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## Dexamethasone and Tofacitinib suppress NADPH oxidase expression and alleviate very-early-onset ileocolitis in mice deficient in GSH peroxidase 1 and 2

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### Abstract

C57BL/6J (B6) mice lacking Se-dependent GSH peroxidase 1 and 2 (GPx1/2-DKO) develop mild to moderate ileocolitis around weaning. These DKO mice have a disease resembling human very-early-onset inflammatory bowel disease (VEOIBD), which is associated with mutations in NADPH oxidase genes. Drugs including dexamethasone (Dex), Tofacitinib (Tofa; a Janus kinase/JAK inhibitor) and anti-TNF antibody are effective to treat adult, but not pediatric IBD.

**Aims:** To test the efficacy of hydrophobic Dex and hydrophilic Dex phosphate (Dex phos), Tofa, anti-Tnf Ab, Noxa1ds-TAT and gp91ds-TAT peptides (inhibiting NOX1 and NOX2 assembly respectively), antioxidant MJ33 and ML090, and pifithrin- $\alpha$  (p53 inhibitor) on alleviation of gut inflammation in DKO weanlings.

**Main Methods:** All treatments began on 22-day-old GPx1/2-DKO mice. The mouse intestine pathology was compared between the drug- and vehicle-treated groups after six or thirteen days of treatment.

**Key findings:** Among all drugs tested, Dex, Dex phos and Tofa were the strongest to suppress ileocolitis in the DKO weanlings. Dex, Dex phos and Tofa inhibited crypt apoptosis and increased

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#### Authors' Contributions

FFC RSE and JHD conceived the study concept. FFC and RSE designed, analyzed, interpreted the data and drafted the manuscript. BS and JHD provided funding and administrative and material support. RSE acquired the data and RSE and QG performed literature search and statistical analysis.

Declaration of competing interests

All authors declare no competing interests.

crypt density. Dex or Dex phos alone also inhibited cell proliferation, exfoliation and crypt abscess in the ileum. Dex, but not Tofa, retarded mouse growth. Both Dex and Tofa inhibited ileum Nox1, Nox4 and Duox2, but not Nox2 gene expression. Noxa1ds-TAT and gp91ds-TAT peptides as well as MJ33 had subtle effect on suppressing pathology, while others had negligible effect.

**Significance:** These findings suggest that NADPH oxidases can be novel drug targets for pediatric IBD therapy, and Tofa may be considered for treating VEOIBD.

## Keywords

Dexamethasone; glutathione peroxidase; NADPH oxidase; Tofacitinib; very-early-onset inflammatory bowel disease

## 1. Introduction

Imbalance in epithelial reactive oxygen species (ROS) is suspected to contribute to very-early-onset inflammatory bowel disease (VEOIBD, diagnosed before 6 years of age) (1). Up to one-half of children with chronic granulomatous disease (CGD) have IBD, and CGD patients often have a mutated *NOX2/CYBB/gp91phox* gene (2). Defects in NOX1 and DUOX2 are linked to VEOIBD (3–6). Furthermore, IBD and VEOIBD patients have elevated *NOX1* and *DUOX2* gene expression (7–10). These results suggest that NOX1 and DUOX2 are involved in IBD pathogenesis.

Mice deficient in GSH peroxidase-1 and -2 (GPx1/2-DKO) have colitis before weaning and ileitis soon after weaning (10–12). The penetrance of ileitis was >95% in 35-day-old C57BL6 (B6) GPx1/2-DKO mice, while B6 non-DKO littermates (GPx1+/-GPx2-/- or GPx1-/-GPx2+/-) are virtually disease-free (10, 11). Gut microbes are essential for disease onset in the GPx1/2-DKO mice (13, 14). Conventionally raised GPx1/2-DKO weanlings have increased expression of *Nox1* in the intestinal epithelium (10, 11). Both *Nox1* and *Duox2* gene expression are induced by bacterial components and inflammatory cytokines (15–17). We have demonstrated that NOX1 and DUOX2 are major contributors to the ileocolitis in GPx1/2-DKO mice by studying Nox1-GPx1/2 and Duoxa2-GPx1/2 triple-knockout mice (10, 11). These observations show parallels between VEOIBD and the GPx1/2-DKO ileocolitis.

We previously tested several inhibitors of NADPH oxidases (NOXs) and antioxidants to treat ileocolitis in the GPx1/2-DKO mice with limited success. Two NOX inhibitors, di-2-thienyliodonium and thioridazine, decreased gut pathology, but with significant side effects (10). Here, we studied four more specific NOX inhibitors, including a NOX2-specific gp91ds-TAT, three NOX1-specific Noxa1ds-TAT, ML090 and MJ33 (via a novel PLA2 dependent pathway), as well as anti-apoptotic pifithrin- $\alpha$  (a p53 inhibitor) on GPx1/2-DKO mice (18–24)

Anti-inflammatory drugs, including Dex, Tofa and anti-TNF Ab, are used to treat adult IBD, but not VEOIBD (25–29). Glucocorticoids are anti-inflammatory mediators used for short-term IBD therapy (30). However, glucocorticoids have both protective and deleterious effects in mouse colitis (31). Glucocorticoids induce annexin A1 (ANXA1 or lipocortin 1)

expression, which induces resolution of inflammation and wound healing via NOX1 activation (32, 33). Both *NOX1* and *DUOX2* gene expression can be induced by TNF- $\alpha$  and interferon- $\gamma$  in the intestinal epithelium (34–36). *NOX1* is also induced by pro-inflammatory IL-4 and IL-13 in colon cells (37). Here, we investigated if Dex alleviates ileocolitis in the GPx1/2-DKO mice. If so, does Dex impact *Nox1* and *Duox2* gene expression?

Tofa, an oral JAK inhibitor targeting mainly JAK1 and JAK3, is effective for adult human IBD and oxazolone-induced colitis (25, 38). Tofa inhibits inflammation via different pathways from Dex (39). Many pro-inflammatory cytokines in IBD use the JAK family of tyrosine kinases for signal transduction. For instance, transcriptional activation of *NOX1* by IL-4 and IL-13 pro-inflammatory cytokines is mediated through JAK1/STAT6 signaling in cancer cells (37). Therefore, we also evaluated the efficacy of Tofa on the very-early-onset ileocolitis in the GPx1/2-DKO mice, and whether Tofa inhibits *Nox1* or *Duox2* gene expression. This work may be useful to develop NOX1-and DUOX2-specific inhibitors for VEOIBD therapy.

