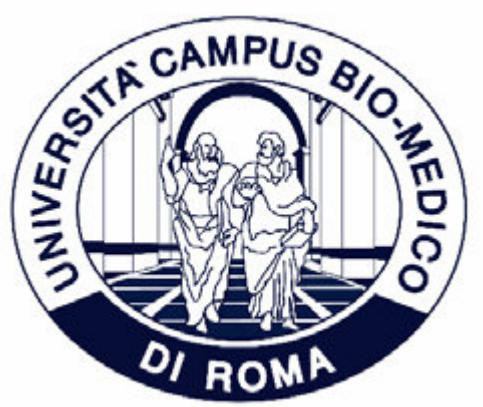

Università Campus Bio-Medico di Roma

Corso di dottorato di ricerca in
Scienze Biochimiche e Tecnologiche Applicate agli Alimenti ed alla Nutrizione
XXV ciclo anno 2010

Intestinal Epithelial Vitamin D Receptor Expression in IBD Patients and in Experimental Colitis

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ABBREVIATIONS

VDR: *Vitamin D receptor*
hVDR: *human VDR*
1,25(OH)2D3: *1,25-dihydroxyvitamin D3*
IBD: *inflammatory bowel disease*
CD: *Crohn's disease*
UC: *Ulcerative colitis*
PUMA: *p53-upregulated modulator of apoptosis*
IECs: *intestinal epithelial cells*
TNBS: *2,4,6-trinitrobenzenesulfonic acid*
DSS: *dextran sulfate sodium*
Tg: *transgenic mice*
WT: *wild-type*
TER: *transephitelial electric resistance*

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ABSTRACT

Vitamin D-deficiency is very prevalent in inflammatory bowel diseases. Global vitamin D receptor (VDR) deletion exaggerates colitis in mice, but the anti-colitic roles of immune and intestinal epithelial VDR are unclear. Here we report that epithelial VDR expression was substantially reduced in patients with Crohn's disease or ulcerative colitis, and VDR up-regulation in intestinal epithelial cells (IECs) prevented mice from developing colitis. In both 2,4,6-trinitrobenzenesulfonic acid (TNBS) and dextran sulfate sodium (DSS) colitis models, transgenic mice expressing human (h)VDR in the IECs were highly resistant to colitis, manifested by a marked reduction in clinical colitis scores and colonic histological damage compared to wild-type mice. Furthermore, reconstitution of the IECs with the hVDR transgene completely rescued VDR-null mice from severe colitis and death in both colitis models. Mechanistically, hVDR overexpression attenuated TNBS- or DSS-induced IEC apoptosis via blocking the induction of PUMA, a key pro-apoptotic regulator, and vitamin D down-regulates PUMA by blocking NF-κB activation. These results indicate that epithelial VDR inhibits colitis by protecting the mucosal epithelial barrier. The observation that hVDR rescues VDR-null mice from colitis despite a VDR-null immune system demonstrates that the anti-colitic activity of epithelial VDR is independent of immune VDR signals.

INTRODUCTION

1.1 Inflammatory bowel diseases

Inflammatory bowel diseases (IBD) also known as Crohn's disease (CD) and ulcerative colitis (UC), are chronic immunologically mediated diseases that are due to a dysregulated immune response to intestinal flora in a genetically susceptible host. While a progressive increase in incidence has been observed worldwide [1], earlier studies identified a significant north–south gradient with the highest incidence rates of CD and UC reported in northern Europe, the UK and North America [2,3].

1.2 Hot Hypothesis: genetics or environmental factors

Despite advances in genetics leading to the identification of 163 loci that account for the variable incidences among different races/ethnicity [4] known risk loci convey less than a third of the risk for CD or UC. [5]. Indeed, epidemiologic data suggest a strong role for the environment in mediating risk of disease [3,6]. The risk of IBD in the immigrant population resembles that of country of residence rather than the country of origin [7]. Also, countries that have witnessed a rapidly changing environment and lifestyle have seen an increase in the incidence of IBD over the past few decades at a rate of change that outpaces what could be attributed solely to genetics [8]. The two most well-established risk factors for IBD, smoking and appendectomy, cannot fully account for all variations in IBD incidence and prevalence. Recent data has suggested novel risk factors for CD and UC (Table 1). In particular, one underexplored area in relation to IBD is that of the greater environment, such as sun exposure, soil, climate or temperature change and air pollution [9], may explain geographical variation. A link between latitude and incidence rates of CD and UC has been supported by a large prospective study from the USA [10]. By tracking the location and lifestyle information of approximately 175 000 female American nurses biennially over 20 years, the authors detected a greater increase in the incidence rates of CD and UC the further subjects lived from the equator. At age 30 years, living in southern latitudes was associated with a roughly

halved risk of developing CD and approximately a 40% reduced risk of developing UC. In that study, most of the patients developed CD in their late 40s or early 50s, suggesting that latitude may play an important role in relatively late-onset CD. The results are consistent with European studies linking latitude with the development of IBD. Latitude differences have also been described for other immune-mediated diseases, especially multiple sclerosis [11]. In addition, temperature might represent the sole mechanistic explanation leading directly or indirectly to a change of microbiota. Studies from Europe suggest that low sunlight exposure may contribute to the pathogenesis of IBD, although the exact mechanism for this is not clear. It has been hypothesized that people in sunnier states may have higher exposure to ultraviolet light, leading to higher vitamin D levels, which help to regulate immunity and inflammation.

Table 1. Environmental risk factors for Crohn's disease and ulcerative colitis [12]

Environmental factor	Effect on disease onset	Effect on disease course	Intervention studies
Smoking–Crohn's disease	+	+	Smoking cessation is beneficial in CD
Smoking–ulcerative colitis	–	–	Equivocal results from trials of nicotine replacement therapy
Appendectomy–CD	?+	None	None
Appendectomy–UC	–	?–	None
Low vitamin D	?+(CD)	?+(CD)	Vitamin D supplementation may reduce risk of relapse in CD
Oral contraceptive	+ (CD)	No data	None
Post-menopausal hormone use	?+(UC)	? –	None
NSAIDs	?+(CD, UC)	+ (CD, UC)	None
Antibiotics	?+(both early childhood and adult exposure)	No data	Trials of antibiotics for the treatment of CD and UC have been mostly null. Antibiotics are effective in preventing post-operative recurrence in CD.
Depression and psychosocial stress	?+CD. Equivocal in UC	Equivocal. May trigger relapses.	Antidepressant use reduced need for steroids and symptomatic relapse in one study. Other psychological interventions have not been shown to be effective.
Air pollution	?	?	None
Dietary fiber	? – (CD; particularly fruits and vegetables)	No data	None
Dietary fat	?+(UC) (n-6 fatty acids increase risk, n-3 fatty acids may confer protection)	No data	None
Dietary protein	?+(UC) (particularly animal protein)	No data	None

2 VITAMIN D

2.1 Vitamin D and Vitamin D receptor

Traditionally recognized for its role in calcium homeostasis and bone mineralization, there has been increasing recognition of the immunologic role of 1,25-dihydroxyvitamin D (1,25(OH)2D3), the hormonal form of vitamin D, including regulation of immune system especially T-cell mediated immune response [13]. The majority of the body's vitamin D content is derived from photosynthesis in the skin following UV light/sunlight irradiation [14]. Vitamin D is converted to the active hormone 1,25(OH)2D3 by 25-hydroxylation in the liver followed by 1α-hydroxylation in the kidney [15]. The biological activity of 1,25(OH)2D3 is mediated by the vitamin D receptor (VDR), a member of the nuclear receptor superfamily [16]. In the presence of 1,25(OH)2D3, VDR regulates transcription by binding to vitamin D response elements on the promoters of targeted genes [16]. In 1983 the VDR was reported to be in cells of the immune system by two different groups [17,18]. Since that time there has been increasing interest in understanding what the targets of vitamin D are in the immune system. Specific polymorphisms in the VDR gene have been also associated with genetic susceptibility to IBD in different ethnic populations [19].

