PPREs (15). We investigated whether Vanin-1 could modulate the degree of PPAR γ activation by analyzing the intracellular distribution of a GFP-tagged PPAR γ molecule transfected in the mIC cells. In unstimulated cells, the localization of the fluorescent tag was predominantly cytoplasmic. Exposure of cells to 15-d-PGJ₂ (5 μ M) for 12 h provoked a considerable nuclear translocation of the GFP-tagged PPAR γ in Vanin-1(-) but less so in Vanin-1(+) mIC cells (Fig. 6 A). To quantify this parameter, we investigated PPAR γ transcriptional activity by transient transfection of a 3XPPRE-luciferase reporter construct in MTE4.14 cells. As already reported in other cellular models (26, 27), 15-d-PGJ₂ induced a threefold increase of the reporter-gene activity in Vanin-1(-) cells. In contrast, this treatment had no detectable effect in Vanin-1(+) mIC cells (Fig. 6 B). Given the modest level of PPRE-mediated transcription in control cells, we combined PPAR γ ligands with a retinoic acid receptor agonist (i.e., all trans-retinoic acid [ATRA]) previously shown to enhance the PPAR γ -dependent effects and to induce maturation of myelomonocytic cells (28). In MTE4.14 epithelial cells, this combination considerably amplified the detectable reporter-gene activity in the Vanin-1(-) cells. In contrast, in Vanin-1(+) cells the reporter gene activity was still barely detectable (Fig. 6 B). This shows that the presence of the Vanin-1 molecule prevents the activation of PPAR γ in response to agonists.

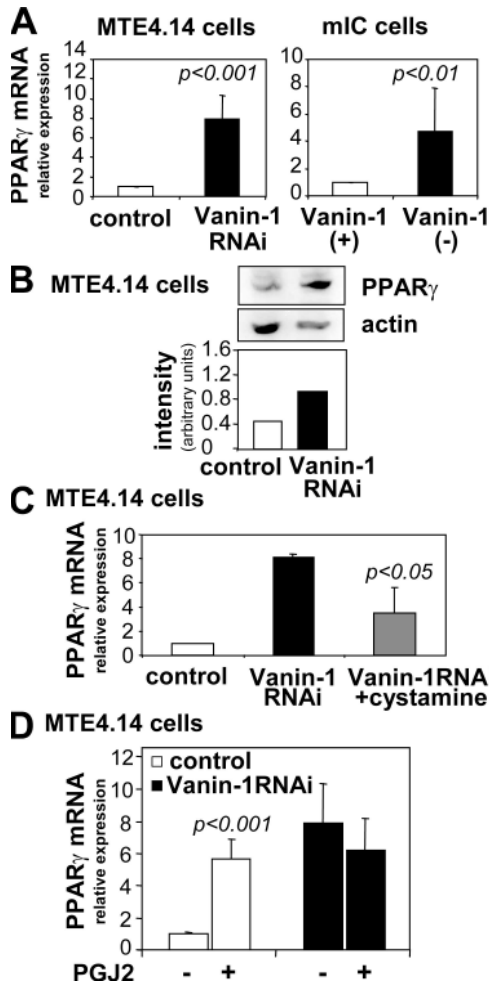


Figure 5. Vanin-1 regulates the expression of PPAR γ in epithelial cells. (A) Real-time PCR analysis of the expression of the PPAR γ mRNA by Vanin-1(+) and Vanin-1(-) MTE4.14 or mIC cells. (B) Western blot analysis of the expression of the PPAR γ protein in lysates from the Vanin-1(+) (control) and Vanin-1RNAi MTE4.14 cells. Quantification is shown in the bottom panel. (C) Real-time PCR measurement of PPAR γ expression in untreated or cystamine-treated MTE4.14 cells. (D) Real-time PCR analysis of PPAR γ expression in MTE4.14 cells treated or not treated with 15-d-PGJ₂.

Vanin-1 regulates IL-1 β -driven chemokine release by epithelial cells via modulation of PPAR γ activation

To demonstrate that Vanin-1 acts autonomously at the level of epithelial cells, we extended in vitro our analysis. We then showed by real-time quantitative PCR analysis that IL-1 β (10 ng/ml), a potent proinflammatory stimulus, induced the production of MCP-1 and MIP-2 in the Vanin-1(+) MTE4.14 or mIC cells, but not in their Vanin-1(-) counterparts (Fig. 7). A similar result was observed with COX-2 in mIC cells (unpublished data). Therefore, Vanin-1 expression licenses the epithelial cell response to a proinflammatory stimulus through the specific transcription of target genes.

