Vitamin D attenuates inflammation in CFTR knockdown intestinal epithelial cells but has no effect in cells with intact CFTR

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Vitamin D is a fat-soluble vitamin that is increasingly recognized for its immunomodulatory and anti-inflammatory properties. Vitamin D deficiency, either caused by genetic or diet-induced means, leads to low-grade intestinal inflammation, increased susceptibility to chemically induced colitis, and, more recently, has been shown to decrease autophagy (4, 17, 45). Conversely, administration of the active form of vitamin D, 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃], reduced the severity of intestinal inflammation in a mouse model with inflammatory bowel disease (49). Mechanisms postulated to account for the protective action of vitamin D include effects on gut epithelial and immune cells (10), integrity of the intercellular tight junctions (48), and gut microbiota composition (32).

Apart from maldigestion and malabsorption, chronic intestinal inflammation is a feature of cystic fibrosis (CF) and has been documented in both patients (33, 40, 43) and mice (31). The pathogenesis of CF intestinal inflammation still remains minimally understood but slow clearance of mucus along with its intestinal accumulation; an increased intestinal permeability, thus allowing a prolonged contact between the luminal contents and the mucosal immune system; and intestinal dysbiosis have all been suggested as contributing factors (29, 33). Recent studies performed on intestinal cells genetically depleted of CF transmembrane conductance regulator (CFTR), the mutated gene in CF, have provided insights on the involvement of other pathogenic factors. CFTR knockdown of intestinal cells induced a proinflammatory status thereby suggesting that the loss of intestinal CFTR is associated with exaggerated inflammatory responses (13). In support of this, Raia et al. (33) have reported the absence of duodenal inflammation in non-CF patients with chronic pancreatitis and pancreatic insufficiency, thus further implying the involvement of a factor intrinsically related to CF. Although CF intestinal inflammation may adversely affect intestinal absorption further compromising nutritional, and hence pulmonary status of CF patients, studies on anti-inflammatory therapies have been limited to the use of laxatives and the synthetic eicosanoid lubiprostone in CF mice (20, 21) as well as probiotics in patients (8, 9). Furthermore, intestinal inflammation is a recognized risk factor for colon cancer in other chronic inflammatory disorders (36) and a higher rate of gastrointestinal cancers has been reported in CF compared with non-CF individuals (26). Whether chronic intestinal inflammation leads to the development of malignancies in CF is unknown but certainly stresses the importance of finding additional means to control this inflammation.

Owing to the malabsorption of fat-soluble vitamins, CF patients are at risk of vitamin D deficiency and are thus routinely supplemented with pharmacological amounts of vitamin D. Given the mounting evidence that vitamin D exerts intestinal anti-inflammatory effects combined with the fact that the intestinal tract of these patients is exposed to significantly larger amounts of vitamin D compounds than any other organ and possesses all the enzymatic machinery to fully metabolize vitamin D into its bioactive form, we believe that vitamin D may prove beneficial in the management of CF intestinal inflammation. Using CFTR knockdown intestinal epithelial cells, we tested the hypothesis that the oral form of vitamin D (vitamin D₃ or cholecalciferol) and its metabolites, 25-hydroxyvitamin D₃ (25OHD₃) and 1,25(OH)₂D₃, attenuate the


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inflammation originally reported in these cells (13). In addition, we explored the intracellular pathways through which vitamin D and its metabolites exert their anti-inflammatory actions on intestinal epithelial cells. Despite the fact that both 25OHD₃ and 1,25(OH)₂D₃ display anti-inflammatory activity by targeting common intracellular pathways, we show that 1,25(OH)₂D₃ is the only compound that ultimately inhibits cytokine-induced nuclear translocation of NF-κB and interleukin-8 (IL-8) secretion of CFTR knockdown intestinal epithelial cells. Intriguingly, the anti-inflammatory effects of vitamin D were only observed in CFTR knockdown Caco-2/15 cells, an observation that was further confirmed in Cfr-null mice.

MATERIALS AND METHODS

Cell culture. The intestinal cell line Caco-2/15, a stable clone derived from the cell line Caco-2 [American Type Culture Collection (ATCC), Manassas, VA] was provided by Dr. Emile Levy [Centre Hospitalier Universitaire (CHU) Sainte-Justine Research Center] while HT-29 cells were purchased from ATCC. Cells were cultured as described previously (13, 24, 25) and were used in a proliferating subconfluent stage (80–90% confluence) for all experiments.

CFTR knockdown. CFTR knockdown of Caco-2/15 and HT-29 cells was described in detail previously (13, 24, 25). Briefly, to produce lentiviral stocks, HEK293FT cells were cotransfected with lentiviral-encoding plasmids and control scrambled short hairpin RNA (shRNA; Addgene, Cambridge, MA) or shRNA directed against CFTR (Open Biosystems, Huntsville, AL). Supernatants containing the lentivirus were collected and centrifuged 48 h posttransfection and concentrated using an ultracentrifugation membrane (Amicon Ultra-15 centrifugal filter device, EMD Millipore, Billerica, MA). Cells were infected with the lentivirus in the presence of hexadimethrine bromide (Polybrene; Sigma, St. Louis, MO), a cationic polynucleotides known to enhance infection efficiency, and grown in the presence of puromycin (Wisent, St-Bruno, QC, Canada) for selection. Gene and protein knockdown were confirmed by quantitative PCR (Q-PCR) and Western blot and were found to be 52 and 39% for Caco-2/15 cells and 88 and 69%, respectively, for HT-29 cells (13). Cells infected with a scrambled shRNA served as controls as we previously demonstrated comparable cell viability and integrity as well as gene and protein CFTR expression between scrambled and noninfected Caco-2/15 cells (Table 1 and Ref. 25).

