The gut microbiota engages different signaling pathways to induce *Duox2* expression in the ileum and colon epithelium

F Sommer[1] and F Bäckhed[1,2]

The epithelium is a first line of defense against microorganisms in the gut. Reactive oxygen species (ROS) have an important role in controlling the normal gut microbiota and pathogenic bacteria. Dual oxidase 2 (DUOX2) is an important source of hydrogen peroxide in the small and large intestine, and the gut microbiota induces *Duox2* expression. Here, we investigated the microbial regulation of *Duox2* expression. We found that *Duox2* was expressed by intestinal epithelial cells mainly in the tip of the epithelium. *Duox2* expression was strongly induced by the presence of a normal microbiota in mice, but not when germ-free mice were colonized with various commensal bacteria. *Duox2* expression was more rapidly induced by the gut microbiota in the colon than in the ileum. Furthermore, we showed that regulation of *Duox2* expression in the ileum involved TIR-domain-containing adaptor protein including interferon-β (TRIF) and canonical nuclear factor-κB p50/p65 signaling, whereas regulation of *Duox2* expression in the colon involved MyD88 and the p38 pathway. Collectively, these data indicate that the gut microbiota uses two distinct signaling pathways to induce *Duox2* expression in the ileum and colon epithelium.

INTRODUCTION

We live in close association with microorganisms (microbiota) that cover all surfaces of the body, with the majority residing within our intestinal tract. It is now recognized that the development of several increasingly abundant diseases such as obesity, atherosclerosis, and inflammatory bowel diseases is associated with dysbiosis of the microbiota.[1] Thus, keeping a stable beneficial symbiosis seems to be a key requirement for health.[2] The gut epithelium has an important role in controlling the normal gut microbiota through production of antimicrobial peptides and reactive oxygen species (ROS).[3–7] The NADPH oxidase family members are the main ROS-producing enzymes[3–7] and can be divided into two classes: NADPH oxidase and dual oxidase (DUOX).[8,9] DUOX enzymes are characterized by their additional peroxidase homology domain, which exerts the catalytic function.[10] Thus, DUOX enzymes produce hydrogen peroxide in a Ca^{2+}-dependent manner, whereas most NADPH oxidase enzymes produce superoxide.[11,12]

Duox2 is expressed by surface epithelia in the lung or the intestine[13,14] and its expression is increased during disease or dysbiosis, e.g., in patients with Crohn's disease or in response to *Helicobacter pylori* infections.[15–17] In *Drosophila*, dDUOX (the homolog of mammalian DUOX2) produced ROS are required for protection from intestinal bacterial infection.[18] However, DUOX2-mediated ROS production needs to be tightly regulated to avoid a nonspecific activation that would cause severe damage to the host tissue, e.g., during chronic inflammation.[19]

In *Drosophila*, dDUOX is regulated by the cooperative action of three signaling pathways:[20] (i) a peptidoglycan pattern recognition receptor pathway, (ii) an unknown nonpeptidoglycan pattern recognition receptor signaling pathway and (iii) phospholipase C–β mediated inhibition of the p38 mitogen-activated protein kinase (MAPK) signaling pathway. In humans, DUOX2 activity is partially regulated via NOD2 signaling[21] as well as by protein kinase C.[22] In the mouse intestine, *Duox2* expression is induced by the microbiota,[23] but it is not known

how host–microbiota interactions regulate *Duox2* in mammals. Here, we investigated how the gut microbiota modulates *Duox2* expression using germ-free (GF) and colonized mice, as well as genetically modified mice lacking components required for innate immune signaling. Finally, we used specific inhibitors of various signaling pathways in an *in vitro* cell culture system to identify molecular mechanisms of the host–microbiota cross-talk regulating *Duox2* gene expression.
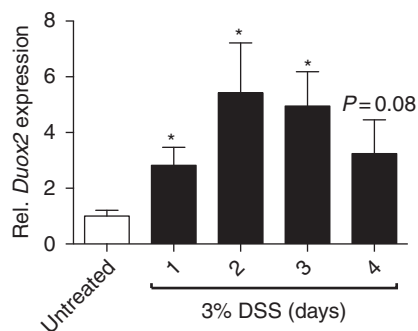
RESULTS

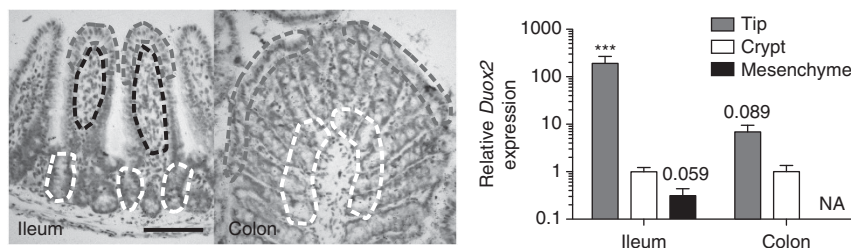### Experimental colitis induced *Duox2* expression