To demonstrate further that PPAR γ is a main target of Vanin-1 proinflammatory activity, we evaluated the effect of

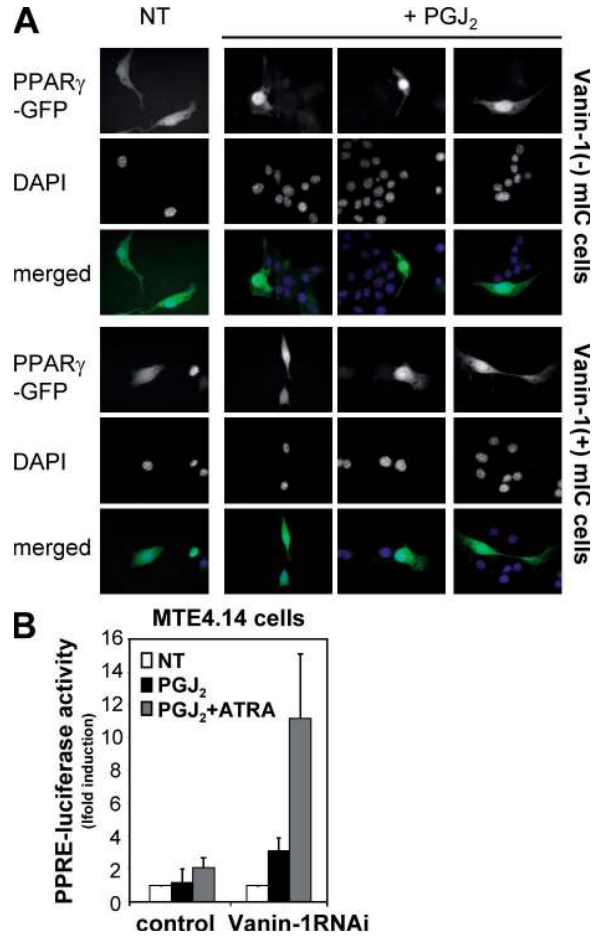


Figure 6. PPAR γ activation is modulated by the expression of Vanin-1 by epithelial cells. (A) Intracellular localization of a GFP-tagged-PPAR γ in Vanin-1(-) control or Vanin-1(+)-transfected mIC cells. Untreated cells (NT) and cells incubated with 15-d-PGJ₂ are shown. The distribution of the fluorescent tag was examined under a fluorescent microscope. In the merged images, green represents PPAR γ -GFP, whereas blue represents the nucleus staining performed with DAPI. PPAR γ can be seen to translocate into the nucleus after 15-d-PGJ₂ treatment most markedly in Vanin-1(-) cells. (B) Vanin-1(+) control and Vanin-1RNAi MTE4.14 cells were transfected with the PPRE-driven luciferase reporter vector 3X-PPRE-luc. Promoter activity was determined in untreated cells (NT) or after exposure of the cells to 15-d-PGJ₂ or a combination of 15-d-PGJ₂ with ATRA.

PPAR γ agonists and antagonists on the production of the MCP-1 or MIP-2 chemokines by Vanin-1(+) or (-) cells (Fig. 7). Stimulation of the Vanin-1(+) cell lines (MTE4.14 and mIC transfectants) by IL-1 β (10 ng/ml for 12 h) resulted in the transcription of MCP-1 or MIP-2 mRNAs (approximately threefold increase), which was abrogated by the simultaneous incubation with the PPAR γ agonist 15-d-PGJ₂ (5 μ M). Interestingly, the deficiency in chemokine production by IL-1 β -stimulated Vanin-1(-) cells (RNAi MTE4.14 or mIC) was almost fully restored by antagonizing PPAR γ activation with BADGE (5 μ M). Hence, modulation of PPAR γ activation by agonists and antagonists influenced the