Cell treatments. To induce proinflammatory responses, serum-starved Caco-2/15 cells were treated with 25 ng/ml of human recombinant TNF-α or IL-1β (both from PeproTech, Quebec, Canada) for short (30 to 90 min) or long durations (8 and 24 h) in the presence or absence of 10⁻⁶ M of cholecalciferol (vitamin D₃), 10⁻⁶ M of 25OHD₃, or 10⁻⁷ M of 1,25(OH)₂D₃ (all from Sigma). Cells treated with 0.4% of ethanol (vehicle) served as negative controls. HT-29 experiments were carried out in the presence of serum, as these cells exhibited higher cell mortality in its absence and were subjected to treatments with 10 ng/ml of TNF-α or IL-1β. Trypan blue staining and cell counting were performed for all experimental conditions to rule out any effect on cell viability. The p38MAPK-selective inhibitor BIRB796 (Selleckchem, Houston, TX) was used in a subset of experiments. BIRB796 was dissolved in DMSO to a final concentration of 19 mM and diluted in the culture medium at the desired concentration. Cells were pretreated with the inhibitor for 30 min before the addition of the proinflammatory cytokines.

RNA extraction, RT-PCR, and Q-PCR. Total RNA was isolated with Trizol and reverse transcribed using the Moloney murine leukemia virus (MMLV) enzyme according to manufacturer’s instructions (Life Technologies, Burlington, ON, Canada). Interleukin-8 (IL-8), vitamin D nuclear receptor (VDR), CYP27B1, and CYP24A1 mRNA expressions were determined by Q-PCR. Briefly, a 15-μl Q-PCR reaction, containing cDNA diluted 100 times, 1X SoFast EvaGreen Supermix with Low ROX (Bio-Rad, Mississauga, ON, Canada) and 0.5 μM of IL-8, VDR, CYP27B1, and CYP24A1 specific and intron-spanning primers, was performed using the MX3000p Q-PCR System (Agilent Technologies, Santa Clara, CA) under the following conditions: 95°C for 30 s, 45 cycles of 95°C for 5 s, 60°C for 30 s, and 72°C for 20 s. Each reaction was performed in duplicate. The relative quantification of each gene was normalized to the 60S ribosomal protein L27 (RPL27) reference gene and fold induction was determined from the average threshold cycle (Ct) using the standard curve method. PCR efficiency was calculated from the slope of the standard curve generated using a fivefold dilution series of cDNA template.

RNA stability. For IL-8 mRNA stability experiments, cells were treated as indicated above before the addition of 5 μg/ml of actinomycin D (Sigma), an inhibitor of RNA polymerase II that blocks gene transcription. Cells were harvested at the onset of actinomycin-D treatment (time 0) and 2 h after actinomycin D, and RNA was isolated and processed as detailed above.

Protein expression. Immunoblotting was carried out according to procedures described previously (25). Briefly, cells were lysed using the cold M-PER mammalian protein extraction buffer (Thermo Scientific, Rockford, IL) containing a cocktail of protease inhibitors (butylated hydroxytoluene 1% and phenylmethylsulfonyl fluoride 100 mM both from Sigma and pepstatin A 25 mg/ml from MP Biomedicals, Santa Ana, CA). Protein extracts were subjected to a 12% SDS-PAGE, transferred to a polyvinylidene fluoride (PVDF) membrane (GE Lifesciences, Baie d’Urfe, QC, Canada), and probed with different primary antibodies: anti-mitogen-activated protein kinase phosphatase-1 and -5 (MKP-1; MKP-5; Santa Cruz Biotechnology, Dallas, TX and MKP-5; 1:1,000; Abcam, Toronto, ON, Canada), or phosphorylated anti-p38, anti-Extracellular signal-regulated kinases

### Table 1. Cell viability of Caco-2/15 cells exposed to the various experimental conditions

<table>
<thead>
<tr>
<th>Experimental Conditions</th>
<th>Uninfected</th>
<th>Scrambled</th>
<th>CFTR knockdown</th>
<th>CFTR knockdown + vit D₃ 10⁻⁶ M</th>
<th>CFTR knockdown + 25OHD₃ 10⁻⁶ M</th>
<th>CFTR knockdown + 25OHD₃ 10⁻⁶ M</th>
<th>CFTR knockdown + 1,25(OH)₂D₃ 10⁻⁷ M</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>96 ± 0.53</td>
<td>94 ± 1.49</td>
<td>95 ± 1.18</td>
<td>94 ± 1.41</td>
<td>95 ± 0.93</td>
<td>95 ± 1.77</td>
<td>92 ± 0.92</td>
</tr>
<tr>
<td>IL-1β</td>
<td>95 ± 1.50</td>
<td>94 ± 1.00</td>
<td>93 ± 2.99</td>
<td>92 ± 1.34</td>
<td>87 ± 3.13</td>
<td>94 ± 1.26</td>
<td>92 ± 2.16</td>
</tr>
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Values are means ± SE. Cell viability was assessed in triplicate with the trypan blue exclusion method. All treatments were 24 h in duration and the dose of TNF-α and IL-1β used was 25 ng/ml. The ethanol concentration used was 0.4%. CFTR, cystic fibrosis transmembrane conductance regulator; vit D₃, vitamin D₃; 25OHD₃, 25-hydroxyvitamin D₃; 1,25(OH)₂D₃, 1,25-dihydroxyvitamin D₃.