2.2 Vitamin D and immune system

The role of vitamin D and VDR in the pathogenesis and course of other T-helper-1 cytokine-mediated immune diseases, such as multiple sclerosis, rheumatoid arthritis, and type 1 diabetes mellitus, has been well recognized [20]. The early experiments treated T cells with 1,25(OH)2D3 to determine the role of vitamin D as an immune system regulator. Experiments done by several different groups of investigators established that 1,25(OH)2D3 suppressed immune responses driven by Th1 cells [21-23]. Th1 cells secrete several signature cytokines including IFN-c, IL-2, and TNF-a. Th1 cells are important in the host's ability to clear intracellular infections and tumor immunity. In conjunction with Th17 cells, the Th1 cells, contribute to the pathogenesis of several autoimmune diseases. Transfer of pathogenic Th1 or Th17 cells induced symptoms of experimental

autoimmune encephalomyelitis (EAE, multiple sclerosis model) and IBD [24-26]. 1,25(OH)2D3 inhibited Th1 and Th17 function including suppressing the production of the signature IL-17 and IFN-c cytokines [20, 27-28]. Th2 cells are important for fighting extracellular infections and are pathogenic T cells in the development of allergy and asthma. The effects of 1,25(OH)2D3 on Th2 cells are more controversial since different groups have shown that IL-4 is increased, decreased or not affected by 1,25(OH)2D3 [29-31]. There are several regulatory T cells that serve to inhibit the induction and function of effector T cells. Natural killer (NK)T cells have been shown to secrete high amounts of cytokines early during an immune response [32]. 1,25(OH)2D3 has been shown to increase cytokine production of NKT cells and to potentiate their functions [31]. 1,25(OH)2D3 suppressed Th1 and Th17 responses, induced cytokine production by NKT cells and induced regulatory T cells suggesting that physiologically vitamin D may be important in the induction of immune-mediated diseases like IBD. Vitamin D deficiency and VDR KO mice have normal numbers of CD4+, CD8+, B cells, macrophage, and NK cells [33,34]. Analysis of activation markers on CD4+ T cells showed that VDR KO mice have fewer naïve CD4+ T cells and more memory and activated T cells [24]. Injection of equal numbers of naïve (either CD45RBhigh or CD25_) VDR KO or wild type (WT) CD4 T cells into T and B cell deficient mice resulted in more severe colitis in mice that received the VDR KO cells [25]. The increased pathogenic potential of the naïve VDR KO CD4 cells was a result of the overproduction of IFN-c and IL-17 [24]. CD4+ T cells from VDR KO mice produced twice as much IFN-c and IL-17 than their WT counterparts [24]. VDR KO mice had T cells that were primed and ready to become Th17 and Th1 cells and these cells are important cells in the pathogenesis of IBD. Experiments showed that CD4+ T cells transferred from WT mice suppressed experimental IBD while CD4+ T cells transferred from VDR KO mice did not [35].

2.3 Vitamin D and animal model of colitis

Animal studies have provided direct evidence for an inhibitory role of vitamin D in the pathogenesis of IBD. Vitamin D-deficiency exacerbated enterocolitis and increased mortality in IL-10-/-mice, a model of spontaneous colitis, whereas dietary vitamin D supplementation ameliorated colitis and decreased mortality in this model [36]. VDR-/-/IL-10-/- mice developed more severe colitis and higher mortality than VDR+/+/IL-10-/- mice [25]. These observations suggest that the VDR signaling in the immune cells plays a protective role against IBD. In addition, global VDR deletion increased mucosal injury that led to high mortality in an experimental colitis model [37]. In VDR-/- mice the colonic trans-epithelial electrical resistance (TER), an indicator of the epithelial barrier integrity, was significantly reduced before clinical symptoms and histological abnormalities of colitis were detected, suggesting that VDR actions in the colonic epithelia may play a key role in protecting the host from developing IBD. The relative anti-colitic contribution of epithelial vs. immune VDR signaling, however, remains to be determined.

2.4 Vitamin D and human IBD

A growing body of epidemiological evidence has demonstrated an association between vitamin D-deficiency and increased risk of IBD [38-40], including both CD and UC [41-48]. A deficiency of vitamin D could be a consequence of IBD itself with reduced physical activity, sunlight exposure, malnutrition, inadequate dietary intake of vitamin D, or lower bioavailability, all contributing to the deficiency [49-51]. In the northern hemisphere, symptomatic onset of UC peaks in December, the month with shortest duration of sunlight and lowest vitamin D production [52,53]. Populations far from the equator have higher prevalence of IBD than those close to the equator. Studies have also examined the association between vitamin D status and disease activity [54,55]. In a retrospective study of 504 IBD patients, Ulitsky et al. found that lower vitamin D was associated with a modest increase in disease activity and a lower health-related quality of life in CD [55]. Vitamin D deficiency is common even in newly diagnosed IBD patients suggests that low vitamin D itself can

contribute to increased risk of IBD [56]. The Nurses Health Study I and II are two large, prospective cohorts of female nurses, initiated in 1976 and 1989, respectively, by the Harvard School of Public Health, to examine environmental influences on chronic disease. Through detailed biennial questionnaires, participants provide information on a spectrum of dietary and lifestyle factors along with information on health status. Two recent studies from these cohorts highlight the role of vitamin D in IBD pathogenesis. Khalili et al. examined the variation in IBD incidence by geography of residence at the time of birth, and at ages 15 and 30 years [57]. Residence at age 30 had a greater impact on disease incidence than that at birth. Women who resided in southern latitudes had a lower risk of CD (Hazard ratio (HR) 0.48, 95 % confidence interval (CI) 0.30–0.77) and UC (HR 0.62, 95 % CI 0.42–0.90) than those residing in northern latitudes. These findings are consistent with data suggesting a similar north–south gradient in disease incidence [58], mimicking exposure to ultraviolet light, a major determinant of vitamin D status. Indeed, a French study modeling surface UV radiation intensity using satellite data found an increased incidence of CD in areas with low sunlight exposure [59]. To more specifically examine the effect of vitamin D, Ananthakrishnan et al. used the NHS cohort and a validated model of predicted plasma 25-hydroxy vitamin D incorporating dietary and lifestyle variables [60]. The mean predicted plasma vitamin D ranged from 22 ng/mL in the lowest quartile to 32 ng/mL in the highest quartile. On multivariate analysis, compared to the lowest quartile, women in the highest quartile had half the incidence of CD (HR 0.54, 95 % CI 0.30–0.99); no effect on UC risk was observed. Dietary vitamin D intake had a more modest effect and attained statistical significance for UC but not CD, likely owing to the weaker contribution of diet to overall vitamin D status. Using microarray analysis on colonic biopsy tissue, Zhu et al. found that several of TNF-α related genes in colonic tissue were down-regulated by administration of 1,25(OH)2D3 [61]. Jorgensen et al. performed a randomized, double-blind placebo controlled trial administering a daily dose of 1,200 IU vitamin D3 or placebo to 94 CD patients in remission [62]. At the end of 1 year, 6/46 patients (13 %) in the vitamin D arm had relapsed compared to 14/48 patients treated with placebo (p=00.06). The prior mechanistic studies

and the Jorgensen clinical trial suggest the need for larger studies of vitamin D as a therapeutic agent in the treatment of IBD, particularly CD, either as monotherapy or in conjunction with existing treatments.

3 INTESTINAL BARRIER AND IBD

The impairment of gut mucosal barrier function is thought to be a significant pathogenic factor leading to intestinal hyperpermeability in IBD [63]. The epithelial barrier consists of a monolayer of gut epithelial cells (IECs) with intercellular junctions between adjacent cells that seal the paracellular space and regulate the permeability of the barrier [64]. This barrier protects the host by preventing harmful solutes, microorganisms, toxins and luminal antigens from entering the body [65]. Compromised barrier function can result in entry of luminal antigens and bacteria into the lamina propria, leading to immune cell activation and ultimately to IBD [66]. Aberrant apoptosis of intestinal epithelia cells (IECs) is thought to be a major pathogenic mechanism leading to increased mucosal permeability and colonic inflammation. Increased IEC apoptosis has been reported in patients with UC and CD [67-69] as well as in murine models of colitis [70,71]. IEC apoptosis causes focal disruption of the mucosal barrier leading to immune cell-mediated proinflammatory cytokine induction that induces more IEC apoptosis. This vicious cycle of events eventually results in clinical symptoms of IBD. Indeed, TNFα and IFNγ, two cytokines critical to IBD pathogenesis, induce IEC apoptosis [72]. Recent studies demonstrated that p53-upregulated modulator of apoptosis (PUMA) is a critical mediator of IEC apoptosis in IBD [73]. PUMA promotes apoptosis in various cell types by p53-dependent and –independent mechanisms. PUMA is a BH-3 domain Bcl-2 family member that binds anti-apoptotic Bcl-2 family members to activate pro-apoptotic members Bax and Bad and triggers mitochondrial dysfunction. This results in the release of several apoptogenic mitochondrial proteins such as cytochrome c, leading to caspase activation and cell death [74]. PUMA is transcriptionally induced by NF-κB in a p53-independent manner to mediate TNFα-induced apoptosis in IECs [73,75].

4 OBJECTIVE OF THE STUDY

Almost all epidemiological and experimental evidence reported in the literature suggests that the vitamin D-VDR signaling pathway plays a protective role against the risk or development of IBD, however, the relative anti-colitic contribution of epithelial vs. immune VDR signaling, remains to be determined. Given the potential importance of colonic VDR in IBD pathogenesis, the VDR status in both CD and UC biopsies in comparison with normal colon samples was examined. To directly address the role of epithelial VDR in the pathogenesis of colitis we also generated transgenic (Tg) mice that overexpress human (h) VDR specifically in intestinal epithelium cells (IECs). Finally, we compared colitis development in wild-type (WT) and Tg mice.