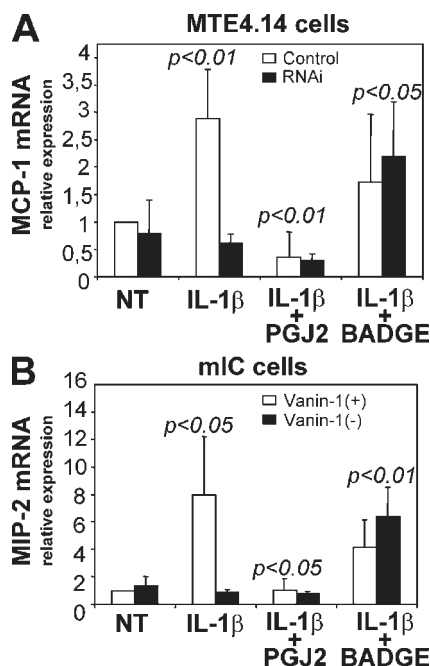


Figure 7. Modulation of PPAR γ activation reflects Vanin-1-dependent control of chemokine release by epithelial cells. (A) Real-time PCR exploration of the expression of the MCP-1 mRNAs in Vanin-1(+) control and Vanin-1RNAi MTE4.14 cells. Analyses were performed on untreated cells (NT) and cells stimulated with IL-1 β alone or submitted to a cocubation of IL-1 β together with 15-d-PGJ₂ or BADGE. (B) A similar experiment was performed to investigate the expression of the MIP-2 mRNA in Vanin-1(-) control and Vanin-1(+)-transfected miC cells.

Vanin-1-dependent cellular response to proinflammatory stimuli. This shows that Vanin-1 acts upstream of PPAR γ in a pathway controlling the production of inflammatory signals by epithelial cells.

DISCUSSION

In healthy individuals, the gut buffers the constant low level of environmental stress originating from the diet and the conventional microflora. A normal epithelium provides an effective barrier against luminal agents. When the integrity of the barrier is compromised, however, innate mechanisms of defense are triggered to eliminate the danger, repair the tissue, and restore a homeostatic state. In this study, we have addressed the role of the epithelial enzyme Vanin-1 in the development of colitis and show that Vanin-1 deficiency reduces colitis severity in the TNBS model. This is clearly demonstrated by an improved survival of Vanin-1^{-/-} mice compared with WT animals after TNBS treatment. Furthermore, treatment of these mice by a PPAR γ antagonist (BADGE) abrogates their protection against TNBS-colitis. Thus, Vanin-1 appears to control TNBS-colitis by antagonizing PPAR γ activity.

Vanin-1 regulates oxidative stress in the gut

Vanin-1 is an epithelial ectoenzyme that provides cysteamine to tissue through the hydrolysis of pantetheine, a vitamin B5

intermediate. In mice, Vanin-1 deficiency confers resistance to systemic oxidative stress associated with ionizing radiations or exposure to xenobiotics (6). Thus, Vanin-1 participates in the response to stress in vivo. In gut, Vanin-1 is expressed by epithelial but not hematopoietic cells. We previously reported that Vanin-1^{-/-} gut shows increased levels of endogenous GSH and that Vanin-1-deficient mice are protected against acute intestinal damages induced by oxidative stress associated with *Schistosoma mansoni* infection or indomethacin administration (7).

In the murine model of colitis, TNBS administration engenders oxidative stress through its metabolism by depleting GSH and generating reactive oxygen species (29). Overproduction of reactive oxygen species by immune cells contributes to tissue injuries observed in colitis, and a mucosal GSH deficiency is observed in IBD (30–32). This deficit is mainly caused by the decreased activities of key enzymes in GSH synthesis like γ -glutamylcysteine synthetase and γ -glutamyltransferase (32, 33). In contrast, antioxidant therapy with *N*-acetylcysteine, a GSH precursor, attenuates the acute colitis through increased mucosal GSH levels (33, 34). Thus, impaired mucosal antioxidant capacity may further promote oxidative damage, whereas increased mucosal antioxidant activity prevents oxidative damage. This might explain the better survival and the mild inflammatory response of Vanin-1^{-/-} mice after induction of colitis by TNBS.

Vanin-1 regulates epithelial cell-driven inflammation

Invasion of the mucosa by leukocytes is a characteristic of the gut inflammation. The role of the IECs in orchestrating this recruitment is however not clearly defined. In vitro, epithelial cell lines respond to proinflammatory signals by secreting cytokines and chemokines. In vivo, specific alteration of the intestinal epithelium was shown to lead to intestinal inflammation (35, 36) and the functional role of epithelium-secreted chemokines was demonstrated by targeting overexpression of MIP-2 in IECs using a transgenic model (37).

We report herein that the absence of Vanin-1 results in a dramatic decrease in the expression of a wide panel of inflammatory cytokines in the colon after TNBS. Consequently, Vanin-1^{-/-} mice exhibit attenuated myeloid cell recruitment and lowered subsequent tissue damage. Using bone marrow-reconstituted chimeras, we definitively show that the decreased susceptibility of Vanin-1^{-/-} mice to experimentally induced colitis was determined at the epithelial level. These findings indicate that IECs may actively alter mucosal immune response.