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bodies (1:10,000; Bio-Rad) for 1 h at room temperature. The protein and incubated with species-specific HRP-conjugated secondary anti-
etins. To confirm equal loading of proteins, membranes were stripped, probed overnight at 4°C with anti-β-actin (1:5,000; Sigma), or with
total p38, ERK1/2, SAPK/JNK, and β-actin (1:5,000; Cell Signaling) and incubated with species-specific HRP-conjugated secondary anti-
odies (1:10,000; Bio-Rad) for 1 h at room temperature. The protein bands were scanned and analysed using the UN-SCAN-IT software (Silk Scientific, Orem, UT).

**Immunofluorescence.** Immunofluorescence staining for nuclear factor-κB (NF-κB) was carried out on scrambled or CFTR knockdown cells grown on Lab-Tek II eight-well chamber slides (Fisher Scientific, Ottawa, ON, Canada) 2–3 days before experiments. Paraformaldehyde-fixed cells were permeabilized with 0.1% Triton X-100 (Sigma) for 10 min then blocked with 1% BSA (WisenB) for 30 min before being incubated with a goat anti-human NF-κB p65 antibody (1:200; Santa Cruz Biotechnology) for 1 h at room temperature. After being washed, to remove unbound primary antibody, cells were labeled with an Alexa Fluor 594 donkey anti-goat IgG (1:1,000; Life) labeled with an Alexa Fluor 594 donkey anti-goat IgG (1:1,000; Life) and incubated for 1 h at room temperature. The protein

**IL-8 secretion.** Cell culture supernatants were collected and centrifuged 24 h after treatment with TNF-α, IL-1β, and/or vitamin D metabolites and kept at −80°C until use. IL-8 concentration was determined in culture medium by ELISA according to the instructions of the supplier (OptEIA antibody set; BD Biosciences, Ontario, Canada). Absorbances were read at 450 nm on a Spectra Rainbow plate reader (Tecan Systems, San Jose, CA). To account for differences in protein concentration, IL-8 levels were normalized to total protein content quantified by the Bradford assay (Bio-Rad).

**Mice.** Cftr heterozygous mice on a BALB/c genetic background, initially provided by Dr. Christina Haston (McGill University, Montreal, Canada), were paired to produce Cftr+/− and Cftr−/− mice. Before weaning, all dams and pups received a standard rodent diet

![Fig. 1](image-url). Effect of vitamin D metabolites on IL-1β-stimulated p38MAPK phosphorylation and MAPK phosphatase-1 (MKP-1) expression in cystic fibrosis transmembrane conductance regulator (CFTR) knockdown and control (scrambled) Caco-2/15 cells. Cells were treated 24 h with either 10−7 M of 1,25-dihydroxyvitamin D3 [1,25(OH)2D3], 10−8 M of cholecalciferol, or 10−6 M of 25-hydroxyvitamin D3 (25OHD3) and incubated 30 min (p38MAPK; A) or 60 min (MKP-1; B) in the absence or presence of 25 ng/ml of IL-1β. Protein expression of phosphorylated and total form of p38, MKP-1, and β-actin was analyzed by Western blotting. A: expression of the phosphorylated form was normalized to that of the total form. Data represent the means ± SE of n = 4–14 independent experiments and are expressed as fold change over the IL-1β-stimulated condition ratio sets as 1. *P < 0.05 vs. IL-1β alone and ††P < 0.001 vs. without any treatment. B: data represent the means ± SE of n = 3–7 independent experiments and are expressed as the ratio of MKP-1 expression to that of β-actin.
containing 1,500 IU/kg of vitamin D₃ (2018 Teklad Global 18% Protein Rodent Diet; Harlan Envigo RMS, Indianapolis, IN). Given the risk of intestinal obstruction in Cfr⁻/⁻ mice resulting in their early death, modifications to the feeding and drinking regimen were instituted from 3 wk of age until death in all mice irrespective of their genotype. Modifications consisted of diet moisturizing with reverse osmotic water combined with 17.8 mmol/l polyethylene glycol 3350 (PEGLYTE; Pharmascience, Quebec, Canada) to drinking water. Upon weaning, which occurred at 4–5 wk of age, mice were randomly kept on the same diet or assigned to a high-vitamin D diet with 5,000 IU/kg of vitamin D₃ (2018 Teklad Global 18% Protein Rodent Diet; no. TD.10949; Harlan) for 8 wk ad libitum, after which mice were euthanized. Diets were of identical composition except for vitamin D content. The human equivalency dose was calculated based on the previously published formula (34). Based on the average food consumption (~5 g) and body weight of 4- to 5-wk-old mice (~21 g), vitamin D₃ daily intakes in the control and high-vitamin D₃ diet were approximately equivalent to human intake of 1,740 IU/60 kg and 5,790 IU/60 kg, respectively. Vitamin D supplementation did not affect body weight gain (data not shown). All procedures were approved by the CHU Sainte-Justine Research Institute Animal Care committee.

**Histological analysis.** Intestinal histological analysis was performed as described previously (13). At death, the whole intestinal tract was removed, flushed with ice-cold PBS to remove fecal matter, and opened longitudinally. The small intestine was cut into three equal segments corresponding roughly to the duodenum, jejunum, and ileon while the large intestine was cut into two regions corresponding to the proximal and distal colon and rectum. None of the resected intestines exhibited signs of intestinal obstruction. The intestinal segments were fixed with buffered formalin (10% final concentration) and paraffin embedded. Four-micrometer sections were stained with hematoxylin eosin safran and eosinophils and apoptotic cells were counted in the intercryptic lamina propria at ×400 magnification from ten randomly selected fields. Male and female mice from each genotype were assessed separately by an experienced pathologist blinded to the experimental conditions.