DUOX2 levels are increased in patients with intestinal chronic inflammation or infections with *H. pylori*.[15–17] To investigate whether *Duox2* expression is also modulated during chemically induced acute colitis, we treated mice with dextran sodium sulfate (DSS) and monitored *Duox2* expression. *Duox2* expression was immediately induced by the DSS treatment already one day after treatment and remained elevated during the course of colitis (**Figure 1**). Thus, *Duox2* expression is rapidly modulated in response to injury and may thus contribute to disease.

Duox2 is predominantly expressed by intestinal epithelial cells in the tip

To address which cells in the epithelium express *Duox2*, we performed laser capture microdissection (LCM) followed by quantitative real-time PCR (qPCR) of different cellular fractions of the mouse intestinal tissue: tip and crypt epithelial

Figure 1 *Duox2* expression is induced by experimental inflammation. *Duox2* expression in the colon of C57Bl6 wild-type mice during the time course of dextran sodium sulfate (DSS)-induced intestinal inflammation; $n = 4$ per group. Data show mean ± s.e.m.; *$P < 0.05$ vs. untreated control.

fractions from the ileum and colon of wild-type (WT) mice. First, we verified the purity of the fractions by assessing the expression of the tip-specific genes *Alpi* (alkaline phosphatase), *Apoa4* (apolipoprotein A4), and *Krt20* (keratin 20), and the crypt-specific genes *Lyz1* (lysozyme 1), *Defa5* (defensing alpha 5), *Ki67* (antigen identified by monoclonal antibody Ki-67), and *Ephb2* (ephrin B2), which were greatly enriched in the respective epithelial fractions (**Supplementary Figure S1** online). *Duox2* expression in the ileum was 190 times higher in the tip than in the crypt and hardly detectable in the mesenchymal fraction (**Figure 2**), which consists of mainly fibroblasts and immune cells. In the colon, *Duox2* expression was seven times higher in the tip than in the crypt (**Figure 2**). These data demonstrate that *Duox2* expression is localized to the epithelium in the gut and predominantly to the differentiated epithelium in the ileum villus tip.

The gut microbiota induces *Duox2* expression in the intestine

In a global microarray-based survey for microbially regulated genes, *Duox2* was identified as one of the genes that were most upregulated by the microbiota in the intestine.[23] Interestingly, we found a significantly increased or trend toward higher peroxidase activity in ileal and colon protein lysates from conventionally raised (CONV-R) mice compared with GF mice, respectively (**Figure 3a**). Using qPCR, we verified that the gut microbiota induced *Duox2* expression: *Duox2* levels were significantly higher in CONV-R compared with GF mice both in the ileum (ninefold) and colon (threefold) (**Figure 3b**). Similarly, *Duox2* expression was increased in CONV-R compared with GF mice in the duodenum and jejunum (**Supplementary Figure S2**), but their absolute expression levels were 10–100-fold lower as compared with those in the ileum and colon. Next, we determined *Duox2* expression changes during colonization of GF mice with a normal microbiota. *Duox2* expression kinetics differed in the ileum and colon (**Figure 3c**). In the ileum, a slight microbial induction of *Duox2* expression was first detected 3 days after colonization) and expression increased to levels similar to CONV-R mice 7 and 14 days after colonization. In contrast, colonic *Duox2* expression was readily induced by the gut microbiota already 1 day after colonization and increased to
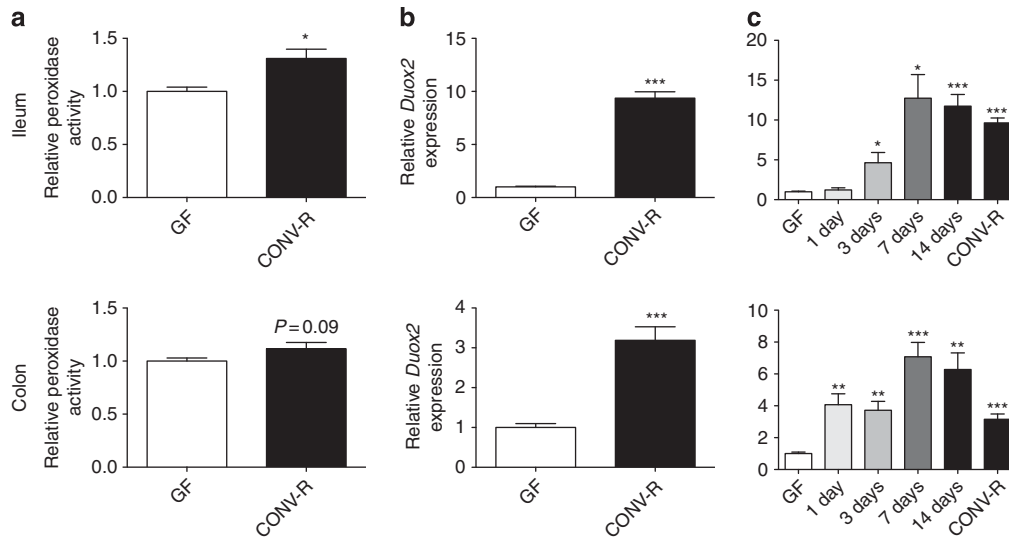