5 MATERIALS AND METHODS

5.1 Human biopsies

Consecutive IBD patients, both CD and UC patients, undergone to colonoscopies for the assess of their inflammatory condition were enrolled. The subjects were recruited in the University of Chicago Medical Center with informed consent. The collection of human biopsies was approved by the Institutional Review Board at the University of Chicago. Non-IBD controls, age- and gender-matched, were patients who underwent screening colonoscopies without active GI pathology. Diagnosis of CD or UC were base on a standard combination of clinical, endoscopical, histological and radiological criteria. Calcium and Vitamin D (25-hydroxyvitamin D) were obtained from all patients and controls. All patients underwent colonoscopy with biopsy specimens from both involved and uninvolved areas. The severity of macroscopic inflammation of the colon mucosal appearances at colonoscopy was graded according to the Mayo score [76] for UC and to the CDEIS sub-score [77] for CD. A minimum of three samples from each site, by using different containers, one tissue sample fixed in buffered formalin for the Immunohistochemical (IS) analysis, one placed in Trizol reagent for RNA preparation and one tissue sample put in dry ice or liquid nitrogen for Western blot (WB) analysis, were taken.

5.1.1 Inclusion and exclusion criteria

The inclusion criteria were (i) age greater than or equal to 18 years; (ii) duration of disease: at last 4 months; (iii) history of colonic involvement for CD patients.

Subjects presenting with any of the following will not be included in the study: (i) pregnant women; (ii) previous proctocolectomy with ileal pouch-anal ansastomisis; (iii) active specific colonic infection: cytomegalovirus infection (CMV) and Clostridium difficile infection.

5.2 Experimental procedures

5.2.1 Production of transgenic (Tg) mice

Flag-hVDR was generated by adding the Flag nucleotide sequence GATTACAAGGATGACGATGACAAG to the N-terminus of hVDR cDNA coding sequence. The Flag-hVDR cDNA (1.3 kb) was placed under the control of the 12.4 kb mouse villin gene promoter [78] (provided by Dr. Gumucio, University of Michigan) followed by SV40 T antigen poly(A) sequence (Fig. 2A). The 14.5 kb PmeI-PmeI DNA construct was purified and used for pronuclear microinjection carried out by the Transgenic Mouse Core Facility at the University of Chicago, using the C57BL/6 genetic background. Pups born from the microinjection were screened by PCR-based genotyping using hVDR-specific primers. Transgene-positive mice were crossed with C57BL/6 mice to obtain germ line transmission. VDR-null mice have been described previously [79]. VDR-null mice that expressed the hVDR transgene (KO-Tg mice) were obtained from crossing Tg and VDR(+/-) mice. These mice were all in C57BL/6 background.

5.2.2 Colitis models

Eight to 12-week old mice were studied using 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced and dextran sulfate sodium (DSS)-induced colitis models as described [80]. For the TNBS model, mice were pre-sensitized by cutaneous expose for one week with TNBS (Sigma) at 40mg/kg dissolved in 50% alcohol. After fasting overnight, the pre-sensitized mice received 10 mg/kg TNBS via rectal injection using a 1-ml syringe fitted with a catheter. For the DSS model, mice were provided 2% DSS (Fisher Scientific) dissolved in the drinking water for one to two cycles, each cycle consisting of 7-day DSS water and 7-day tap water. Body weights, stool consistence and GI bleeding were monitored daily. Clinical scores and colonic damage scores were estimated as detailed previously [81-83]. Colons were collected immediately after sacrifice, and mucosa scraped to isolate total RNA or proteins. Colonic histological analyses were carried out using the “Swiss roll” method [84] or using bread loafing cross sections, and histological scores were graded

according to a previously published system [85]. Colonic TER was determined in freshly harvested colons using Ussing chamber as described [37,85]. All animal studies were approved by the Institutional Animal Care and Use Committee at The University of Chicago.

5.2.3 TUNEL staining

TUNEL staining was performed with ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Millipore, Temecula, CA) according to manufacturer's instruction. Apoptosis was quantified by assessing TUNEL-positive cells in randomly chosen 100 crypts in each mouse.

5.2.4 Cell culture and treatment

HCT116 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Cells were usually treated with or without 100 ng/ml TNFα for 0-72 hours in the presence of 20 nM 1,25(OH)2D3 or ethanol (vehicle) followed by isolation of total RNA or lysate proteins for analyses.

5.2.5 RT-PCR

Total RNAs were extracted using TRIzol reagents (Invitrogen, Carlsbad, CA). Firststrand cDNAs were synthesized using a ThermoscriptRT kit (Invitrogen). Conventional PCR was carried out in a BioRad DNA Engine (BioRad). Real time PCR was performed in a Roche 480 Real-Time PCR System, using SYBR green PCR reagent kits (Clontech). Relative amount of transcripts was calculated using the 2-ΔΔCt formula as described previously [86]. PCR primers are provided in Supplementary Table 1.

5.2.6 Western blot

Proteins were separated by SDS-PAGE and electroblotted onto Immobilon-P membranes. Western blotting analyses were carried out as previously described [87]. The antibodies used in this study

included: VDR, IκBα, IKKβ, IKKα/β (Santa Cruz), β-actin (Sigma), PUMA (Abcam), caspase 3 and p53 (Cell signaling).

5.2.7 Kinase assay

IKK kinase assays were performed as described [88]. Briefly, lysates prepared from HCT116 cells or colonic mucosa were immunoprecipitated with anti-IKKγ antibodies (Santa Cruz). The precipitant was incubated with recombinant GST-IκBα (1-54) (Clontech) in the presence of γ-32P-ATP, and 32P-labelled GST-IκBα (1-54) was detected by autoradiography.

5.2.8 Luciferase reporter assays

HCT116 cells were transfected with PUMA κB luciferase reporter plasmid or its mutant or co-transfected with this plasmid and IKKβ-expressing plasmid, using Lipofectamine 2000 (Invitrogen). After overnight culture the transfected cells were treated with or without TNFα (100 ng/ml) in the presence of 1,25(OH)2D3 (20 nM) or ethanol as indicated in the experiment. Luciferase activity was determined using Luciferase Assay Systems (Promega) as reported previously [89].

5.2.9 Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed as described previously [89], using anti-p65 antibodies. The assays were quantified by real time PCR using primers (see Sup. Table 1) flanking the κB site in the promoter of the *PUMA* gene as described [75].

5.3 Statistical analysis

Data values were presented as means ± SEM. Statistical comparisons were carried out using unpaired two-tailed Student's *t*-test or AVOVA as appropriate, with $P< 0.05$ being considered statistically significant.

6. RESULTS

6.1 Demographics and Disease Characteristics

33 patients (13 F, median age 41 years, range 18-61), 15 with CD, 10 with UC and 8 controls were enrolled. The colonoscopy was performed by two different endoscopists (S.H and M.B.) after premedication with propofol i.v. in 2 cases and with midazolam i.v in the other 31. The endoscopic localization of disease is shown in table 2. Of the 25 IBD patients, only 3, affected by CD, had active disease at colonoscopy: 1 presented with ulcers, 1 with inflammatory stenosis and 1 with erithema (Table 2). In these 3 active CD patients additional specimens from involved area were taken. A large proportion of patients had been treated with immunomodulators and almost half (45%) had received anti-TNF biologic agents.

Table 2 Disease activity according to Mucosal appearance in IBD patients

Pts	Disease	Gender	Disease activity	Mucosal appearence
1	UC	M	inactive	quiescent
2	CD	M	inactive	quiescent
3	CD	F	inactive	stenosis
4	CD	M	inactive	quiescent
5	CD	M	active	ulcers
6	CD	M	inactive	quiescent
7	UC	M	inactive	quiescent
8	UC	M	inactive	quiescent
9	CD	F	inactive	quiescent
10	CD	F	inactive	quiescent
11	UC	F	inactive	quiescent
12	CD	F	inactive	quiescent
13	UC	M	inactive	quiescent
14	CD	F	inactive	quiescent
15	CD	F	inactive	quiescent
16	CD	M	inactive	erithema
17	UC	M	inactive	quiescent
18	CD	F	inactive	quiescent
19	UC	M	inactive	quiescent
20	CD	M	inactive	quiescent
21	UC	F	inactive	quiescent
22	UC	M	inactive	quiescent
23	CD	F	active	quiescent
24	UC	F	inactive	quiescent
25	CD	M	active	deep ulcers

6.2 Colonic epithelial VDR levels are markedly reduced in CD and UC patients

Given the potential importance of colonic VDR in IBD pathogenesis, we examined the VDR status in both CD and UC biopsies in comparison with normal colon samples (Fig. 1A). Massive cDNA microarrays revealed that VDR expression was markedly reduced in the UC biopsies (n>10) relative to normal colon samples (Fig.1B). Consistently, Western blot analyses showed that VDR protein levels were significantly reduced in both CD and UC specimens compared to normal controls (Fig. 1C and 1D). In fact, VDR levels were even lower in active CD biopsies than in quiescent CD biopsies (Fig. 1C), suggesting that inflammation down-regulates epithelial VDR expression. Immunostaining with anti-VDR antibodies revealed strong VDR expression in normal colonic epithelial cells, whereas, epithelial VDR levels were markedly decreased in the UC biopsies (Fig. 1E). These human data demonstrated that colonic epithelial VDR levels were substantially reduced in both CD and UC lesions. We postulated that reduced epithelial VDR in IBD diminishes epithelial barrier function, thus contributing to the development of colitis.