## 2. Materials and Methods

### 2.1. Mice

Generation and breeding of B6 GPx1/2-DKO (Gpx1<sup>tm1Ysh</sup>/Gpx2<sup>tm2Coh</sup>, MGI: 2651587) colony in the Animal Resources Center at City of Hope (COH) has been reported previously (10, 11). Briefly, male GPx1/2-DKO mice were bred with female non-GPx1/2-DKO mice with a wild-type (WT) GPx1 or GPx2 allele (GPx1<sup>+/+</sup>-GPx2<sup>-/-</sup> or GPx1<sup>-/-</sup>-GPx2<sup>+/+</sup>) to produce GPx1/2-DKO and non-GPx1/2-DKO littermates. The B6 non-DKO mice rarely have pathology and are virtually indistinguishable from WT. The non-DKO littermates were better controls than WT mice since they shared the same bedding, being exposed to the same microflora. Breeders were fed LabDiet 5062 (9% fat, St. Louis, MO 63144), and 21-day-old pups were weaned onto LabDiet 5061 (5% fat). Both male and females were studied. All mice had free access to food and water.

### 2.2. Drug treatment

All treatments began on 22-day-old mice, since the ileum pathology of GPx1/2-DKO mice increases from 24 to 35 days of age (10). Mice were administered drugs commencing around 6 A.M. after weighing and inspection for disease signs. The regimen for drug administration is shown in Supplementary Table 1. Effects of drugs were analyzed on 35-day-old mice, except for some mice treated a hydrophilic dexamethasone phosphate (Dex phos), which were analyzed on day 28 along with respective controls. Dex was dissolved in DMSO, Dex phos in PBS; both were obtained from Sigma-Aldrich, St. Louis, MO 63178. The vehicle controls were DMSO (Sigma-Aldrich) and PBS (I.P.; Mediatech, Inc. Manassas, VA, 20109), respectively.

Other drugs used here are: Anti-TNF $\alpha$  Ab (XT22, rat anti-mouse monoclonal neutralizing antibody; Thermo Scientific, Waltham, MA, 02451); gp91ds-TAT (blocks p67phox recruitment to NOX2) and Noxa1ds-TAT (YGRKKRRRQRREPM DALGKAKV-[CONH<sub>2</sub>]; modified to mouse Noxa1 sequence with a TAT motif; blocks NOX1 enzyme assembly)

peptides were manufactured by Synthetic and Biopolymer Chemistry Core Facility COH (19, 24); MJ33 (inhibits NOX1 activity indirectly by inhibiting the PLA2 activity of peroxiredoxin-6)(21), ML090 (SID-26535836; 5,11-Dihydroquinoxalino [2,3-b] quinoxaline; a putative NOX1–4 inhibitor)(22) and pifithrin- $\alpha$  (p53 inhibitor)(23) were obtained from Cayman Chemical (Ann Arbor, Michigan, 48108). Tofacitinib citrate (Tofa) was obtained from LC laboratories (Woburn, MA 01801). No morbidity was observed for all drugs. The vehicle for anti-TNF, gp91ds-TAT and Noxa1ds-TAT was PBS and all were administered I.P. The vehicle for MJ33 and pifithrin- $\alpha$  was DMSO, which were administered I.P. ML90 and Tofa were suspended in 1.2% methyl cellulose, 0.1% polysorbate 80 in water (oral vehicle; Sigma-Aldrich) and administered by gavage (10). Their control mice received vehicle only by gavage.

Doses for most compounds were selected based on the literature for rodent testing, which are referred in Supplementary Table 1. We used 1 mg/Kg Dex based on a mouse study by Ho et al. (40). A higher dose, 2 mg/Kg was used for Dex phos since Ren et al. treated mice with 5 mg/Kg Dex and did not observe adverse effects (41). The dose of 30 mg/Kg Tofa was used following Beattie's report that oral dosing of 10 and 15 mg/Kg three times a day decreased disease activity index in oxazolone-induced colitis (25). For Noxa1ds-TAT, 5 mg/kg was used initially, and was repeated at 8.5 mg/kg without any sign of morbidity (24). For ML090 since no published data on rodents were available, we used the middle dose after testing 3 doses (6 mg/kg, 12 mg/kg and 30 mg/kg) and did not observe morbidity.

The number of mice studied for each treatment and its control is listed below: 21 Dex phos-treated and 23 PBS controls were analyzed on 28-day-old mice. Analyzed on 35-day-old mice included 20 Dex-treated, 21 DMSO controls, IP; 18 Tofa-treated, 12 vehicle controls; 18 anti-TNF Ab-treated, 21 gp91ds-TAT-treated, 20 low-dose Noxa1ds-TAT-treated, 15 high-dose Noxa1ds-TAT-treated, 21 PBS controls, IP; 21 MJ33-treated, 14 ML090-treated, 15 pifithrin- $\alpha$ -treated, and 12 oral-vehicle controls. All treatments were approved by the COH IACUC.

### 2.3. Data and tissue collection

Mice were weighed daily. At necropsy the length of the small and large intestine (excluding cecum) was recorded. One cm each of the distal ileum at the ileocecal junction and rectum were harvested and immersed in RNALater (Qiagen, Valencia, CA, 91355) for 24 hours at 4°C then stored at -80°C for RNA isolation. A section of distal ileum (1–5 cm above the cecum), cecum and mid-to distal colon were processed for histological analysis.

### 2.4. Histopathology

The histopathological features of mouse ileum and distal colon were evaluated on sections stained with hematoxylin and eosin (H&E) by the Solid Tumor Core at COH. Quantification of ileal and colonic pathology was described previously (10). For colon, we counted apoptotic figures, exfoliation and gland abscesses. Since only 2 gland abscesses were seen among all of the mice studied, no analysis is presented. Histopathological scoring was performed on slides with concealed identification and mixing of experimental groups with

the respective control groups to ensure the blind scoring. Scoring was performed on all samples listed in the Drug Treatments section.

Immunohistochemistry (IHC) was performed with anti-Ki-67 antibody (Ventana 30–9; no dilution; Roche diagnostics, Fishers, IN, 46037) to assess proliferation. Scoring of Ki-67 positive cells was done by counting the most normal portions of each section. Nine to 24 crypts were counted for each slide depending on the availability of normal-appearing tissue. Six DKO mice were tested in each of Dex/DMSO-, DMSO-, Tofa- and oral-vehicle-treated groups, and 4 non-DKO mice were tested as normal control. TUNEL (ApopTag Peroxidase In Situ Apoptosis Detection Kit; EMD Millipore, Burlington, MA 1803) and lysozyme Ab (1:1,000, ab108508, Abcam, Cambridge, MA 02139–1517) were performed as described previously (10).

## 2.5. Real-time quantitative PCR (rt-qPCR)

RNA was isolated using a Qiagen RNeasy kit. The cDNA was prepared using 2 µg total RNA with Promega reagents (Madison, WI, 53711). The rt-qPCR was performed using Taqman primer sets (Thermo Fisher Scientific, Waltham, MA) on a BioRad CFX96 (Irvine, CA, 92618) for 40 cycles (Supplementary Table 2). The Ct values were determined using the CT method and quantification was normalized against β-actin.