The TNBS-colitis model is usually considered as a Th1-driven inflammatory model recapitulating clinical aspects of the human Crohn's disease. However, several arguments highlight the importance of innate immunity in the development of colitis: (a) in human IBD as in mouse experimental models, recruitment and activation of macrophages is a constant feature of the immunopathology; (b) mice devoid of Stat3 specifically in macrophages and neutrophils develop chronic enterocolitis (38); (c) murine colitis is dependent on

continuous macrophage migration inhibitory factor production by the nonlymphocyte immune system (39); (d) the $\alpha 1$ -mediated inhibition of TNBS-colitis is accredited to monocytes as key $\alpha 1\beta 1$ integrin-expressing cell type involved in the effector phase of intestinal inflammation (40); and (e) SCID mice submitted to TNBS develop colitis indicating that this syndrome can be induced in the absence of lymphocytes, so that the disease can be driven by innate immune mechanisms. Here we reported that Vanin-1^{-/-} mice display a protection against colitis-causing death associated with an observable mild infiltration of macrophages and granulocytes in the colonic mucosa. In addition, Vanin-1^{-/-} SCID mice exhibit the same protection. Collectively, all this clearly highlights the contribution of the IECs to the regulation of the innate immune responses.

Vanin-1 regulates PPAR γ in epithelial cells

PPAR γ is a nuclear receptor activated by fatty acid ligands and involved in the control of the cellular response to metabolic and nutritional signals (16). A broad recent study about PPAR γ describes a key role of this nuclear factor in the maintenance of mucosal integrity in the intestine and a powerful intestinal antiinflammatory effect (10, 41). PPAR γ agonists decrease the severity of colitis (9, 11). Thus, modulation of PPAR γ has become of great interest for therapeutic strategies in human IBD (42). PPAR γ is expressed by several cell types in the colon, including hematopoietic cells like macrophages or lymphocytes and epithelial cells. The antiinflammatory function of PPAR γ is attributed to a transcriptional attenuation of the expression of cytokines, chemokines, or COX-2 by these cells (9). Recently, it has been reported that targeted inactivation of PPAR γ in colonic epithelial cells abrogates its protective role in colitis (43), but the regulation of PPAR γ in epithelial cells remains poorly understood.

One of our main results is that in vivo a functional PPAR γ antagonist (BADGE) abrogates the protective phenotype of the Vanin-1^{-/-} mice against colitis. This clearly positions PPAR γ downstream of a Vanin-1 effect. We next demonstrated that in vivo the expression of PPAR γ in IECs is modulated by the presence of Vanin-1 or the administration of cystamine. This was confirmed in vitro using in parallel cellular models based on transfectants and RNA interference. The absence of Vanin-1 is correlated with an increase in PPAR γ expression, whereas cystamine down-regulates its expression. In addition, our results indicate that Vanin-1 directly affects PPAR γ activation. As shown using PPARE reporter assays, the activation of PPAR γ by its natural agonist ligand 15-d-PGJ₂ was improved in Vanin-1(-) epithelial cells compared with Vanin-1(+) counterparts, both at the level of nuclear translocation and transcriptional activity. On the other hand, we showed that Vanin-1(+) epithelial cells significantly respond to IL-1 β proinflammatory stimulation by expressing inflammatory genes including MCP-1, MIP-2, or COX-2. In addition, we showed that 15-d-PGJ₂ considerably antagonized the effect of IL-1 β by inhibiting the expression of these target genes in cells. A similar result was

described for pancreatic stellate cells in which PPAR γ activation by 15-d-PGJ₂ results in the inhibition of inducible MCP-1 expression in IL-1 β -stimulated cells (27). Of interest, Vanin-1(-) cells do not respond to IL-1 β stimulation, but in contrast, exposure of these cells to BADGE restored the cellular capacity to respond to the IL-1 β proinflammatory signal.

Conclusion

Collectively, these findings argue for a Vanin-1-dependent regulation of the function of PPAR γ in epithelial cells. This might account for the protective phenotype of Vanin-1-deficient mice against colitis. The absence of Vanin-1 and the consequently decreased levels of cysteamine in tissue will promote PPAR γ antiinflammatory effects by inhibition of the transcription of epithelial signals intended to alert the innate effectors of the mucosal immune system. Our data support the idea that the presence of Vanin-1 at the epithelial level is determinant for the perception of stress by innate immune cells. Thus, targeting inflammatory pathways in epithelial cells might help to control the development of colitis. Finally, the manipulation the Vanin-1/pantetheinase activity represents an attractive therapeutic strategy for the treatment of IBD.