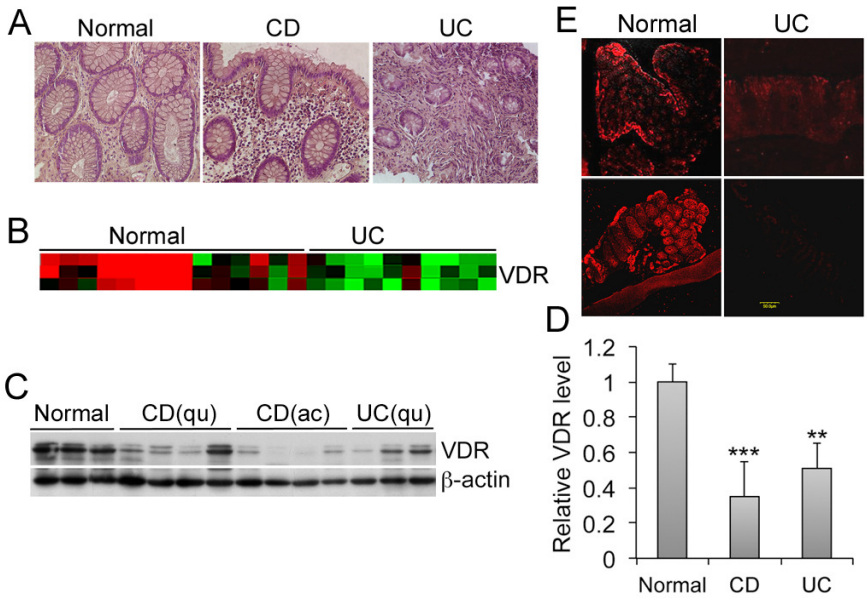

Figure 1. Reduced VDR expression in patients with IBD. (A) Representative H&E histology of colonic biopsies obtained from normal control, CD and UC patients. (B) cDNA microarray heatmap of VDR expression levels in normal and UC biopsies. (C and D) Western blot analyses (C) and densitometric quantitation (D) of VDR protein levels in normal and active (ac) or quiescent (qu) CD and UC lesions. ** P<0.05, *** P<0.001 vs. normal, n=5-12. (E) VDR immunostaining of normal and quiescent UC lesions.

6.3 Generation of transgenic mice that overexpress hVDR in intestinal epithelium

To directly address the role of epithelial VDR we generated transgenic (Tg) mice that overexpress human (h) VDR specifically in gut IECs. We used the 12.4 kb villin promoter to drive a Flag-tagged hVDR transgene (Fig.2A). The villin promoter has been used to target various transgenes to the intestinal epithelium [78]. The Flag-tag, added to the N-terminus of hVDR, had no effect on the transactivating activity of hVDR, but distinguished the human transgene from the endogenous mouse VDR. Pronuclear injection of the 14.5 kb PmeI construct (Fig. 2A) resulted in 4 transgene positive founders. Three founder lines were mated with wild-type (WT) mice to obtain germline transmission. Transgenic lines 3 and 14 were used in this study with comparable results. Western analyses with anti-Flag antibody revealed high expression levels of Flag-hVDR from the jejunum to distal colon in Tg mice. As reported the transgene expression in the duodenum was low [78]. As expected WT littermates were negative for the Flag tag (Fig. 2B). Immunostaining with anti-Flag antibodies confirmed that hVDR was highly expressed in the epithelial cells in the small intestine (Fig.2C) and colon (Fig. 2D), with higher hVDR levels observed in the luminal side of the crypt (Fig. 2D). No differences were detected in crypt morphology or cellular proliferation rates between WT and Tg colons, as revealed by histology and BrdU labeling (Fig. 2E).

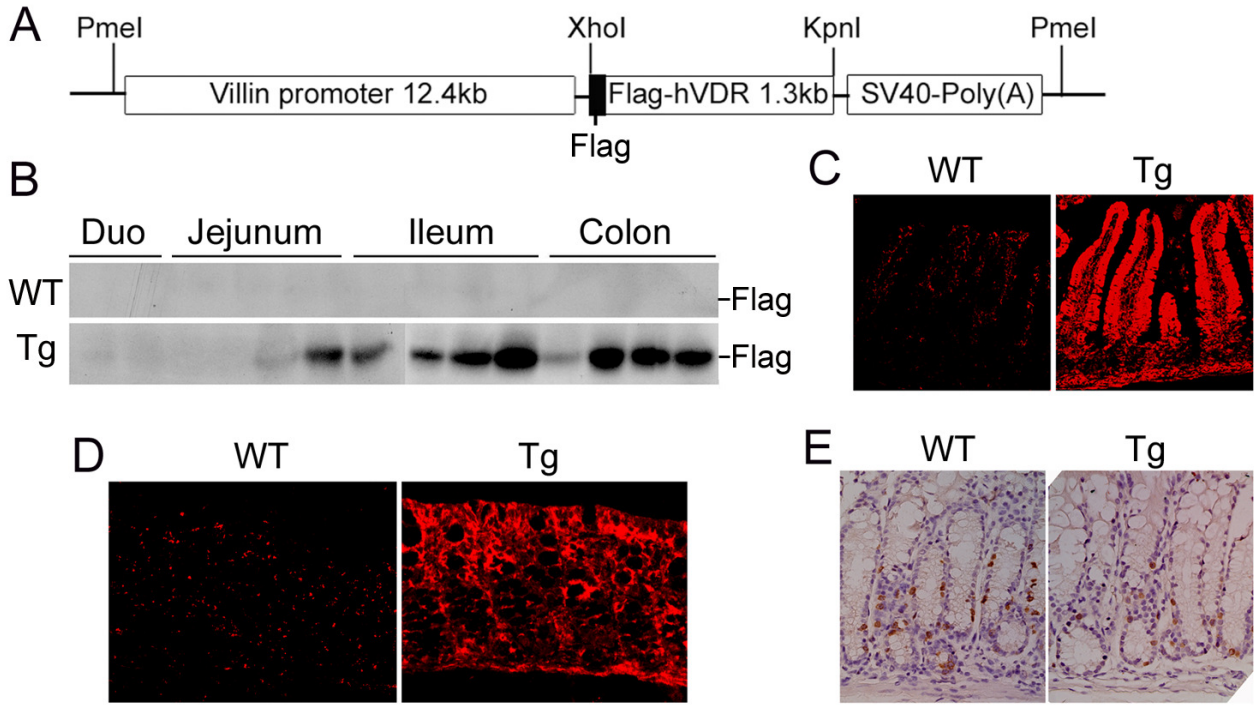

Figure 2. Characterization of hVDR transgenic mice. (A) DNA construct used for pronuclear microinjection to generate hVDR transgenic mice. (B) Western blot using anti-Flag antibodies to detect Flag-hVDR in small and large intestines in wild-type (WT) and transgenic (Tg) mice. Each lane represents 2-3 cm of intestine in the regions indicated. Duo, duodenum; Flag, Flag-hVDR. (C) Immunostaining of small intestine (Jejunum) with anti-Flag antibody; (D) Immunostaining of large intestine with anti-Flag antibody. (E) BrdU staining of large intestine from WT and Tg mice.