Figure 2 *Duox2* is expressed by intestinal epithelial cells predominantly in the tip epithelium. *Duox2* expression in laser capture microdissection (LCM)-harvested tip (dashed red line) and crypt epithelial fractions (dashed white line) of the ileum and colon; $n = 3–4$ per group. Data show mean ± s.e.m. and all values were normalized to expression in the ileum crypt; ***$P < 0.001$ vs. crypt in the same tissue. A full color version of this figure is available at the *Mucosal Immunology* jounal online.

Figure 3 The gut microbiota induces *Duox2* expression in the ileum and colon. (**a**) Relative peroxidase activity in the ileum and colon protein lysates of germ-free (GF) and conventionally raised (CONV-R) mice. *$P < 0.05$. (**b** and **c**) *Duox2* expression (quantitative PCR) in the ileum and colon of (**b**) GF and CONV-R mice and (**c**) during colonization of GF mice with a normal microbiota; $n = 6–7$ (**a**) and $n = 4–5$ (**b** and **c**) per group. Data show mean ± s.e.m.; *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. GF.

levels higher than in CONV-R mice 7 and 14 days after colonization (**Figure 3c**). By measuring *Tnfa* expression, we tested whether *Duox2* expression changes during colonization follow a transient inflammatory reaction, but no increases in *Tnfa* were detected (**Supplementary Figure S3**). We also investigated if *Duox2* expression could be induced by different commensals of the gut microbiota. None of the 13 tested bacterial strains (3 single bacteria or an assembly of 10 strains), including both Gram-positive and -negative bacteria, induced *Duox2* expression (**Supplementary Figure S4A and B**) despite successful colonization (data not shown), suggesting that commensal-derived patterns are not sufficient to elicit *Duox2* expression.

Induction of *Duox2* expression in the ileum involves NF-κB signaling

Next, we aimed to determine the signaling pathways engaged to induce *Duox2* expression in the intestinal epithelium. Therefore, we investigated *Duox2* expression levels in mice deficient in different components of innate immune signaling, specifically the NF-κB pathway with the receptor adaptors TIR-domain-containing adaptor protein including interferon-β (TRIF) or MyD88 and the transcription factors p50 and p65. In the ileum, *Duox2* expression was increased in *Myd88*$^{-/-}$ mice and decreased in *Trif*$^{-/-}$ and *Myd88*$^{-/-}$*Trif*$^{-/-}$ compared with WT mice (**Figure 4a, b**). Furthermore, *Duox2* expression involved canonical NF-κB signaling as expression was decreased in *p50*$^{-/-}$ and IEC *p65*$^{-/-}$ (VillinCre/p65$^{f/f}$) mice (**Figure 4c**). In contrast, *Duox2* expression in the colon was decreased in *Myd88*$^{-/-}$ mice compared with WT mice (**Figure 4a**), but increased in *Trif*$^{-/-}$ mice and unchanged in *Myd88*$^{-/-}$*Trif*$^{-/-}$, *p50*$^{-/-}$, and IEC *p65*$^{-/-}$ mice (**Figure 4b, c**). These data indicate that *Duox2* expression

is partially induced via TRIF and p50/p65 in the ileum, and through the adaptor protein MyD88 in the colon.