## 2.6. Statistical analysis

GraphPad Prism 7.01 was used for statistical analysis. Each data set was checked for a parametric distribution except for the pathology score, which is not a continuous distribution. For the main figures parametric sets were analyzed with ANOVA and Tukey's multiple corrections and nonparametric sets with the Kruskal-Wallis test and Dunn's multiple corrections. Multiples testing results are indicated by letters where a>b>c>d; groups with shared letters indicate no difference. Results from pairwise testing are mentioned to indicate suggestive results not found after correction for multiple groups. Data testing analysis for the remaining drugs involved pairwise comparisons vs. respective controls and Bonferroni post-test correction for multiple samples. Since the post-test correction left only a few significant differences, we indicate those few with an asterisk and bracket. Supplementary Table 3 contains results of complete pairwise testing for Figures 2 and 3.

## 3. Results

### 3.1. Dexamethasone (Dex) and Dex phos alleviated ileocolitis in the GPx1/2-DKO ileum

Glucocorticoids have both protective and deleterious effects on experimental colitis in mice (31). It was shown that glucocorticoids inhibited inflammation and induced wound healing via induction of annexin A1 (ANXA1 or lipocortin 1) expression and activation of NOX1 (32, 33). Therefore, we tested whether Dex, a synthetic hydrophobic glucocorticoid, would prevent inflammation analyzed in 35-day-old GPx1/2-DKO mice. The Dex-treated GPx1/2-DKO mice had apparently slower weight gain (even though not statistically significant) than the DMSO-treated mice (Figure 1A, males; 1D, females). The ratio of small intestine length over body weight (to adjust the slower weight gain by Dex treatment) was significantly

greater in the Dex mice vs. the DMSO control analyzed by pairwise comparison ( $p=0.02$ ; Mann-Whitney, although not different when adjusted for multiple comparisons; Figure 1G).

Ileum histology was analyzed to quantify the extent of pathology and inflammation (Figure 2A–2F). DMSO- and Dex-treated DKO ileum sections stained by H&E are shown in Figure 2G, 2K. The GPx1/2-DKO ileal histopathology shows rampant apoptosis, exfoliation and loss of Paneth cells in the crypt. The pathology score is obtained by combining four sub-scores excluding exfoliation as shown in Figure 2B, 2D–2F. IHC of TUNEL assay and anti-Lysozyme were performed on some slides to confirm the identity of apoptotic cells and Paneth cells, respectively (Figure 2J, 2N). The Dex-treated 35-day-old DKO mice had lower pathology scores in all subcategories compared to DMSO-treated DKO ileum. DMSO did not affect pathology analyzed on 35-day-old DKO mice.

The pathology parameters of untreated DKO and non-DKO mice are shown in Supplementary Figure 1. We have noted that the GPx1/2-DKO mice have peak ileal pathology by 30–35 days of age having increased TNF $\alpha$  mRNA levels, crypt abscesses and submucosal MPO-positive cell infiltration (Supplementary Figure 1A–1F) (10). *Duox2* mRNA levels are relatively high in 24-day-old B6 GPx1/2-DKO mice and remain so throughout the study interval (Supplementary Figure 1G)(11).

To examine the short-term effect of glucocorticoid before clear infiltration of inflammatory cells, we used Dex phos to treat GPx1/2-DKO mice and analyzed at 28 days of age along with a PBS control. Dex phos was used since we detected a possible beneficial DMSO effect at 28 days, which was not evident at 35 days (not shown). The short-term Dex phos treatment also showed growth retardation (albeit not significant; Figure 1C, 1F). The ratio of small intestine length over body weight was significantly greater in the Dex phos-treated mice than PBS control that supported the anti-inflammatory effect of Dex Phos (Figure 1G). Even with the lower pathology in the 28-day-old than the 35-day-old DKO mice, Dex phos had likely decreased total pathology score ( $p<0.0001$ ; pairwise, Mann-Whitney test), clearly inhibited apoptosis, and probably better preserved Paneth cells ( $p=0.0008$ ; pairwise, Mann-Whitney test) (Figure 2A–2D).

B6 GPx1/2-DKO colon has milder pathology than ileum. The colon apoptotic pathology starts early ( $<17$  days of age) and occurs at about half the levels of the ileum, while colon exfoliation is at one-sixth the levels of ileum. Similar to the ileum, Dex and Dex phos significantly suppressed colon gland apoptosis (Figure 3A). Dex also moderately decreased the levels of exfoliation in the colon ( $p=0.012$ ; pairwise, Mann-Whitney test; Figure 3B). No gland abscesses were found in either the control or Dex-treated colon.

### 3.2. Tofacitinib (Tofa) had a significant impact on ileocolitis in the GPx1/2-DKO mice

Tofacitinib (Tofa, a Janus kinase/JAK inhibitor) has anti-inflammatory activity and has shown robust efficacy in treating adult human IBD and oxazolone-induced colitis in mice (25). Here, we tested its effect in very-early-onset ileocolitis in the GPx1/2-DKO mice. Tofa did not affect mouse growth (Figure 1B, male; 1E, female), nor affect the length of small intestine compared to the vehicle-treated GPx1/2-DKO mice (Figure 1G). Tofa significantly inhibited crypt apoptosis and increased crypt density compared to the vehicle-treated



GPx1/2-DKO ileum (Figure 2B, 2E). Although Tofa-treated ileum did not lower total pathology score when analyzed with a post-test correction for multiple samples, pairwise comparison showed that Tofa-treated ileum had lower pathology scores than the vehicle control ( $p=0.015$  pairwise, Mann-Whitney test). Similar to Dex, Tofa significantly inhibited colon gland apoptosis (Figure 3A).

### 3.3. Dex, but not Tofa, suppressed cell proliferation in ileum crypt epithelial cells

We have reported that GPx1/2-DKO mice have high levels of proliferation in the crypt epithelium compared to the non-DKO mice, which could counter the high level of apoptosis and exfoliation (10, 13). Glucocorticoid inhibition of cell proliferation was reported in DSS-treated colon (31). Tofa regulates lymphocyte activation and proliferation (42), but it is unclear if it affects intestinal epithelial cell proliferation. We analyzed the effect of Dex and Tofa on cell proliferation in the 35-day-old GPx1/2-DKO ileum on sections stained with anti-Ki-67 Ab (Figure 4). Counting the Ki-67+ cells in the relatively undistorted areas, we found Dex, but not Tofa, suppressed cell proliferation in the crypt epithelium.