6.4 Epithelial VDR overexpression protects mice from colitis

We compared colitis development in WT and Tg mice using two widely employed models of experimental colitis, 2,4,6-trinitrobenzene sulfonic acid (TNBS)- and dextran sulfate sodium (DSS)-induced colitis. The TNBS colitis model is thought to resemble Crohn's disease because the model involves TH1-mediated mucosal inflammation [90]. We subjected WT and Tg littermates to TNBS treatment as previously described [80]. Intrarectal instillation of TNBS resulted in gradual weight loss in WT mice during the following 6 days, whereas the weight loss was ameliorated in Tg mice in the first 2 days and quickly recovered in the following days (Fig. 3A). By day 6, WT mice were symptomatic with severe colitis disease activity scores, and the colon appeared markedly shortened and thickened. In contrast, gross examinations of the large bowel from Tg mice showed few signs of colitis (Fig. 3B). Histological examinations confirmed loss of crypts, severe focal ulceration and inflammation with infiltration of immune cells in WT colons, along with thickened colonic walls,

whereas the Tg colons showed little abnormalities (Fig. 3C). Tg mice had significantly lower colon to body weight ratios (Fig. 3D), lower colonic damage scores (Fig. 3E) and lower histological inflammation scores (Fig. 3F) compared to WT mice on day 6. To gain insight into the permeability of the colonic mucosa after TNBS injury, we used the Ussing chamber to measure trans-epithelial electric resistance (TER) of the colon obtained from WT and Tg mice two days after TNBS treatment. At this time point no clinical symptoms or histological damage were observed in either genotype. While there was marked reduction of TER in WT mice in both the distal (Fig. 3G) and proximal (Fig. 3H) colon, the TER in Tg mice was preserved. This result indicates that the epithelial hVDR provides protective effects on mucosal barrier integrity. We also examined colonic epithelial tight junctions. Immunostaining revealed diminished ZO-1 in focal regions in WT mice, whereas Tg mice maintained a normal ZO-1 pattern in the colonic epithelium (Fig. 4A). Real time PCR quantitation showed that Tg mice maintained relatively higher ZO-1, occludin 1 and claudin-5 transcript levels compared to WT mice, whereas transcripts of claudin-2, known to be induced by inflammatory cytokines and to impair tight junction function [91], was suppressed in Tg mice (Fig. 4B). Consistently, mucosal transcript levels of pro-inflammatory TNFα, IL-1β, IL-12, MCP-1 and MIP-1 were substantially lower in Tg mice on day 6 (Fig. 4C), confirming that Tg colons were less inflamed than WT colons.

The DSS-induced experimental colitis resembles human ulcerative colitis with respect to loss of barrier function [92]. We subjected WT and Tg mice to DSS treatment to further assess the anti-colitic activity of the epithelial VDR signaling. WT and Tg mice received two cycles of DSS treatment, each cycle consisting of 7-day 2% DSS in the drinking water followed by 7-day tap water alone. In the second DSS cycle, clinical scores were assessed daily [81] (Fig. 5A). WT mice developed clinical symptoms of colitis with increasing severity in the second cycle starting on day 3 and peaking on day 9, whereas symptoms in Tg mice were delayed and significantly reduced in severity (Fig. 5B). Histological examination of the colon confirmed a marked decrease in immune cell infiltration and ulceration in Tg mice (Fig. 5C), with significantly lower histological scores on

day 4 and 10 in the second DSS cycle (Fig. 5D). Transcript levels of inflammatory cytokines were also lower in colons of Tg mice compared to wild type mice (not shown). Taken together, the data obtained from the TNBS and DSS models provide strong evidence that epithelial VDR signals protect the colonic epithelial mucosal barrier and inhibit the development of colitis.

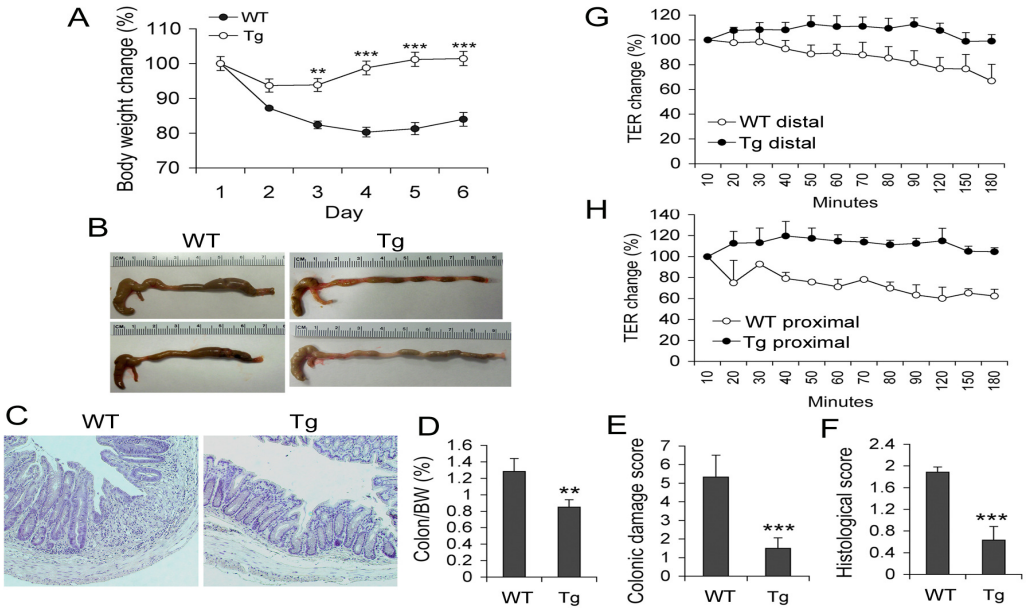

Figure 3. Epithelial cell hVDR protects against TNBS colitis. (A) Changes of body weight (% of original body weight) in WT and Tg mice following TNBS treatment. (B) Gross morphology of the large intestine from WT and Tg mice on day 6 after TNBS treatment. (C) H&E staining of colons from WT and Tg mice on day 6 after TNBS treatment. Note the mucosal ulceration in WT colon. (D-F) Colon weight to body weight ratio (D), Colonic damage score (E), and Histological score (F), of WT and Tg mice on day 6 after TNBS treatment. (G and H) Time courses of trans-epithelial resistance (TER), determined by Ussing chamber, in the distal (G) or proximal (H) colon on day 2 after TNBS treatment in WT and Tg mice.

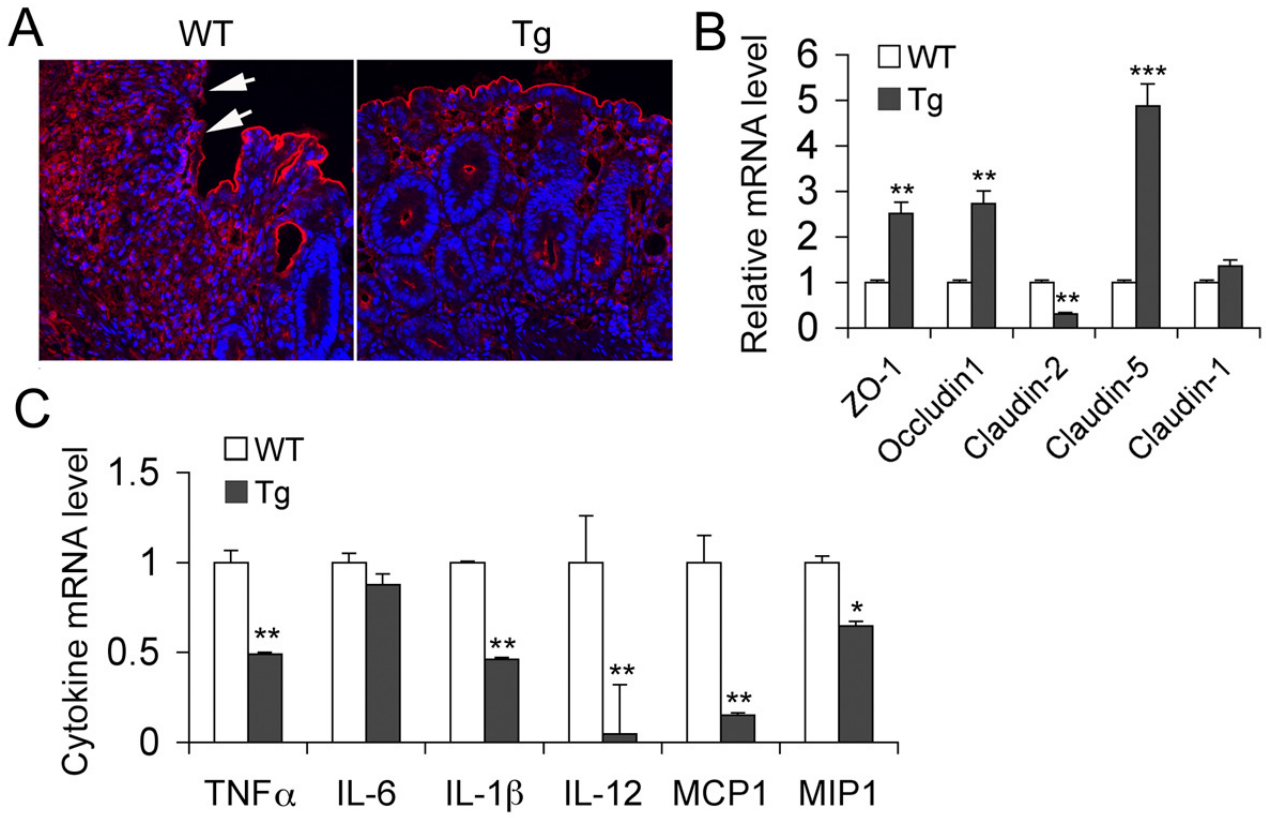

Figure 4. Expression of hVDR in intestinal epithelial cells preserves colonic epithelial tight junction and suppresses colonic inflammation in TNBS model. (A) ZO-1 immunostaining (red) in WT and Tg colons on day 6 after TNBS treatment. Arrows indicate the loss of ZO-1 protein in the luminal epithelia in WT mice. (B) Real time RT-PCR quantitation of transcripts of colonic tight junction proteins in WT and Tg mice. (C) Real time RT-PCR quantitation of pro-inflammatory cytokines and chemokines in colonic mucosa from WT and Tg colons on day 6 after TNBS treatment. *P<0.05; ** P<0.01; *** P<0001 vs. WT. n=5.