**Fig. 2.** Effect of vitamin D metabolites on NF-κB p65 nuclear translocation in IL-1β-treated CFTR knockdown and control (scrambled) Caco-2/15 cells. Cells were incubated 24 h with either 10⁻⁷ M of 1,25(OH)₂D₃, 10⁻⁶ M of cholecalciferol, or 10⁻⁶ M of 25(OH)D₃ in the absence or presence of 25 ng/ml of IL-1β for 30 min. A: nuclear and cytoplasmic fluorescence intensities of NF-κB p65 were calculated following the quantitative analysis of digitized immunofluorescence images. Data represent the means ± SE of n = 3–5 independent experiments and are reported as fold change over the IL-1β-stimulated ratio of nuclear to cytoplasmic fluorescence intensity. ***P < 0.001 vs. IL-1β alone and ††P < 0.01, ‡P < 0.001 vs. without any treatment. B and C: Immunofluorescence images representing NF-κB p65 nuclear and cytoplasmic fluorescence intensity (in red) and DAPI nuclear staining (in blue) from scrambled and CFTR knockdown cells incubated 24 h with either vehicle, 10⁻⁷ M of 1,25(OH)₂D₃, 10⁻⁶ M of cholecalciferol, or 10⁻⁶ M of 25(OH)D₃ in the absence or presence of 25 ng/ml of IL-1β for 30 min. Contrast and luminosity were standardised for all images. Phosphorylated IκBα/β-actin was measured by Western blotting. Data represent the means ± SE of n = 3–5 independent experiments and are expressed as the ratio of IκBα to β-actin. *P < 0.05 vs. without any treatment.
Statistical analysis. Data are presented as means ± SE. Mean differences between experimental groups were tested using unpaired Student’s t-test or ANOVA. Post hoc comparisons were made with the Tukey’s test when appropriate. The level of significance was set at P < 0.05.

RESULTS

We tested three forms of vitamin D: cholecalciferol (vitamin D3), the form found in most over-the-counter supplements; 25OHD3, the precursor of the bioactive compound and the most abundant circulating form; and 1,25(OH)2D3, the biologically active form. Intestinal cells, including Caco-2 cells, express the enzymes required for the local conversion of vitamin D3 to 25OHD3 and to 1,25(OH)2D3 (5, 42).

To exclude the possibility that our observations may have resulted from cytotoxic effects, we first assessed the impact of the various experimental conditions on cell viability. As shown in Table 1, none of the treatments caused significant cytotoxicity with percentages of viability exceeding 87% in all conditions.

Effect of vitamin D metabolites on inflammatory transducing pathways. Limited information exists regarding the intracellular signaling pathways involved in the anti-inflammatory activity of vitamin D in intestinal epithelial cells. When proinflammatory cytokines interact with their respective cell surface receptors, several cytoplasmic transducing pathways, such as the MAPK pathways, are activated (39). To test the hypothesis that activation of these pathways is modulated by the addition of vitamin D, we treated scrambled (control) and CFTR knockdown cells for 30 min with the proinflammatory cytokine IL-1β in the presence of different vitamin D metabolites and measured the activation of p38MAPK. As illustrated in Fig. 1A, IL-1β treatment elicited a rapid and transient phosphorylation of p38MAPK as indicated by an average 20-fold increase in the levels of phosphorylated p38 upon addition of IL-1β. Coadministration of vitamin D metabolites and IL-1β to scrambled cells did not affect the expression of phosphorylated p38MAPK. Conversely, concomitant addition of IL-1β and 1,25(OH)2D3 or 25OHD3 resulted in a significant 56% and 62% decline in the phosphorylation of p38MAPK in CFTR knockdown cells whereas cholecalciferol treatment had no impact. In contrast, 25OHD3 or 1,25(OH)2D3 treatment had no apparent effect on IL-1β-induced SAPK/JNK and ERK1/2 activation in either scrambled or knockdown cells (data not shown).

Effect of vitamin D metabolites on the expression of MKP-1. MKP-1 is a negative regulator of p38, SAPK/JNK, and ERK MAPK (22) and has been shown to be upregulated by 1,25(OH)2D3 in other cell types (46). We then hypothesize that vitamin D increases MKP-1 expression in intestinal cells, which in turn mediates its inhibitory effect on p38MAPK phosphorylation. As shown in Fig. 1B, the addition of 1,25(OH)2D3 or 25OHD3 did not affect MKP-1 expression of scrambled and knockdown cells regardless of the presence of IL-1β. These data suggest that the effect of 1,25(OH)2D3 or 25OHD3 on p38MAPK phosphorylation is not mediated by an increase in MKP-1 expression. We also assessed the expression of MKP-5 in response to vitamin D metabolites, and, similarly to MKP-1, we failed to detect differences between any of the experimental conditions (data not shown).

Effect of vitamin D metabolites on NF-κB nuclear translocation. In response to proinflammatory cytokines, NF-κB translocates from the cytoplasm into the nucleus (6). We indeed observed a significant increase in nuclear translocation of NF-κB following the addition of IL-1β. To verify whether vitamin D metabolites impact on NF-κB nuclear translocation, we calculated the ratio of nuclear to cytoplasmic fluorescence of NF-κB and reported it as fold increase or decrease over IL-1β-treated cells. As shown in Fig. 2, A and B, vitamin D metabolites did not influence the translocation of NF-κB in scrambled cells whereas nuclear translocation of NF-κB was reduced by 53% upon 1,25(OH)2D3 treatment in CFTR knockdown cells (Fig. 2, A and C). Incubation with cholecalciferol or 25OHD3 did not significantly affect NF-κB translocation in knockdown cells.