### 3.4. Both Dex- and Tofa-treated GPx1/2-DKO mice inhibited *Nox1*, *Duox2* and *Nox4* but not *Nox2* gene expression in the GPx1/2-DKO ileum

We have shown that the very-early-onset ileocolitis in the GPx1/2-DKO mice is driven by NOX1 or DUOX2, since GPx1/2-DKO mice with inactivated NOX1 or DUOX2 enzyme have no or mild inflammation (10, 11). Therefore, we analyzed if Dex and Tofa inhibited *Nox1* and *Duox2* gene expression in 35-day-old ileum. Dex-treated ileum has dramatically decreased *Nox1* and *Duox2* mRNA levels, 8-fold and 5.7-fold, respectively compared to the DMSO-treated GPx1/2-DKO mice (Figure 5A, 5B). Similar to Dex, Tofa also inhibited *Nox1* and *Duox2* gene induction in the GPx1/2-DKO ileum (7.4- and 4.5- fold lower than the vehicle-treated, respectively). Although it is desirable to have IHC to confirm the suppression of NOX1 and DUOX protein expression, our monoclonal Abs against human NOX1 and DUOX (it cannot distinguish DUOX1 and DUOX2) did not work on mouse proteins (43, 44).

We also analyzed *Nox2* and *Nox4* gene expression in the GPx1/2-DKO ileum. *Nox2* is considered as a marker for inflammation since it is highly expressed in the leukocytes and also expressed in lymphocytes and non-hematopoietic cells (45). Apparently, the slightly lower *Nox2* mRNA levels in Dex- or Tofa-treated GPx1/2-DKO mice were not significantly different from the vehicle-treated controls (Figure 5C).

The *Nox4* gene is widely expressed in various tissues and cell types including the intestinal epithelium (46, 47). We reported that B6 GPx1/2-DKO ileum had elevated *Nox4* gene expression compared with the WT control (11). Here, we found that *Nox4* gene expression level was also significantly decreased in Dex- or Tofa-treated GPx1/2-DKO mice, and the Tofa-treated ileum had significantly lower *Nox4* mRNA levels than Dex-treated by pairwise comparison (Figure 5D). We noted that the relative *Nox4* mRNA levels were much lower than *Nox1* or *Duox2* mRNA levels.

Heme oxygenase-1 (*Hmox1*) gene expression is regulated by Nrf2, a redox-sensitive transcription factor which is activated by oxidative stress (48). We analyzed the levels of

*Hmox1* mRNA levels as an indicator for oxidative stress. Both Dex- and Tofa-treated groups had lower levels of *Hmox1* mRNA than in the respective controls, although only the Dex-treated reached statistical significance (Figure 5G).

### 3.5. Dex but not Tofa inhibited *Il1b* mRNA induction in the GPx1/2-DKO ileum

We have reported that GPx1/2-DKO ileum had elevated Tnf( $\alpha$ ) cytokine protein levels and elevated *Il1b* mRNA levels compared to non-GPx1/2-DKO mice, whereas GPx1/2-DKO ileum had the same *Ifng* (interferon- $\gamma$ ) mRNA level as in non-GPx1/2-DKO controls (11). Since *Nox1* and *Duox2* gene expression can be induced by TNF (and Ifng) in the intestinal epithelium (34–36), here we analyzed *Tnf* and *Il1b* mRNA levels mouse ileum. Dex-treated, but not Tofa-treated, GPx1/2-DKO ileum had significantly reduced *Il1b* mRNA levels than DMSO-treated ileum (Figure 5E). Tofa, but not Dex, decreased Tnf mRNA levels in GPx1/2-DKO ileum compared to vehicle-treated mice when analyzed by pairwise comparison (Figure 5F). We also analyzed mRNA levels of other cytokines *Il4* and *Il13*, which were shown to induce *Nox1* mRNA levels in colon cell lines (37). Both mRNAs were undetectable in the GPx1/2-DKO ileum. These results suggest that Dex suppresses *Il1b* mRNA levels while Tofa has a greater impact on *Tnf* mRNA levels.

### 3.6. Nox inhibitors had a mild effect on suppressing ileal pathology in the GPx1/2-DKO mice

Because NOX1 and DUOX2 are major contributors to ileocolitis in the GPx1/2-DKO mice, inhibition of these NOX enzymes should alleviate gut pathology. Although two NOX inhibitors, di-2-thienylidonium and thioridazine, decreased gut pathology (10), they have significant side effects. Here, we tested four more NOX inhibitors, namely gp91ds-TAT (Nox2ds-TAT), Noxa1ds-TAT, ML090 and MJ33 on the GPx1/2-DKO mice. TAT (trans-activating transcriptional activator), a cell-penetrating peptide found in HIV-1, was added to facilitate cell entry. None of the drugs lowered the total ileal pathology scores (Supplementary Figure 2A). However, peptide Noxa1ds-TAT significantly decreased crypt apoptosis (at 5 mg/kg dose) and increased the crypt density in the ilea (at both 5 and 8.5 mg/kg doses) (Supplementary Figure 2B, 2E). Peptide gp91ds-TAT and MJ33 also significantly increased ileal crypt density in the DKO mice compared to vehicle-treated DKO mice (Supplementary Figure 2E). Other drugs tested including anti-Tnf Ab and pifithrin- $\alpha$  (p53 inhibitor) did not have any effect. High-dose NOXA1ds-TAT may have aggravated colon apoptosis, but none of the drugs tested had other effects on colon (Supplementary Figure 3A, 3B).

## 4. Discussion

Elevated *NOX1* and *DUOX2* gene expression is observed in both pediatric and adult ulcerative colitis and Crohn's disease subjects with crypt or gland expression of DUOX2 in some cases (7–10). Defects in *NOX1* and *DUOX2* genes are linked to VEOIBD (3–6). In GPx1/2-DKO mice, the *Nox1* and *Duox2* genes are major contributors to gut inflammation (10, 11). Here, we evaluated the effect of anti-inflammatory drugs to determine their effect on *Nox* gene expression.



Among the anti-inflammatory drugs tested, we found Dex has the strongest impact on the GPx1/2-DKO mice. Dex, having immunosuppressive effects, has been used for decades in the treatment of IBD flares (30). However, Dex has the undesirable side effect of inhibition of weight gain (49), thus may limit its application to VEOIBD patients. An aspect of the anti-inflammatory effect of glucocorticoids was reported to be activation of NOX1 via annexin A1 induction during wound healing (32, 33). In this study, we found Dex suppressed *Nox1* and *Duox2* gene expression. Absence of *Nox1* expression (*Nox1*-TKO mice) is associated with a decrease in apoptosis and proliferation of crypt epithelium (11). Unlike the DSS-induced colitis with erosion of epithelium, the ileocolitis in the B6 GPx1/2-DKO mice does not have erosion and so may involve less extensive wound healing. This may explain why Dex alleviated mild inflammation in the DKO mice but aggravated DSS-induced colitis (29, 50).

Tofacitinib (Tofa, a Janus kinase/JAK inhibitor) is recently approved for adults with moderate to severe ulcerative colitis and marketed as Xeljanz®. Many inflammatory cytokines, including IL6, IL13, IL15, IL22–24, IL27 and IFN $\gamma$  are elevated in either ulcerative colitis or Crohn's disease, rely on the JAK family of tyrosine kinases for signal transduction (51, 52). Tofa administered orally appeared to modulate intestinal inflammation without systemic immunosuppression (25). Here, we found that Tofa (at 30 mg/kg orally) is effective to suppress the crypt apoptosis in the post-weaning GPx1/2-DKO mice without any impact on growth.