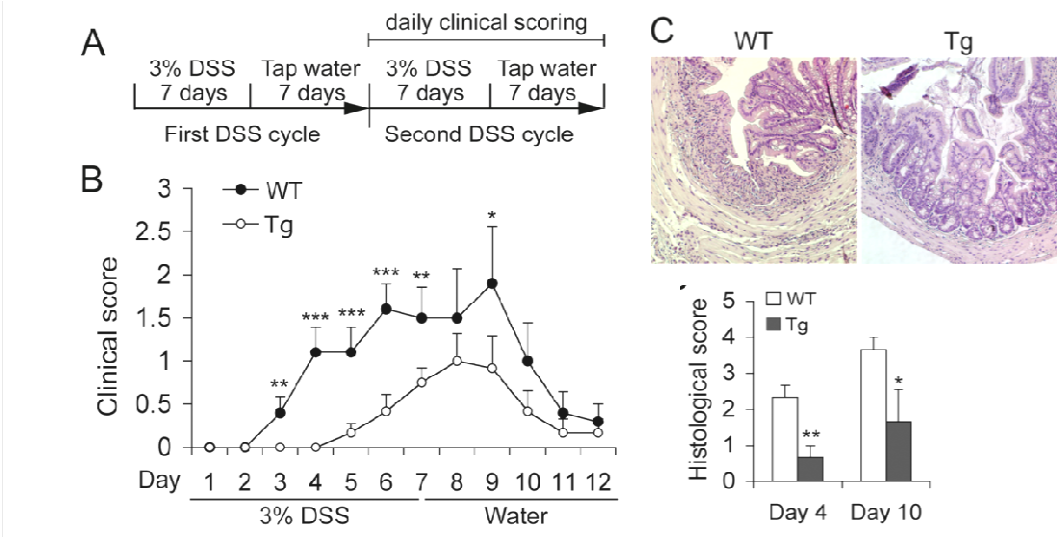

Figure 5. Epithelial hVDR expression inhibits DSS-induced colitis. (A) Schematic illustration of DSS treatment protocol; (B) Time course of disease activity index in WT and Tg mice during the second DSS cycle. (C) H&E stained colonic sections from WT and Tg mice on day 10 of the second DSS cycle. Note the colonic mucosal ulceration in WT mice. (D) Histologic scores of WT and Tg colons on day 4 and day 10 in the second DSS cycle. * P<0.05; ** P<0.01 vs. corresponding WT; n=5-6.

6.5 Reconstitution of intestinal epithelium with hVDR rescues VDR-null mice from colitis and death

To further assess the anti-colitic activity of the epithelial VDR signaling, we asked whether reconstituting the IECs of VDR-null mice with the hVDR transgene would prevent VDR-null mice from developing colitis. To this end, we generated, through breeding, VDR-/- (KO) mice that expressed hVDR only in the IECs (designated as KO-Tg mice). We examined WT, Tg, VDRKO and KO-Tg mice in parallel using TNBS and DSS colitis models. In the TNBS model, VDRKO mice developed dramatic weight loss and all died by day 5 (Fig. 6A and 6B). Colons from VDRKO mice were markedly shortened and swollen (Fig. 6C). Histological examination revealed severe ulcerations with complete crypt depletion in the distal colon (Fig. 6D). As expected, this phenotype was more severe than that of WT mice. Importantly, KO-Tg mice showed little weight loss, only mild colitis and no deaths, and their colons had almost normal gross and histological morphologies (Fig. 6A-C). Consistently, among these four genotypes, VDRKO mice had the highest colonic damage score (Fig. 6E), histological score (Fig. 6F) and myeloperoxidase (MPO) activity (Fig. 6G). Again, these severe colonic injury and inflammation phenotypes were almost completely "corrected" in KO-Tg mice (Fig. 6D-F). Similar results were obtained in the DSS colitis model. In this model, the hVDR transgene was able to substantially reduce animal mortality and clinical scores and markedly attenuated the colonic injury relative to VDRKO mice. Taken together, these data demonstrate that the hVDR transgene was able to rescue the severe colitis phenotype of VDRKO mice despite the absence of VDR in immune cells, demonstrating a critical role for epithelial VDR signaling to protect against experimental colitis.

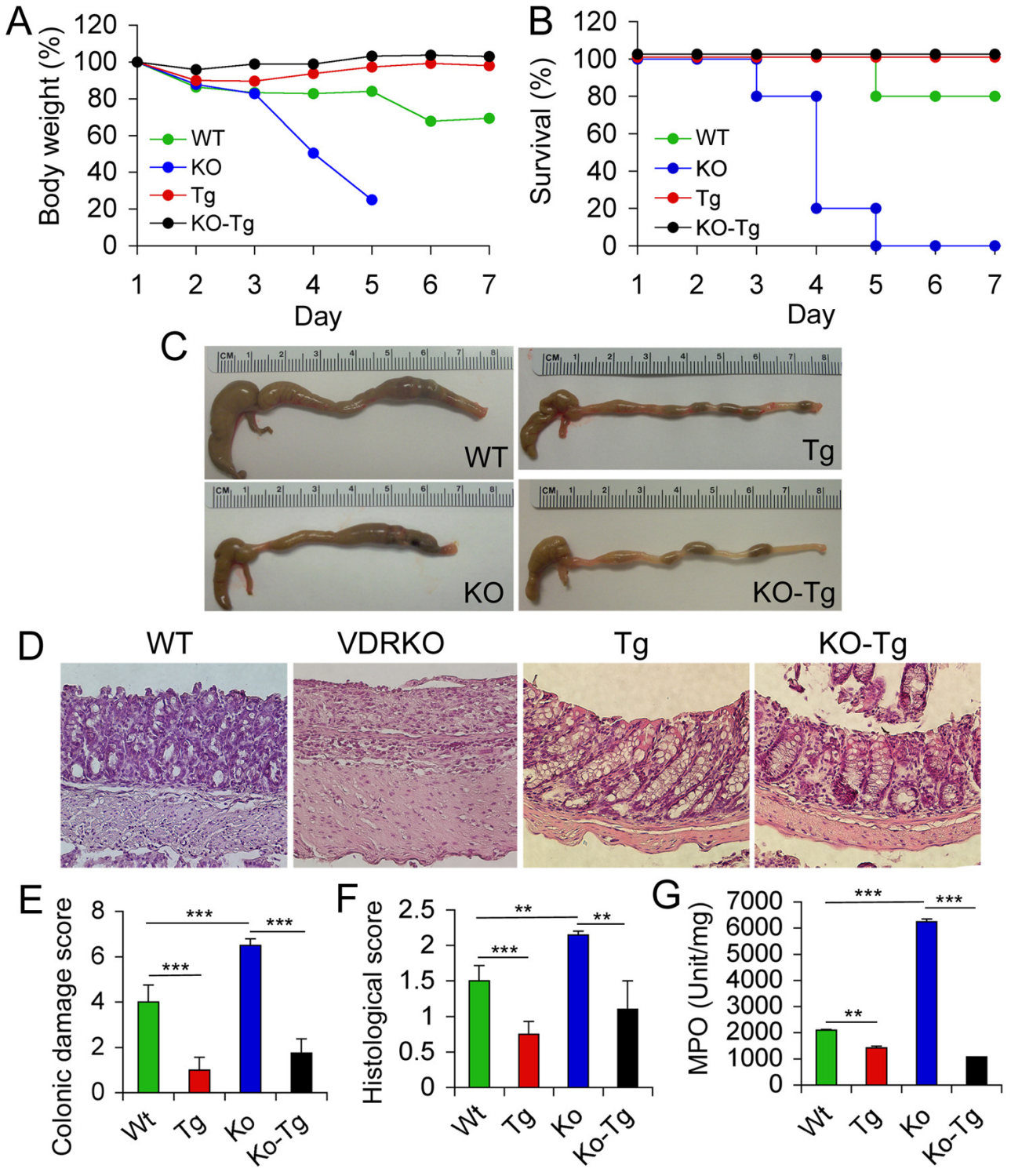

Figure 6. Reconstitution of intestinal epithelial cells with the hVDR transgene rescues VDR-null mice from colitis and death. WT, VDRKO, Tg and KO-Tg mice were studied in parallel using the TNBS colitis model. (A) Body weight changes. (B) Survival curve. (C) Gross morphology of the large intestines on day 7 after TNBS treatment. (D) H&E histology of the colons on day 7 after TNBS treatment. (E) Colonic damage score; (F) Histological score; and (G) Myeloperoxidase (MPO) activity on day 7. **P<0.01, *** P<0.001. n=5-7 in each genotype.