It was postulated that the inhibitory effect of 1,25(OH)2D3 on nuclear translocation of NF-κB was due to the inhibition of upstream cellular events required for NF-κB activation. NF-κB activation is initiated by cytokine-induced phosphorylation of the inhibitory protein IκBα, which is then targeted for proteasomal degradation. IκBα degradation frees NF-κB and exposes a

![Image](http://ajpgi.physiology.org/)

**Fig. 3.** Effect of vitamin D metabolites on IL-1β or TNF-α-stimulated IL-8 secretion in Caco-2/15 cells. CFTR knockdown or control (scrambled) cells were incubated 24 h with either 10−7 M of 1,25(OH)2D3, 10−6 M of cholecalciferol, or 10−5 M of 25OHD3 in the absence or presence of 25 ng/ml of either IL-1β (A) or TNF-α (B) for 24 h. Supernatants were then collected to quantify IL-8 secretion by ELISA and its concentration was normalized to total protein concentration. Results represent the means ± SE of n = 4–12 independent experiments and are expressed as fold change over the cytokine-stimulated condition secretion sets as 1. *P < 0.05, ***P < 0.001 vs. cytokine-stimulated condition alone and ‡P < 0.0001 vs. without any treatment.
nuclear localization sequence, enabling its translocation to the nucleus (6). To verify whether 1,25(OH)_{2}D_{3} affected IkB_{α}, we assessed the level of phosphorylated and total IkB_{α} in scrambled and CFTR knockdown Caco-2/15 cells treated with 1,25(OH)_{2}D_{3}. Expectedly, IL-1β induced IkB_{α} phosphorylation and degradation within 30 min (Fig. 2, D and E). However, the addition of 1,25(OH)_{2}D_{3} did not affect these events, indicating that the inhibitory effect of 1,25(OH)_{2}D_{3} on nuclear translocation of NF-κB is not mediated by the regulation of IkB_{α} turnover.

Effect of vitamin D metabolites on IL-8 gene expression, mRNA stability, and protein secretion. Given that IL-8 is a cytokine whose expression is controlled by NF-κB, we next determined whether a treatment with cholecalciferol, 25OHD_{3} or 1,25(OH)_{2}D_{3} impact upon basal or cytokine-stimulated IL-8 secretion of Caco-2/15 cells. Figure 3A shows that basal secretion of IL-8 remained the same regardless of the metabolites involved. However, we observed that IL-1β-induced IL-8 secretion was reduced by 40% upon treatment with 1,25(OH)_{2}D_{3}, Consistent with the above findings, the effect of 1,25(OH)_{2}D_{3} was only observed in CFTR knockdown cells. In contrast, neither cholecalciferol nor 25OHD_{3} inhibited IL-8 release of Caco-2/15 cells. To confirm these results, we measured IL-8 release in response to a different cytokine stimulus (Fig. 3B). Similarly, TNF-α-stimulated IL-8 secretion was only decreased in CFTR knockdown cells treated with 1,25(OH)_{2}D_{3}.

The large release of IL-8 following cytokine stimulation implies activation of IL-8 gene expression as well as enhancement of IL-8 mRNA stability (19). Reduced IL-8 expression combined or not to decreased mRNA stability may therefore represent additional mechanisms through which 1,25(OH)_{2}D_{3} reduces IL-8 secretion. We previously showed that TNF-α and IL-1β both induced IL-8 mRNA expression in Caco-2/15 cells (13). Incubation of cells with either TNF-α or IL-1β, and 1,25(OH)_{2}D_{3} did not affect steady-state IL-8 mRNA expression in scrambled and knockdown cells (Fig. 4, A and B). Conversely, concomitant treatment of TNF-α or IL-1β, and 1,25(OH)_{2}D_{3} led to a significant decrease in mRNA stability compared with cells treated with the cytokines but in the absence of 1,25(OH)_{2}D_{3} (Fig. 4, D and F). Once again, these results were seen only in CFTR knockdown cells.

To further confirm that the effect of 1,25(OH)_{2}D_{3} on IL-8 secretion was mediated primarily through down regulation of
p38MAPK phosphorylation, we treated the cells with the p38MAPK inhibitor BIRB796 and assessed the amount of IL-8 secreted. As illustrated in Fig. 5A, we demonstrated that concentrations of BIRB796 ranging from 0 to 10 μM caused a dose-dependent inhibition of p38MAPK phosphorylation of noninfected Caco-2/15 cells. We then selected two doses corresponding to a partial (1 μM) and complete (10 μM) inhibition of p38MAPK phosphorylation. To validate the specificity of BIRB796, we also examined the levels of total and phosphorylated forms of ERK1/2 and SAPK/JNK MAPK upon treatment with the selected concentrations of the inhibitor. Pretreatment with either 1 or 10 μM of BIRB796 neither altered the phosphorylation status nor the total protein level of ERK1/2 and SAPK/JNK MAPK (data not shown). Treatment with BIRB796 significantly and dose dependently reduced IL-8 secretion in scrambled cells whereas only the highest dose resulted in a significant decrease in IL-8 output from CFTR knockdown cells (Fig. 5B). Addition of 1.25(\(\text{OH}\))\(_2\)D\(_3\) did not further decrease IL-8 secretion in cells with complete inhibition of p38MAPK suggesting that an intact p38MAPK is required for 1.25(\(\text{OH}\))\(_2\)D\(_3\) to fully exert its actions. Accordingly, partial inhibition of p38MAPK did not completely abolish the effect of 1.25(\(\text{OH}\))\(_2\)D\(_3\) on IL-8 secretion.