Consistent with our hypothesis that ROS-generating NOX1 and DUOX2 are contributors of ileocolitis in the GPx1/2-DKO mice, both Dex and Tofa inhibited *Nox1* and *Duox2* gene expression in the ileum. Interestingly, both Dex and Tofa also suppressed *Nox4*, but not *Nox2* (a marker for inflammatory cells) gene expression. It suggests that NOX4 also plays a role in ileocolitis in the GPx1/2-DKO mice. Since very little is known about *Nox4* function in the intestine, the role of NOX4 in the GPx1/2-DKO mice should be evaluated in the future. While Dex and Tofa had a similar effect on *Nox* gene expression, the reason that Dex was more effective than Tofa might be that Dex, but not Tofa, significantly inhibited *Il1b* gene expression. Other inflammatory responses besides ROS also play an important role in ileocolitis in the GPx1/2-DKO mice. These additional actions of Dex and Tofa complicate any interpretation of action through suppression of enzyme levels.

We suspect that *Nox1*, which is expressed in the crypt epithelium, is a major contributor to crypt apoptosis (37, 53). Crypt apoptosis is a hallmark of human IBD (54). GPx1/2-DKO ilea deficient in *Nox1* gene expression do not have rampant crypt apoptosis as observed in the GPx1/2-DKO mice (11). In contrast, GPx1/2-DKO ilea deficient in *Duox2* expression still have similarly high level of apoptosis as the GPx1/2-DKO mice (10). The timing of increasing crypt apoptosis corresponds with the increase of *Nox1* gene expression in the GPx1/2-DKO mice (10). Here, both Dex and Tofa decreased *Nox1* gene expression and had lowered the crypt apoptosis in the GPx1/2-DKO mice. This is consistent with the notion that NOX1 strongly impacts crypt apoptosis.

Although NOX1 also can promote cell proliferation in colon cell lines, it is puzzling that Dex, but not Tofa, inhibited cell proliferation while both drugs had decreased *Nox1* gene

expression in the epithelium of the GPx1/2-DKO mice (53). It is possible that the amount of ROS required for proliferation is much lower than that for apoptosis; the residual NOX1 activity is sufficient to induce proliferation. Furthermore, regeneration of inflamed intestine is regulated by multiple pathways, including JAK/STAT, Wnt/Wingless and EGFR pathways. The interplay of the ligands in different pathways may outweigh the effect of NOX1-produced ROS (55).

In this study, we found three more NOX inhibitors have small but significant impact, suppressing one or more aspects of ileal pathology in the GPx1/2-DKO mice. Two are peptides, Noxa1ds-TAT and gp91ds-TAT as well as MJ33. Although *Nox2* gene expression was not significantly affected by Dex or Tofa, the ROS generated by NOX2 could still contribute to the gut pathology exhibited in the GPx1/2-DKO mice, which is highly sensitive to ROS. A possible reason for the limited drug effect is that unlike genetically modified mice which are completely devoid of NOX1 or DUOX2 activities, drugs can only partially inhibit enzymes due to limited access and constant enzyme turnover. It is also possible that the gut pathology in the DKO mice once initiated from the ROS produced by NOX1 and DUOX2 it is amplified by inflammatory responses, thus inhibitors of NOX/DUOX alone become less effective with age. Since we observed no side-effects, dose escalation is possible and might yield a better outcome.

Loss of redox balance is involved in the pathogenesis of many diseases including IBD (56). Increase of oxidative stress markers are often detected in the intestinal mucosa of IBD patients, such as overproduction of ROS, increase in 4-hydroxynonenal or malondialdehyde (markers for protein and lipid oxidation) levels, and oxidative DNA damage, etc. Increased NOX1 and DUOX2 gene expression was reported in the gut of IBD patients (5, 9). However, recent studies have shown that partial inactivation of NOX1 and DUOX2 activities may link to VEOIBD (3, 4). Since NOX1 and DUOX2 activation clearly promotes ileocolitis in the DKO mice, which are deficient in antioxidant activity, it is possible that the gut microbes modulated by NOX1 and DUOX2 are the link to IBD. Both NOX1 and DUOX2 gene expression is induced by bacterial colonization as a part of host responses to bacterial signal transduction, which may lead to dysbiosis in humans (57). DKO mice (having elevated *Nox1* gene expression) may also have dysbiosis with *Escherichia coli* overgrowth (10, 58). Thus, gut microbes should be monitored when using NOX/DUOX inhibitors for IBD therapy.

## 5. Conclusion

Dex and Tofa, two anti-inflammatory drugs used in adult IBD, suppressed markers of pathology in the post-weaning GPx1/2-DKO mice. Tofa did not impact on the growth of weaning mice, and thus may be safe for VEOIBD clinical trials. Dex and Tofa also have an antioxidant effect by suppressing *Nox1*, *Duox2* and *Nox4* gene expression in the ileum, suggesting that ROS are a contributing factor to ileocolitis in the GPx1/2-DKO mice. The lowered oxidative stress in the ileum of Dex-treated mice is supported by the low levels of *Hmox1* mRNA, which is regulated by the redox-sensitive Nrf2 (48). Although antioxidants and anti-NOX drugs tested thus far had modest effects in suppressing gut pathology in the

GPx1/2-DKO mice, it is possible that combining such drugs with conventional therapies will enhance the efficacy of therapy for IBD or VEOIBD.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations:

<b>B6</b>	C57BL6/J
<b>COH</b>	City of Hope
<b>Dex Phos</b>	dexamethasone phosphate
<b>DKO</b>	double knockout
<b>DMSO</b>	dimethyl sulfoxide
<b>Duox2</b>	dual oxidase-2
<b>GPx</b>	glutathione peroxidase
<b>Hmox1</b>	heme oxygenase-1
<b>IBD</b>	inflammatory bowel disease
<b>Ifng</b>	gamma-interferon
<b>non-GPX1/2-DKO</b>	mice with a wild-type allele of GPx1 or GPx2
<b>Nox</b>	NADPH oxidase
<b>ROS</b>	reactive oxygen species
<b>TAT</b>	a cell-penetrating peptide named trans-activating transcriptional activator from HIV-1
<b>Tnf</b>	tumor necrotic factor-alpha
<b>Tofa</b>	Tofacitinib, or Xeljanz®
<b>VEO</b>	very-early-onset
<b>WT</b>	wild-type