MATERIAL AND METHODS

Mice. Mice were kept in a specific pathogen-free mouse facility and handle according to the rules of Décret no. 87-848 du 19/10/1987, Paris. All experiments were performed on 6–8-wk-old Vanin-1^{-/-} mice or WT BALB/c littermate controls. Vanin-1^{-/-} lymphocyte-deficient mice used for experiments were generated by introducing the Vanin-1 deficiency on a BALB/c SCID background. To generate chimeras, Vanin-1^{-/-} and BALB/c mice were total body irradiated (10 Gy) before receiving 10⁶ T cell-depleted bone marrow cells obtained from Vanin-1^{-/-} or BALB/c donors.

TNBS-colitis. Acute colitis was induced by an intrarectal administration of 100 μ l containing 3 mg of TNBS (Sigma-Aldrich) in 30% ethanol. Cystamine (Sigma-Aldrich) or BADGE (Sigma-Aldrich) treatments were performed by a daily intraperitoneal administration of a 120 or 30 mg/kg dose, respectively, starting 2 d before TNBS treatment. Acute colitis was assessed 2 d after infusion of TNBS. Colons were examined by two independent individuals to evaluate the macroscopic lesions according to the Wallace criteria (44). Colon tissue samples located precisely 2 cm above the anal canal were prepared for histological staining with hematoxylin and eosin. Histologic grades were assigned in a blind manner by one pathologist according to the Ameho criteria (45).

Cytokine antibody array. Mouse cytokine antibody array membranes (RayBiotech) were incubated overnight at 4°C with 500 μ g of total protein lysates prepared from colon tissues according to the manufacturer's instructions. Detection of signal intensities was performed using a chemiluminescence imaging system (Intelligent Dark Box II; FUJIFILM). Relative cytokine expression levels were determined using the Multi Gauge V2.3 software (FUJIFILM). Internal controls included in the array were used to normalize the results from different membranes. Experiments were performed in duplicate for each mouse genotype.

Mouse epithelial cell lines. MTE4.14 and mIC cells are Vanin-1-expressing mouse thymic epithelial cells and non-Vanin-1-expressing IECs, respectively. We used a lentiviral-based strategy to generate both Vanin-1(+) mIC cells and MTE4.14-derived stable clones with \sim 80% reduction of the cell

surface expression of Vanin-1 by means of the RNA interference technology (named herein MTE4.14-Vanin-1RNAi). In brief, full-length Vanin-1 coding sequences were inserted into pRRLpgkEGFPsin18 (provided by D. Trono, University of Geneva, CH-1211, Geneva, Switzerland) by replacing the enhanced green fluorescent protein (EGFP) gene (pRRLpgkVanin1) (46). In parallel, Vanin-1 oligonucleotide encoding for shRNA was selected following the criteria described by Rubinson et al. (46) (5'-TGTGGTATCTATGCACCCGATTCAAGAGATCGGGTGCATAGATACCACTT-TTTTC-3' combined with the following complementary strand 3'-ACAC-CATAGATACGTGGGCTAAGTTCTCTAGCCACGTATCTATGG-TGAAAAAGAGCT-5') and placed under the control of the U6 promoter into the Lentilox 3.7 vector (LL3.7; provided by Luke van Parijs, Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, MA) to generate LL3.7shRNAvanin1. Lentiviral particle stocks were produced according to a previously described protocol (46). Viral titers were assessed by quantifying the percentage of EGFP or Vanin-1(+) HUH7 target cells by flow cytometry (FacsCalibur; Becton Dickinson). Membrane expression of Vanin-1 was assayed using the 407 monoclonal antibody (4). The lentiviral preparations showed viral titers ranging from 2 to 5×10^8 IU/ml. No replication competent virus was detected in any of viral stocks. MTE4.14 cells were transduced with LL3.7shRNAvanin1 or LL3.7 vector as control in a final volume of 500 μ l of 8 μ g/ml polybrene with a multiplicity of infection of 40. EGFP expression was used to sort transduced cell populations. We obtained one MTE4.14-Vanin-1RNAi clone showing a stable \sim 80% reduction of the cell surface expression of Vanin-1. In parallel, mIC cells were transduced with pRRLpgkVanin1 or pRRLpgkEGFP as control using a multiplicity of infection of 100.