Expression of genes involved in vitamin D metabolism. In an attempt to elucidate potential mechanisms underlying why 1.25(\(\text{OH}\))\(_2\)D\(_3\) exerts anti-inflammatory effects solely in CFTR knockdown cells, we compared the status of three genes associated with vitamin D metabolism between scrambled and knockdown cells treated with 1.25(\(\text{OH}\))\(_2\)D\(_3\) in the absence or presence of two proinflammatory cytokines. Data regarding the gene expression of the vitamin D nuclear receptor (VDR), as well as CYP27B1 and CYP24A1, two enzymes responsible for the synthesis and catabolism of 1.25(\(\text{OH}\))\(_2\)D\(_3\) respectively, are presented in Fig. 6. Neither CFTR disruption nor the cellular inflammatory status influenced the expression of VDR or CYP27B1. As expected, CYP24A1 expression was induced only upon exposure to 1.25(\(\text{OH}\))\(_2\)D\(_3\). Interestingly, CFTR knockdown cells exhibited a 35–57% decrease in CYP24A1 induction compared with control cells regardless of the presence of cytokines. Taken together these observations suggest that the reduction of CYP24A1 expression in CFTR knockdown cells may decrease the catabolism of 1,25(\(\text{OH}\))\(_2\)D\(_3\) thereby increasing its bioavailability and hence providing an explanation for its greater anti-inflammatory activity. To complement these findings, we documented the expression of CYP27B1 and CYP24A1 in another intestinal cell line, HT-29. Patterns of expression were strikingly different in HT-29 compared with Caco-2/15 cells. Irrespective of the presence of 1.25(\(\text{OH}\))\(_2\)D\(_3\), CYP27B1 expression was significantly decreased in scrambled and CFTR knockdown HT-29 cells while mRNA levels of CYP24A1 were markedly elevated by 14- and 85-fold in HT-29 cells with normal and reduced expression of CFTR, respectively (data not shown). Consistent with our hypothesis, treatment of control and CFTR knockdown HT-29 cells with 1.25(\(\text{OH}\))\(_2\)D\(_3\) failed to exert any anti-inflammatory effects. Of note, none of the treatments caused significant HT-29 cytotoxicity with percentages of viability exceeding 90% in all conditions.

In vivo effects of vitamin D. To determine whether our in vitro findings have physiological relevance, we compared the number of eosinophils in the intestinal lamina propria of Cfr\(^{+/-}\) and Cfr\(^{-/-}\) mice fed a high-vitamin D\(_3\) or control diet (Fig. 7A). Eosinophil counts were low in Cfr\(^{+/-}\) and Cfr\(^{-/-}\) females maintained on the control diet and remained unaffected by the addition of extra dietary vitamin D\(_3\). Similar results were obtained in Cfr\(^{+/-}\) females. In contrast, duodenal mucosa of Cfr\(^{-/-}\) females fed the control diet exhibited the largest number of infiltrating eosinophils, which tended to decrease in Cfr\(^{+/-}\) females fed the high-vitamin D\(_3\) diet (7.2 ± 2.5 vs. 2.2 ± 1.1; \(P < 0.083\)). The effect of vitamin D\(_3\) supplementation was observed only in the duodenum, as eosinophil counts in all other intestinal segments did not differ between mice given the control or the supplemented diet (data not shown). Since there is evidence suggesting that apoptosis may enhance inflammatory processes or, alternatively, occurs secondarily to inflammation, we also assessed the number of apoptotic cells in the duodenal mucosa. Once again, the high-vitamin D\(_3\) diet exerts an effect only in Cfr\(^{-/-}\) females with a significant decrease in the counts of apoptotic cells compared with Cfr\(^{-/-}\) females fed the control diet (Fig. 7B). Such an antiapoptotic effect was also observed in CFTR knockdown females fed the control diet (Fig. 7B).
find the jejunal mucosa of Cfrt<sup>−/−</sup> females but not in more distal segments (data not shown).

DISCUSSION

Although the immunomodulatory properties of vitamin D have been documented in genetic and chemically induced intestinal inflammation (11, 16), its anti-inflammatory potential, especially in intestinal absorptive epithelial cells, remains poorly characterized. Furthermore, vitamin D has been exclusively studied in experimental models mimicking inflammatory bowel diseases but has never been investigated with respect to intestinal inflammation associated with CF. CF patients display features of moderate intestinal inflammation and are instructed to ingest pharmacological amounts of vita-

The present findings demonstrate that Caco-2/15 cells respond to two different vitamin D metabolites: 25OHD<sub>3</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub>, confirming earlier work showing that the parental Caco-2 cells expressed CYP27A1 and CYP27B1, the cyto-

chromes catalyzing the conversion of cholecalciferol to 25OHD<sub>3</sub> and of 25OHD<sub>3</sub> to 1,25(OH)<sub>2</sub>D<sub>3</sub> (14, 42). We found that 25OHD<sub>3</sub> significantly reduced p38MAPK activation in a similar manner to that observed with ten times less of the bioactive form 1,25(OH)<sub>2</sub>D<sub>3</sub>. A lower dose of 25OHD<sub>3</sub> (10<sup>−8</sup> M) also resulted in a significant and dose-dependent inhibition of p38MAPK phosphorylation (data not shown). These data suggest that Caco-2/15 cells are capable of converting 25OHD<sub>3</sub> to 1,25(OH)<sub>2</sub>D<sub>3</sub> in a sufficient amount to produce anti-inflammatory effects at both pharmacological and physiological concentrations. However, a pharmacological amount of 25OHD<sub>3</sub> may also exert independent biological activity as previously reported in bone and parathyroid cells (35, 41). Although attractive, this explanation is unlikely for two reasons: the affinity of 25OHD<sub>3</sub> for the vitamin D receptor is lower than that of 1,25(OH)<sub>2</sub>D<sub>3</sub> (7) and we observed that CYP24A1 expression, a well-known 1,25(OH)<sub>2</sub>D<sub>3</sub>-inducible gene, was enhanced by increasing concentrations of 25OHD<sub>3</sub> in Caco-

2/15 cells (data not shown). On the whole, these observations suggest that the inhibitory effect of 25OHD<sub>3</sub> on p38MAPK likely arises from its local conversion to 1,25(OH)<sub>2</sub>D<sub>3</sub>. Its lack of effect on nuclear translocation of NF-κB and IL-8 secretion may imply that reaching an optimal threshold cellular concentration of 1,25(OH)<sub>2</sub>D<sub>3</sub> is a prerequisite for the achievement of specific biological effects.