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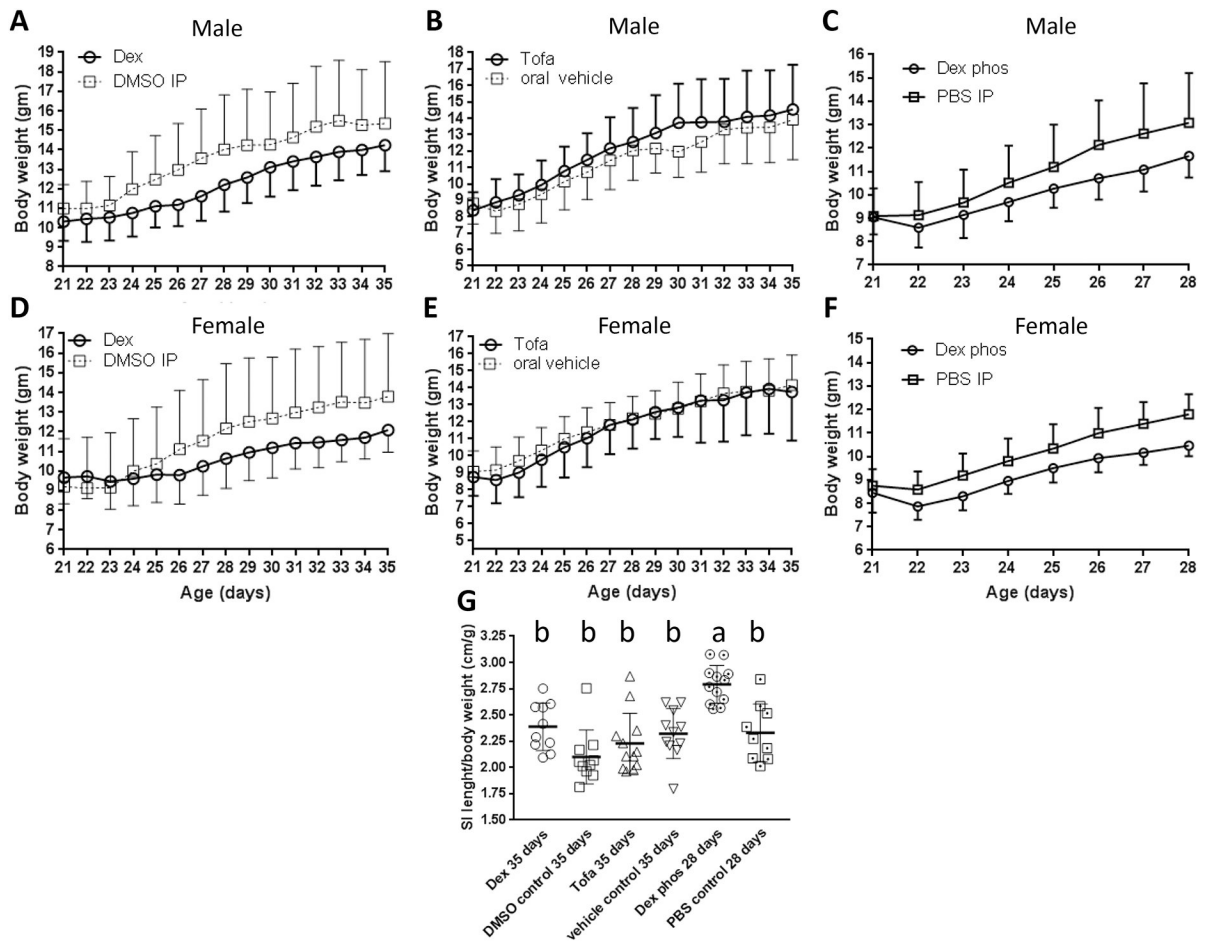
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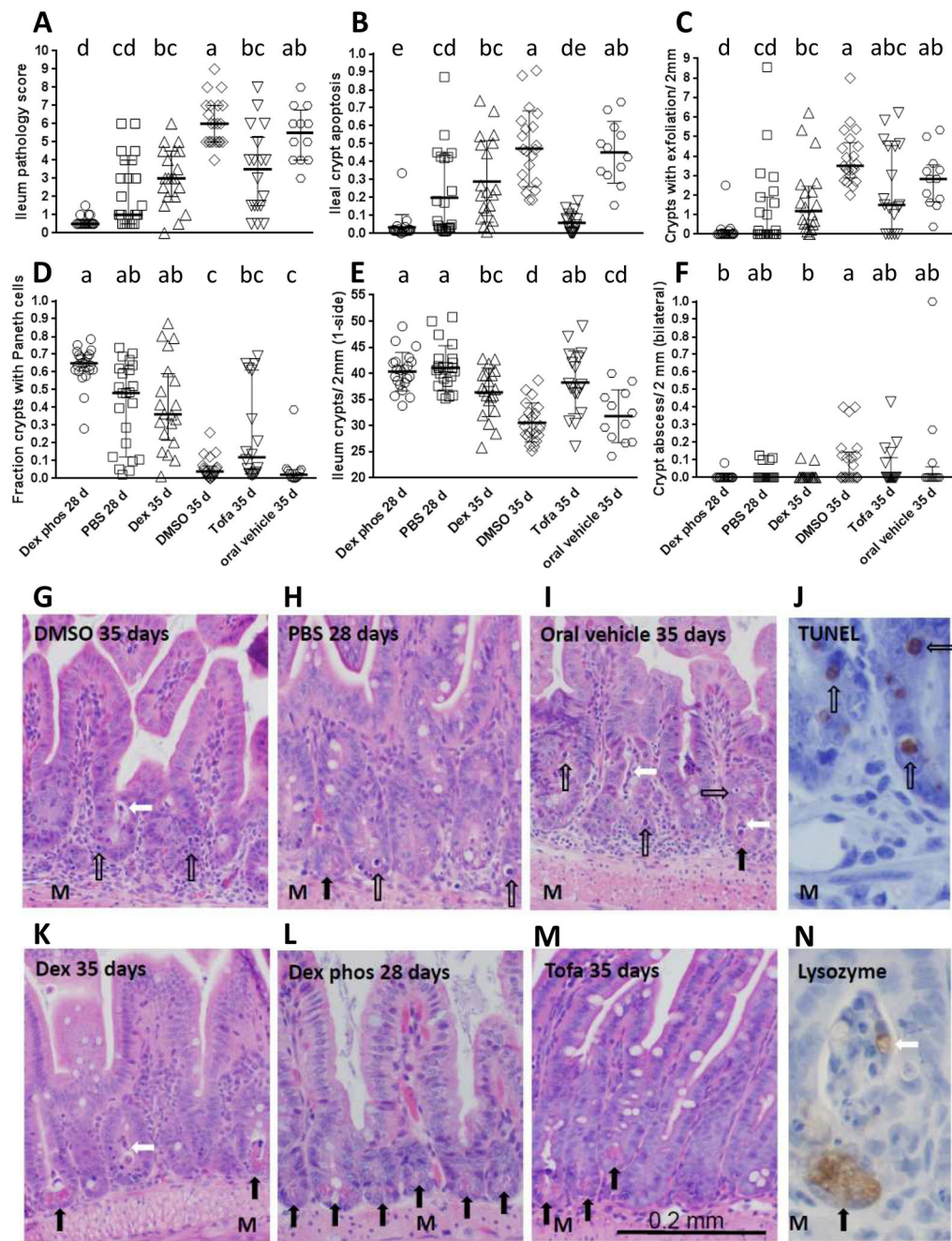