6.6 Epithelial VDR signaling inhibits IEC apoptosis by suppressing PUMA

Increased apoptosis in gut epithelial cells compromises the mucosal barrier leading to colonic inflammation. By TUNEL staining we observed abundant apoptotic colonic epithelial cells in WT mice that were further increased in VDRKO mice after TNBS insult, whereas apoptotic epithelial cells were markedly reduced in Tg and KO-Tg mice (Fig. 7A and 7B). In agreement with these observations, caspase 3 cleavage was increased in colonic mucosal lysates from WT and VDRKO mice compared to Tg and KO-Tg mice (Fig. 7C and 7D). PUMA, a key mediator of IEC apoptosis, was up-regulated in WT mice and even more so in VDRKO mice compared to Tg and KO-Tg mice (Fig. 7C and 7D). In contrast, levels of p53, another important regulator of apoptosis, were not altered in these mice (Fig. 7C and 7D). In the DSS model, PUMA induction and caspase 3 activation were also attenuated in Tg mice. Supporting the relevance of this apoptotic pathway in colitis, PUMA was also up-regulated in human CD biopsies. Taken together, these observations suggest that epithelial VDR signals reduce colonocyte apoptosis by down-regulating PUMA in a p53-independent manner.

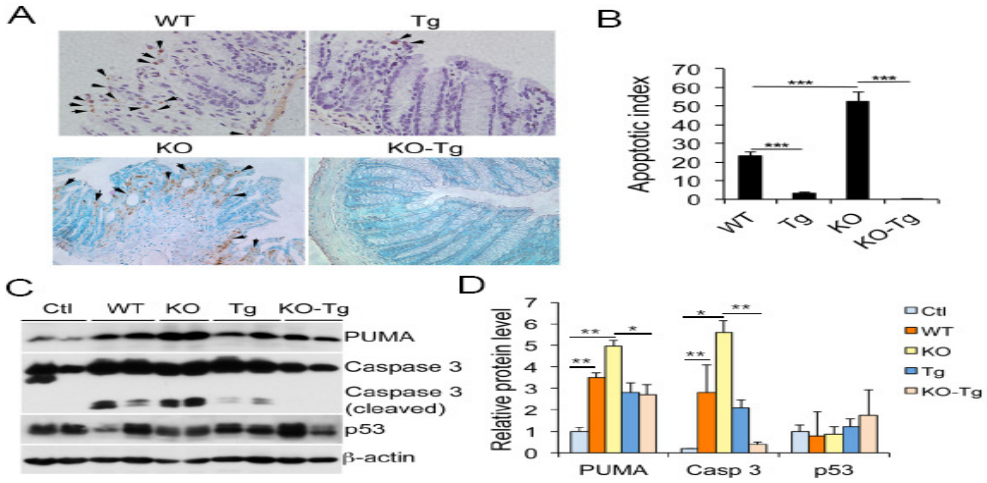

Figure 7. Epithelial VDR signaling attenuates IEC apoptosis. WT, VDRKO, Tg and KO-Tg mice were studied in parallel using the TNBS colitis model. (A) Representative TUNEL staining of WT, Tg, VDRKO and KO-Tg colon at day 4 after TNBS treatment. (B) Semi-quantitative assessment of IEC apoptosis. Apoptotic index is the number of TUNEL-positive crypts among 100 crypts randomly chosen in each genotype. *** P<0.001. (C and D) Western blot analyses (C) and densitometric quantitation (D) of colonic mucosal levels of PUMA, caspase 3 and p53 proteins in TNBS-treated mice on day 2. Ctl, non-TBNS-treated control. * P<0.05, ** P<0.01.

6.7 Epithelial VDR signaling down-regulates PUMA by blocking NF-κB activation

We used a cell culture systems to explore the VDR-dependent mechanism involved in PUMA regulation. In HCT116 cells, a human colonic cancer cell line, TNFα markedly induced PUMA that was abrogated by 1,25(OH)2D3 (Fig. 8A). PUMA is regulated by NF-κB, and a functional *cis*-κB site has been identified in the *PUMA* gene promoter [73,75] (Fig. 8B). ChIP assays showed that 1,25(OH)2D3 blocked TNFα- induced p65 binding to this κB site in HCT116 cells (Fig. 8C), and electrophoretic mobility shift assays confirmed that 1,25(OH)2D3 attenuated TNFα-induced NF-κB binding to the PUMA κB probe in HCT116 nuclear extracts. In luciferase reporter assays, when HCT116 cells were transfected with canonical NF-κB luciferase reporter plasmid, 1,25(OH)2D3 suppressed TNFα-induced NF-κB activity. In HCT116 cells transfected with a luciferase reporter that contains the PUMA κB site or its mutant (Fig. 8B) [75], 1,25(OH)2D3 treatment inhibited TNFα-induced luciferase activity for the wild-type reporter. For the mutant reporter, neither TNFα nor 1,25(OH)2D3 had any effects on the luciferase activity (Fig. 8D). To validate that the luciferase activity was mediated by the PUMA κB element, we co-transfected the cells with the PUMA κB reporter or its mutant and an IKKβ- expressing plasmid. IKKβ dramatically induced luciferase activity of the wild-type reporter, but not the mutant reporter, and this induction was also markedly abrogated by 1,25(OH)2D3 (Fig. 8E). IKK kinase assays showed that 1,25(OH)2D3 blocked TNFα-induced IKK activity to phosphorylate IκBα in HCT116 cells (Fig. 8F). To demonstrate that the epithelial VDR signaling inhibits NF-κB activation *in vivo*, we compared the colonic mucosal IKK kinase activity between WT and Tg mice. TNBS induced the mucosal IKK kinase activity, which was accompanied by PUMA induction and caspase 3 activation in WT mice, but these events were substantially attenuated in Tg mice, (Fig. 8G). Taken together, these *in vitro* and *in vivo* data provide compelling evidence that the epithelial VDR signaling inhibit inflammation-induced PUMA via blockade of NF-κB activation, leading to reduction in colonic epithelial cell apoptosis.