In accordance with our findings, downregulation of p38MAPK phosphorylation by 1,25(OH)<sub>2</sub>D<sub>3</sub> has been reported in adipocytes (27), prostate cells (30), and monocytes (46). Although the inhibitory effect of 1,25(OH)<sub>2</sub>D<sub>3</sub> on the p38MAPK pathway has been attributed to upregulation of members of the MAPK phosphatase family in two studies (30, 46), we failed to detect any difference in the expression of MKP-1 or MKP-5 following a treatment with 1,25(OH)<sub>2</sub>D<sub>3</sub>. It has been shown that p38MAPK pathway may enhance the recruitment of NF-κB to the promoters of some of its target genes such as IL-8 (38) and may regulate mRNA stability of several proinflammatory proteins including also IL-8 (44). We found that NF-κB nuclear translocation was significantly depressed by 1,25(OH)<sub>2</sub>D<sub>3</sub> but remained unaffected by cholecalciferol and 25OHD<sub>3</sub>. Similar findings have been reported in other cell models for which many mechanisms, both direct and indirect, have been proposed. For instance, it has been shown in macrophages that 1,25(OH)<sub>2</sub>D<sub>3</sub> inhibits NF-κB signaling by increasing the expression of the sequestering protein IkB (12). It is well-documented that IkB phosphorylation, ubiquitina-

tion, and subsequent proteasomal destruction release NF-κB and allow its translocation across the nuclear membrane. Despite many reports indicating increased stabilization of IkBα in the presence of 1,25(OH)<sub>2</sub>D<sub>3</sub> (37, 47), we failed to demonstrate any effect of 1,25(OH)<sub>2</sub>D<sub>3</sub> on IkBα phosphorylation and degradation. The underlying reasons for this discrepancy are unknown but may relate to the use of different cell types as well as to alterations in the process of nucleocytoplasmic

Fig. 6. Effect of 1,25(OH)2D3 and inflammation on vitamin D nuclear receptor (VDR), CYP27B1, and CYP24A1 mRNA levels in CFTR knockdown and control (scrambled) Caco-2/15 cells. Scrambled and CFTR knockdown cells were incubated 24 h with 10<sup>−7</sup> M of 1,25(OH)2D3 in the absence or presence of 25 ng/ml of either TNF-α or IL-1β for 8 h. Total RNA was collected and subsequently analyzed by Q-PCR using intron-spanning primers for VDR (A), CYP27B1 (B), and CYP24A1 (C). mRNA levels are normalized to that of the housekeeping gene RPL27. Data are illustrated as fold change over scrambled cells for the same condition (sets as 1) and are represented as means ± SE of n = 3–6 independent experiments. Given that CYP24A1 expression is undetectable under basal condition but strongly induced in the presence of 1,25(OH)2D3, only data on 1,25(OH)2D3-treated cells are shown. *P < 0.05 vs. scrambled cells for the same condition.

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transport of NF-κB. Degradation of IκBα unmasks nuclear localizing signals on the NF-κB subunits, allowing its transport to the nucleus. Due to the large size of the NF-κB subunits, the translocation requires the assistance of importins, which are proteins that mediate the docking and transport through nuclear pore complexes (1). Downregulation of importin α3 has been reported in bronchial smooth muscle cells in response to increasing doses of 1,25(OH)2D3 (2). Our observations thus suggest that 1,25(OH)2D3 may exert its effects through modulation of nuclear import of NF-κB rather than IκBα stability.

Consistent with the above findings, we demonstrated that TNF-α- and IL-1β-induced IL-8 secretions were similarly decreased by 1,25(OH)2D3 (2). These results indicate that 1,25(OH)2D3 may exert its effects through modulation of nuclear import of NF-κB rather than IκBα stability.

In light of the previous report demonstrating the involvement of the p38MAPK pathway in the stabilization of IL-8 mRNA in response to proinflammatory stimuli (44), we found that CFTR knockdown cells treated with 1,25(OH)2D3 exhibited reduced IL-8 mRNA stability. Suppression of IL-8 secretion following partial and total inhibition of p38MAPK phosphorylation further confirmed the involvement of this signaling pathway in IL-8 release by Caco-2/15 cells. The observation that the inhibitory effect of 1,25(OH)2D3 on IL-8 secretion was abolished by the complete inhibition of p38MAPK phosphorylation indicates that 1,25(OH)2D3 likewise targets the p38MAPK pathway in CFTR knockdown Caco-2/15 cells.

Although we failed to detect an effect of cholecalciferol on any of the outcomes studied, we cannot exclude the possibility that doses larger than 10−6 M may induce anti-inflammatory effects. In fact, 10−6 M of cholecalciferol represents roughly 16 international units, which is less than the amount found in most over-the-counter vitamin supplements and far less than the amount recommended to CF patients. Treatment of Caco-2/15 cells with higher concentrations of cholecalciferol (10−4 M) resulted in significant cell mortality and precludes us from drawing any firm conclusion regarding the anti-inflammatory potential of this compound in vitro. On the other hand, our in vivo findings suggest that dietary cholecalciferol, when ingested in large amounts, exerts anti-inflammatory and antiapoptotic actions in Cftr-null females only. It remains unclear why Cftr−/− females responded to cholecalciferol while Cftr−/−

![Image](http://ajpgi.physiology.org/)