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**Figure 1: Comparison of body weight and length of small intestine of GPx1/2-DKO mice from 21 to 35 days of age treated with immune suppressants.**

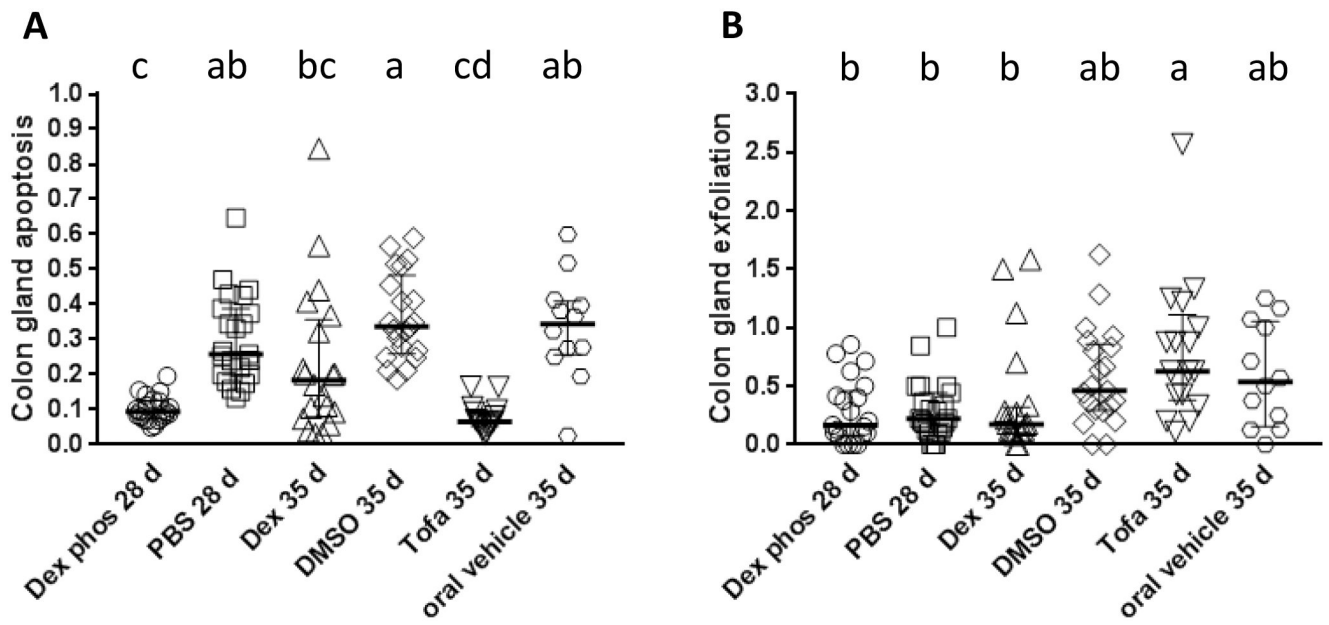
Male mice treated with Dex (Panel A) or Tofa (Panel B) daily and analyzed at 35 days of age, or treated with Dex phos (Panel C) analyzed at 28 days of age vs. the corresponding control set. Differences in weight at the endpoints were not significant. Females showed similar trends (Panels D, E and F). Panel G: Comparison of the lengths of small intestine (SI) adjusted for body weight at either 28 or 35 days of age.



**Figure 2: Comparison of total and sub-pathology scores of GPx1/2-DKO ilea treated with immune suppressants.**

Panel A shows the total pathology scores compiled from the sub-scores obtained from Panel B and D-F. All scores were done on H&E sections. Each symbol represents one mouse. Panel A, C, D and F were plotted to show median and interquartile range, and Panel B and E for apoptosis and crypt density count were shown as mean and Stdev. Analysis of parametric data is by ANOVA with Tukey's multiple correction test and non-parametric data by Kruskal-Wallis test with Dunn's multiple correction test. The letters assigned to each group according to the mean or medium, where  $a > b > c > d$ . The groups are significantly different

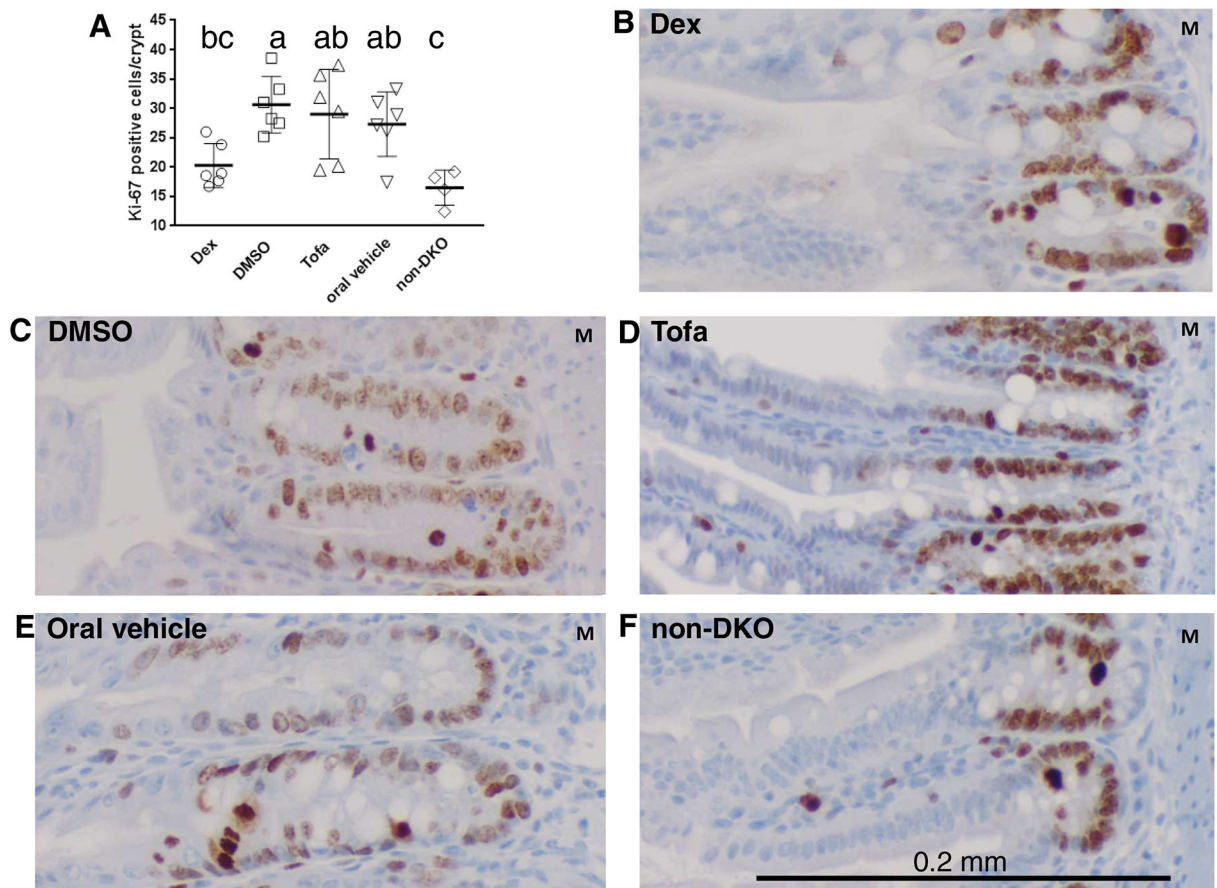
only when they do not share the same letters; i.e. group bc is not different from b or c. The complete results of pairwise comparisons to show suggestive effects are shown in Supplementary Table 3. Panels G-I show H&E histology of GPx1/2-DKO ileum without drug treatment and Panels K-M shows with Dex or Tofa treatment. Panel J is IHC of control DKO ileum stained by TUNEL to verify crypt apoptosis, and Panel N is IHC of ileum labeled with anti-lysozyme antibody to illustrate Paneth cell exfoliation. The open arrows point to apoptotic cells, black arrows point to Paneth cells and white arrows point to an exfoliated Paneth cell. Panels are labeled as vehicle or drug treated and the age of mice analyzed. Original images were 20× for H&E and 40× for IHC. M (muscle layers) indicates the orientation.