Real-time quantitative PCR. Total RNAs were prepared from tissue samples or harvested cell cultures using the TRIzol reagent (Invitrogen). Reverse transcription was performed using the QuantiTect Reverse Transcription kit (Qiagen). Expression of mouse MCP-1, MIP-2, COX-2, and PPAR γ mRNAs was measured by the real-time quantitative RT-PCR method using SYBR green Master Mix (ABI) and the ABI PRISM 7700 sequence detection system (PE Applied Biosystems). Relative mRNA levels were determined by the comparative threshold cycle ($2^{-\Delta\Delta CT}$) method (47). The expression of all mRNAs was normalized to that of actin mRNA. All data shown here result from at least three independent experiments. Sequences of all primers used in this study are the following: MCP-1, forward 5'-ctcactgctgctactcattc-3' and reverse 5'-gcttgaggtggttctggaaa-3', MIP-2, forward 5'-accctgccaaggttgacttc-3' and reverse 5'-ggcacatcaggtacgatccag-3', COX-2, forward 5'-tcaaaagaagtctggaaggtt-3' and reverse 5'-tctactgag-tatcttctgactgtg-3', and PPAR γ , forward 5'-gatcaaggttcttccggagaac-3' and reverse 5'-tggtgattgtctgtctcttc-3'.

Western blot analysis. 30 μ g of total protein lysates derived from either harvested epithelial cell cultures, colonic tissue samples, or purified IECs were loaded on SDS-PAGE gels. Membranes were probed overnight at 4°C with antibodies against PPAR γ (Santa Cruz Biotechnology, Inc.), COX-2 (Cayman), or actin (Sigma-Aldrich) antibodies followed by the appropriate species-specific horseradish peroxidase conjugate (Sigma-Aldrich) and developed using the enhanced chemiluminescence detection system (GE Healthcare). Quantification of band intensities was performed using the Multi Gauge V2.3 software (FUJIFILM).

Isolation of IECs. Colons were cut open longitudinally, and feces was removed by washing with PBS. Colons were then cut in small pieces (2 mm) and carefully rocked in RPMI 10% FBS solution for 10 min at 25°C. Colon pieces were then successively incubated under shaking in collagenase mix (RPMI containing 0.2 mg/ml collagenase, 20 mM Hepes, 2% FCS) for 15 min at 30°C, collagenase mix supplemented with 0.2 mg/ml of dispase plus 25 μ g/ml DNase I for 25 min at 30°C, and finally trypsin mix (PBS containing 0.05% trypsin, 0.5 mM EDTA, 0.3% BSA, 50 μ g/ml DNase I) for 25 min at 36°C. Resulting preparations were filtered on cell strainers (BD Falcon) and centrifuged. Pelleted cells were then resuspended, and IECs

were isolated from lymphoid cells using a gradient of Percoll. Cells were counted and checked for viability.

Luciferase reporter assay. The 3XPPRE-TK-luc plasmid (48) was provided by ADDGENE. MTE4.14 control and MTE4.14 Vanin-1RNAi clones were cultured in 24-well plates and cotransfected with 250 ng of 3XPPRE-TK-luc plus 50 ng of pRL-TK-RN control vector (Promega) using the lipofectamine 2000 (Invitrogen). 24 h later, cells were treated for 12 h with 10 μ M 15-d-PGJ₂ (Sigma-Aldrich) alone or in combination with 10 μ M of ATRA (Sigma-Aldrich). After treatment, cells were lysed in PLB buffer, and detection of the luciferase transcription was performed using the Dual luciferase assay kit (Promega).

PPAR γ translocation. Vanin-1(+) and (-) mIC cells were seeded on coverslips in 24-well plates at a density of 2×10^5 cells/well. After 24 h, cells were transfected with 1 μ g of GFP-tagged PPAR γ plasmid (provided by J.P. Desreumaux, Institut National de la Santé et de Recherche Médicale U795, Lille, France) using lipofectamine 2000 (Invitrogen). 24 h after transfection, cells were treated overnight with 20 μ M of 15-d-PGJ₂ (Sigma-Aldrich) and then PBS washed and fixed in 4% paraformaldehyde. The nuclei were stained with DAPI (Sigma-Aldrich) after permeabilization of cells with 0.1% Triton X-100. Coverslips were mounted with mowiol and visualized with a Zeiss Axiovert fluorescence microscope (Carl Zeiss MicroImaging, Inc.).

Statistical analysis. Data are expressed as means \pm SD. Values from experimental and control groups were compared using the Student's *t* test. Probability values of $P < 0.05$ were considered statistically significant.

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