**Fig. 7.** Histology of the duodenum of Cftr+/+ and Cftr−/− mice fed a high-vitamin D3 diet or maintained on a control diet. Representative images (A), eosinophil (B), and apoptotic cell counts (C) in the duodenal mucosa of Cftr+/+ and Cftr−/− males and females after a 2-mo control diet (black bars) or high-vitamin D3 diet (white bars). Data represent the means ± SE of 4–5 mice for each sex and genotype. Magnification: ×200. Inset: higher magnification (×400) of the lamina propria. Black arrows: eosinophils. White arrows: apoptotic cells. *P < 0.05 vs. Cftr−/− females fed the control diet.
males did not; however, such finding is not completely unexpected, as CF female patients are known to have a worse prognosis (18, 28). Moreover, the fact that the effects were seen only in the more proximal intestinal segments suggests that the intraluminal concentration of cholecalciferol should be above a certain level to exert local biological effects on the intestinal mucosa, given that its concentration is expected to decrease throughout the intestinal tract as a result of the absorptive process. We found that a high-vitamin D3 diet reduced the number of apoptotic cells in the duodenal and jejunal mucosa of Cfrt−/− females, which is in line with the hypothesis that excessive accumulation of proinflammatory cytokines induced apoptosis of intestinal epithelial cells, which in turn disrupts the mucosal barrier integrity and drives chronic mucosal inflammation (23). However, from our results, it is unclear whether the antiapoptotic effects of vitamin D3 lead to, are a consequence of, or coincide with the anti-inflammatory actions. Nevertheless, these observations provide additional support for a link between apoptosis and inflammation and the regulating role of vitamin D in that respect.

The reasons underlying why vitamin D metabolites exerted anti-inflammatory actions only in CFTR knockdown cells are unclear. We postulated that alterations in vitamin D metabolism secondary to reduced CFTR expression might have influenced its cellular bioavailability and bioactivity. Scrambled and CFTR knockdown cells did not differ in terms of the expression of CYP27B1, the P450 cytochrome responsible for the synthesis of 1,25(OH)2D3. Thus increased production of 1,25(OH)2D3 was not the mechanism accounting for the superior anti-inflammatory activity of 1,25(OH)2D3 in knockdown cells. We observed a blunted induction of CYP24A1 expression by 1,25(OH)2D3 in cells with reduced expression of CFTR, a finding that could not be attributed to a decreased expression of the 1,25(OH)2D3 nuclear receptor. Since CYP24A1 is the enzyme involved in the catabolism of 1,25(OH)2D3 and an inverse relationship has been documented between CYP24A1 mRNA and 1,25(OH)2D3 (3), one might speculate that a reduction in CYP24A1 expression may translate into an increase in the cellular level of 1,25(OH)2D3. In support of this hypothesis are the findings that 1,25(OH)2D3 did not attenuate inflammation in both scrambled and CFTR knockdown HT-29 cells, a cell line characterized by the combination of a high and low expression level of CYP24A1 and CYP27B1 respectively. An alternative explanation for the higher effectiveness of 1,25(OH)2D3 in CFTR knockdown cells may be that a certain threshold of inflammation must be reached in order for 1,25(OH)2D3 to fully exert its biological effects. We have indeed shown that CFTR knockdown of intestinal cells exhibited exaggerated responses to proinflammatory cytokines compared with cells with intact CFTR (13).

One limitation to this study is the use of pharmacological concentration of vitamin D compounds. Plasma level of 1,25(OH)2D3 is in the range of 10−10 M whereas the concentrations of 1,25(OH)2D3 associated with biological effects in vitro are 10 to 1,000 times higher (e.g., 10−9 to 10−7 M). The reasons for this discrepancy are unclear but may relate to the rapid inactivation of 1,25(OH)2D3 in vitro. Another limitation is the residual expression of CFTR in our knockdown cells, which makes data extrapolation to a certain class of CF patients, characterized by the lack of functional CFTR, difficult. However, our in vivo findings in Cfrt−/− female mice suggest that large amounts of dietary vitamin D3 may have biological effects, at least in the proximal intestine, in the complete absence of CFTR. Finally, all experiments have been performed on proliferating Caco-2/15 cells, which have been found to display a blunted transcriptional responsiveness to 1,25(OH)2D3 (15). Therefore, we cannot exclude the possibility that vitamin D metabolites may induce more anti-inflammatory effects in differentiated Caco-2/15 cells, although additional experiments are needed to fully test this hypothesis.

Collectively, our study provides additional evidence that vitamin D exerts anti-inflammatory actions through the regulation of p38MAPK activity, NF-κB nuclear translocation, and ultimately release of the proinflammatory cytokine IL-8. However, these findings cannot be generalized as the effects of vitamin D were restricted to CFTR knockdown Caco-2/15 cells and Cfrt−/− females. Our data led us to postulate that vitamin D requires a certain threshold of inflammation to be fully effective or alternatively is effective under conditions characterized by changes in its metabolism resulting in a higher cellular concentration of the bioactive vitamin D metabolite. In either case, it may exert beneficial effects in CF intestinal inflammation, although large amounts appear to be required in vivo.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
G. Morin, V.O., and K.S.-M.C. performed experiments; G. Morin, V.O., K.S.-M.C., N.P., and G. Mailhot analyzed data; G. Morin, V.O., K.S.-M.C., N.P., and G. Mailhot interpreted results of experiments; G. Morin, V.O., K.S.-M.C., N.P., and G. Mailhot prepared figures; G. Morin and G. Mailhot drafted manuscript; G. Morin, N.P., and G. Mailhot edited and revised manuscript; G. Morin, V.O., K.S.-M.C., N.P., and G. Mailhot approved final version of manuscript; G. Mailhot conception and design of research.

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