**Figure 3: Comparison of apoptosis and gland exfoliation in the GPx1/2-DKO colon treated with immune suppressants.**

Each symbol represents one mouse. Panel A and B show the median and interquartile range of number of apoptosis in each gland and exfoliated cells per 10× field, respectively. When groups do not share letters, they are significantly different. The complete results of pairwise comparisons to show suggestive effects are shown in Supplementary Table 3.

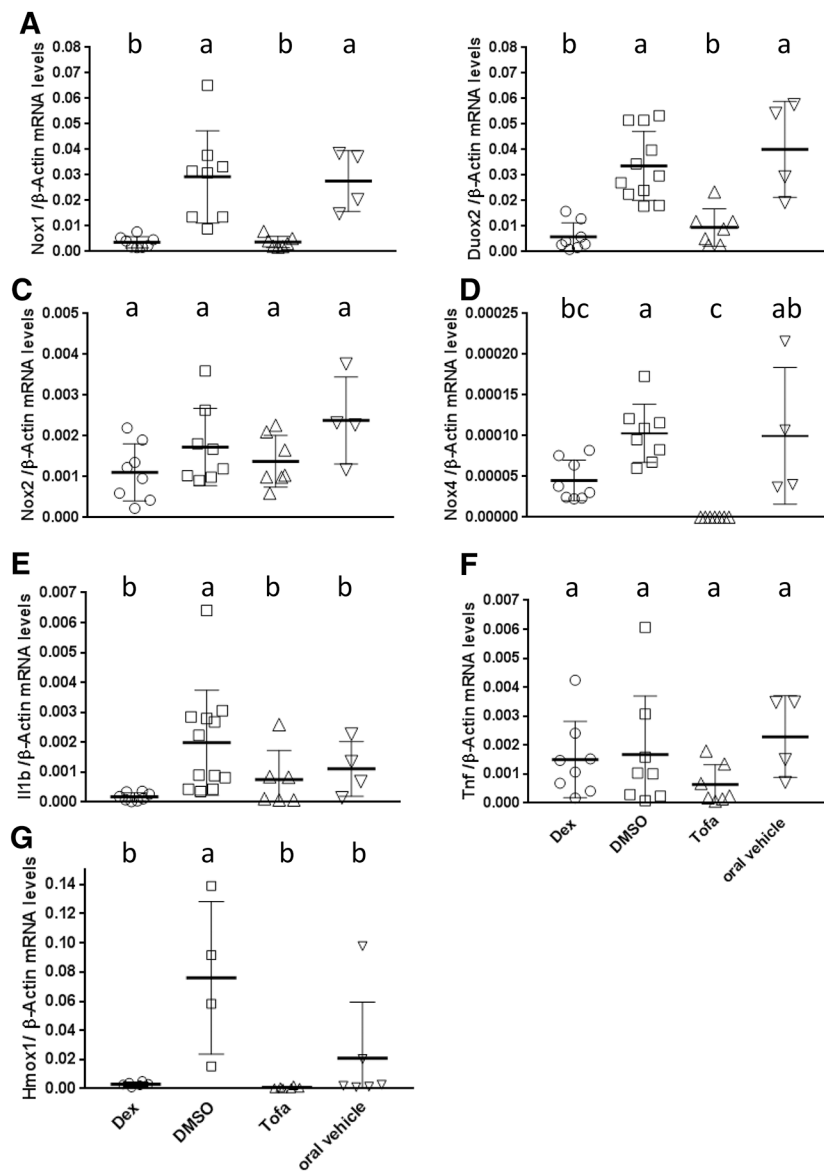




**Figure 4: Comparison of cell proliferation with IHC of anti-Ki-67 Ab treated with Dex or Tofa in 35-day-old GPx1/2-DKO ilea.**

Panel A shows the number of Ki-67 positive cells in the crypt of GPx1/2-DKO mice treated with Dex, DMSO, Tofa or Tofa vehicle and untreated non-GPx1/2-DKO mice; the non-GPx1/2-DKO ileum has a background level of proliferating cells. Each symbol represents one mouse, six mice in each group except 4 in non-DKO group. The letters above each groups indicate their values in the order of a>b>c. Groups are different when they don't share a same letter; i.e. ab is not different from a or bc; analyzed by ANOVA with Tukey's multiple correction test. Panel F shows a non-DKO control and Panels B-E show GPx1/2-DKO ileum. The images show the representative areas in each group with the original magnification at 20X. The positively stained cells (brown color) are abundant in the crypt, and the section was counterstained with hematoxylin (blue color). M indicates the muscle layer.





**Figure 5: Comparison of the mRNA levels in GPx1/2-DKO ileum affected by Dex and Tofa analyzed at 35 days of age.**

Panels A-G show the mRNA levels of *Nox1*, *Duox2*, *Nox2*, *Nox4*, *Il1b*, *Tnf* and *Hmox1* altered by Dex or Tofa. All mRNA levels were normalized by  $\beta$ -actin mRNA levels. Each symbol represents one mouse (the number of mice in each group is 8, 8–11, 8 and 4 for Dex, DMSO, Tofa and oral vehicle, respectively; except *Hmox1*: 6, 4, 6, 6) and the group is shown as mean  $\pm$  Stdev. The letters above each groups indicate the value of mRNA levels, where  $a > b > c$ . When groups share a same letter, they are not different; i.e. ab is not different from a or bc; analyzed by ANOVA with Tukey's multiple correction test.