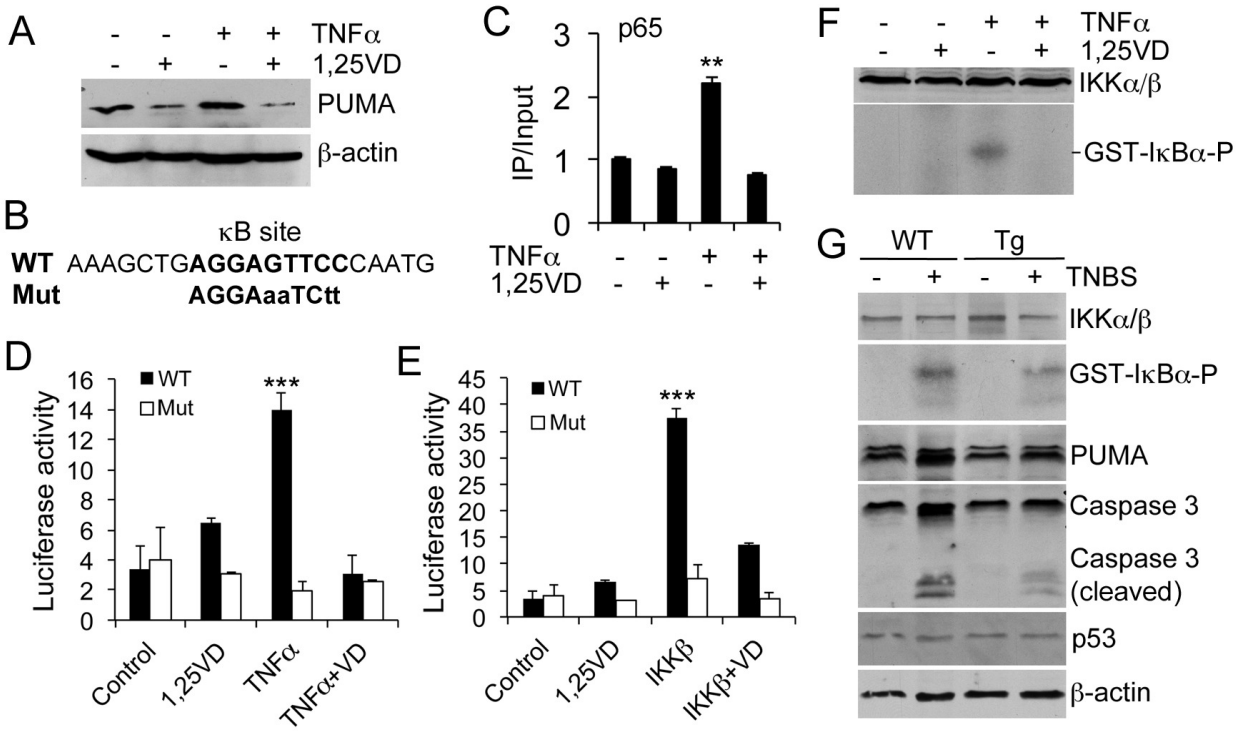

Figure 8. VDR signaling abrogates PUMA induction by blocking NF-κB activation. (A) Western blot analysis of PUMA in HCT116 cells treated with or without TNFα (100 ng/ml) overnight in the presence of 1,25(OH)2D3 (20 nM) or ethanol (vehicle). (B) Sequence of the κB cis-element in the PUMA gene promoter and its mutant. (C) ChIP assay in HCT116 cells with anti-p65 antibody. *** P<0.001 vs. the rest. (D) HCT116 cells transfected with the PUMA κB-reporter wild-type (WT) or mutant (Mut) plasmid were treated with or without TNFα (100 ng/ml) overnight in the presence of 1,25(OH)2D3 (20 nM) or ethanol (vehicle). PUMA promoter activation was then assessed by luciferase activity. (E) HCT116 cells were co-transfected with the WT or Mut PUMA κB-reporter plasmid and IKKβ-expressing plasmid or empty vector. After treatment with 1,25(OH)2D3 or ethanol (vehicle) overnight luciferase activity was determined. *** P<0.001 vs. other conditions. (F) IKK kinase assays in HCT116 cells treated with or without TNFα in the presence of 1,25(OH)2D3 or vehicle. (G) Colonic mucosa were isolated from TNBS-treated mice on day 2 and the lysates were subjected to IKK kinase assays and Western blot analyses for IKKα/β, PUMA, caspase 3 and p53 proteins. Each lane represents a pool of 4-5 mice of the same genotype and treatment.

7. DISCUSSION

In this study we reported that the epithelial VDR levels were substantially reduced in patients with CD and UC and demonstrated in experimental colitis models that the intestinal epithelial VDR signaling has potent anti-colitic activity that is independent of the immune VDR signaling. The anti-colitic mechanism of the epithelial VDR signaling, at least in part, is due to inhibition of colonocyte apoptosis through down-regulation of PUMA, a key pro-apoptotic protein. This action results in protection of the colonic mucosal barrier. Vitamin D-deficiency is prevalent in IBD. As vitamin D synthesis relies on sunlight and vitamin D can be obtained from dietary sources, it is thought that vitamin D might be an environmental factor affecting the development of IBD; however, whether low vitamin D status plays a causative role in the pathogenesis of IBD in humans is unclear. Data from animal models indicate that vitamin D can inhibit the development of colitis [25,36,37].

As VDR is expressed in both the epithelial and immune compartments of the colon, the relative anti-colitic contribution of the epithelial and immune VDR signaling is unknown. Given the distinct roles of the epithelial and immune components in colonic biology, this is an important question that warrants careful investigation. Our observation that epithelial VDR is substantially reduced in the diseased colon in both CD and UC patients provides a strong rationale to pursue the role of the epithelial VDR signaling. By taking a genetic approach, we generated transgenic mice that expressed Flag-hVDR in gut epithelial cells of both small and large intestines. This transgene did not appear to alter crypt morphology or cellular proliferation. We showed that this epithelial hVDR expression rendered mice highly resistant to colitis in both TNBS and DSS colitis models. Compared to their WT counterparts, Tg mice showed much less colonic injury as assessed at clinical, morphological, histological and molecular levels. While at the early stage WT mice exhibited increased mucosal permeability even prior to detectable morphological and histological abnormalities, Tg mice maintained relatively normal permeability. This colitis-resistant phenotype induced by the hVDR transgene includes increased expression of colonic tight junction proteins. Taken together, these data suggest that the potent anti-colitic activity of epithelial VDR, at least in

part, derives from protecting the integrity of the colonic epithelial barrier. As the TNBS and DSS models resemble CD and UC in humans, these transgenic data have potential clinical relevance.

Kong et al. showed that mice with global VDR inactivation developed very severe colitis in the DSS model [37]. If the anti-colitic activity of the epithelial VDR is an essential protective mechanism, we reasoned that reconstitution of VDR-null IECs with the hVDR transgene might rescue the severe colitic phenotype of VDR-null mice. Indeed, we showed that while VDR-null mice developed very severe colitis leading to high mortality following either TNBS or DSS insult, the VDR-null mice that expressed hVDR only in the gut epithelial cells (KO-Tg) were resistant to mucosal ulcerations in both experimental colitis models. In contrast to the VDR-null mice, the KO-Tg mice not only maintained relatively normal crypt architecture, but also demonstrated reduced colonic inflammation. Since the KOTg mice still have a VDR-null immune system, these results indicate that the epithelial VDR signaling is sufficient to suppress colitis regardless of the VDR status in the immune cells. It is well established that vitamin D has potent immune-modulatory activities [93,94], and previous studies suggested that the VDR-null immune system is the main cause for the robust colitic phenotype seen in IL-10/VDR double mutant mice [25]. IBD is a chronic inflammatory disorder, and there is little doubt that, at least in animal models, vitamin D can exert anti-colitic effects by downgrading pro-inflammatory immune reactions, mediated by the immune VDR. As such, in vitamin D-deficiency or when VDR is inactivated in the immune system, the colonic immune components are more reactive to invading colonic antigens or bacteria leading to more severe colitis.

Our study suggests that the epithelial VDR suppresses immune response to colonic antigens and bacteria by maintaining an intact and functional mucosal barrier. As such, in KO-Tg mice the reconstituted epithelial hVDR preserves the integrity of the mucosal barrier, thereby preventing the hyper-immune response otherwise seen in VDRnull mice. These data demonstrate for the first time that the anti-colitic activity of the epithelial VDR is, to some extent, independent of the immune VDR status. Therefore, the reduction of epithelial VDR observed in IBD patients is predicted to

compromise the epithelial barrier and thus contribute to the development of IBD. Therefore, vitamin D-deficiency is likely pathogenic for IBD as it not only potentiates the immune system for hyper-responsiveness but also weakens the mucosal barrier.

A key question is how the epithelial VDR protects the mucosal barrier? Our investigations demonstrated that the epithelial VDR signaling inhibits colonocyte apoptosis by down-regulating proapoptotic PUMA. It is well understood that increased gut epithelial apoptosis compromises the mucosal barrier and increases mucosal permeability. In fact, increased IEC apoptosis has been documented in patients with IBD [67-69]. As reported previously [73], we detected marked elevation in caspase 3 activation and colonocyte apoptosis in the models of experimental colitis. The increased colonic epithelial apoptosis depended on PUMA induction, but appeared to be p53-independent [73]. Consistent with the anti-colitic effects, epithelial expression of hVDR in either VDR wild-type or null mice markedly suppresses PUMA up-regulation and caspase 3 activation, leading to reduction in IEC apoptosis.

Since PUMA is a key inducer of gut epithelial apoptosis, we embarked on studies to dissect the molecular mechanism whereby 1,25(OH)2D3 down-regulates PUMA expression. In intestinal epithelial cells NF-κB mediates inflammation-induced PUMA up-regulation [75]. Here we demonstrated that 1,25(OH)2D3 abrogates PUMA up-regulation by blocking NF-κB activation. This conclusion is based on several lines of *in vitro* and *in vivo* evidence. In HCT116 cells 1,25(OH)2D3 inhibits TNFα-induced PUMA protein and *PUMA* promoter activity, and this inhibition is mediated by the κB *cis*-DNA element in *PUMA* gene promoter. To directly link vitamin D actions with blockade of NF-κB activation, we showed that 1,25(OH)2D3 inhibits IKKβ-induced *PUMA* promoter activity and TNFα-induced IKK kinase activity, and disrupts TNFα-induced p65 binding to the PUMA κB site. Importantly, we showed that hVDR overexpression in colonocytes significantly inhibited IKK kinase activity in colonic mucosa, together with inhibition of PUMA expression and caspase 3 activation. These data confirm that VDR signaling suppresses

epithelial NF-κB activity *in vivo*, which provide new insights into the function of epithelial VDR in colonic homeostasis.

In conclusion, in this report we provide strong evidence that epithelial VDR signals inhibit colitis by protecting the mucosal epithelial barrier, and this anti-colitic activity is independent of VDR status in the immune system. Given the relatively deficient VDR status observed in IBD patients, the new knowledge obtained from this study is relevant and significant. This knowledge might be useful for designing new preventive and/or therapeutic strategies for more effective treatment of IBD.

Acknowledgements

This study was supported by NIH grant HL085793 and research grants from Gastrointestinal Research Foundation (GIRF) Associate Board, Foundation for Clinical Research in Inflammatory Bowel Disease (FCRIBD) and International Organization for the Study of Inflammatory Bowel Disease (IOIBD). The production of villin-hVDR transgenic mice was supported in part by CTSA grant UL1 RR024999 from National Center for Research Resources (NCRR). The University of Chicago Transgenic Mouse Core Facility was supported in part by NIH grant 5P30CA014599-36.

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