P38 pathway is required for IL-1β-mediated *Duox2* expression induction in the colon

To investigate how *Duox2* is regulated in the colonic epithelium, we treated ileal and colonic explants with the proinflammatory stimuli lipopolysaccharide (LPS) and interleukin (IL)-1β, both of which are known to activate MyD88 signaling pathways. Only IL-1β induced *Duox2* expression in the ileum explants (**Figure 5a**), whereas both LPS and IL-1β induced *Duox2* expression in the colon explants (**Figure 5b**). IL-1β and mouse intestinal content, but not LPS, also induced *Duox2* expression in the human colon carcinoma cell line Caco-2 (**Figure 5c, d**). To confirm our qPCR data that NF-κB is not involved in the regulation of *Duox2* expression in the colon, we generated luciferase-based reporter constructs with different stretches of the *Duox2* promoter containing either WT or mutated NF-κB binding sites and transfected Caco-2 cells with these vectors (**Supplementary Figure S5A**). Stimulation with IL-1β increased luminescence for the reporters containing the 1 or 3 kb constructs. However, mutation of the NF-κB binding sites did not abolish the increase in luminescence by treatment with IL-1β (**Supplementary Figure S5B**), further demonstrating that *Duox2* regulation in the colonic epithelium is independent of NF-κB.

In an attempt to identify the signaling pathway leading to *Duox2* expression in the colonic epithelium, we tested the effect of specific inhibitors of different signaling pathways downstream of MyD88 signaling on IL-1β-induced increases in *Duox2* expression in Caco-2 cells. Pretreatment with the specific p38 MAPK inhibitor SB203580 (10 μM for 60 min) abolished IL-1β-mediated *Duox2* expression (**Figure 5e**). No effect was observed
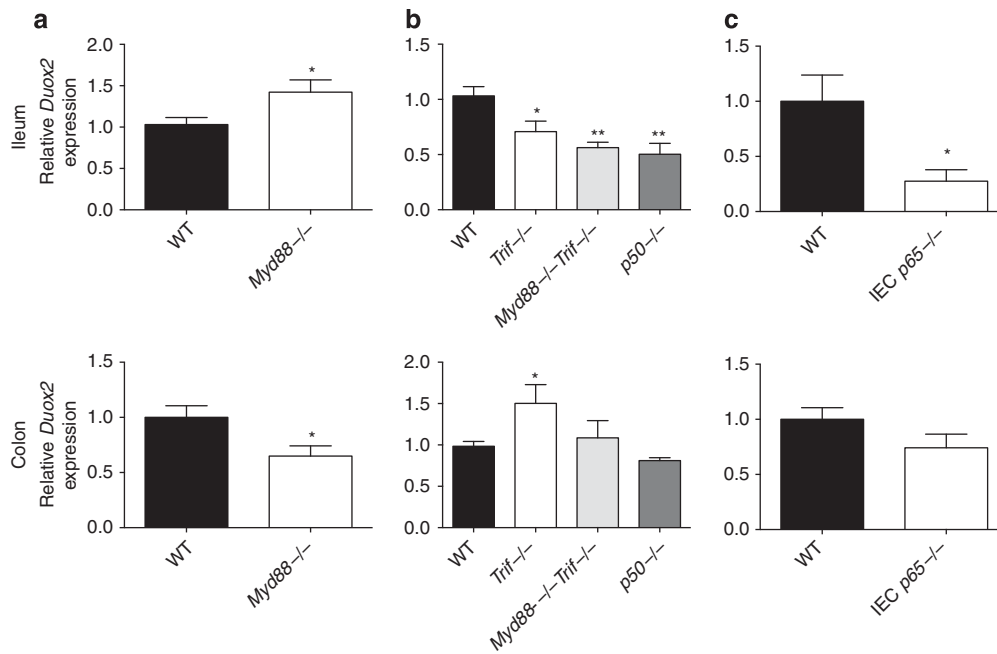
Figure 4 Microbial induction of *Duox2* expression in the ileum involves nuclear factor-κB (NF-κB) signaling. *Duox2* expression in the ileum and colon of (**a**) wild-type (WT) and *Myd88*^{−/−} mice, (**b**) WT, *Trif*^{−/−}, *Myd88*^{−/−} *Trif*^{−/−} compound homozygous knockouts, and *p50*^{−/−} mice and (**c**) WT and IEC *p65* (VillinCre/p65[f/f]) mice; *n* = 4–15 per group. Data show mean ± s.e.m.; *P < 0.05 and **P < 0.01 vs. WT.

with any of the other inhibitors (wedelolactone, IKK inhibitor; JSH-23, NF-κB activation inhibitor; SP600125, JNK-1/2/3 inhibitor; and U0126, MEK1/2 inhibitor). Importantly, pretreatment with SB203580 also abolished IL-1β-mediated *Duox2* expression in the colon explants (**Figure 5f**). Thus, our data suggest that *Duox2* expression involves NF-κB in the ileum and p38 in the colon.
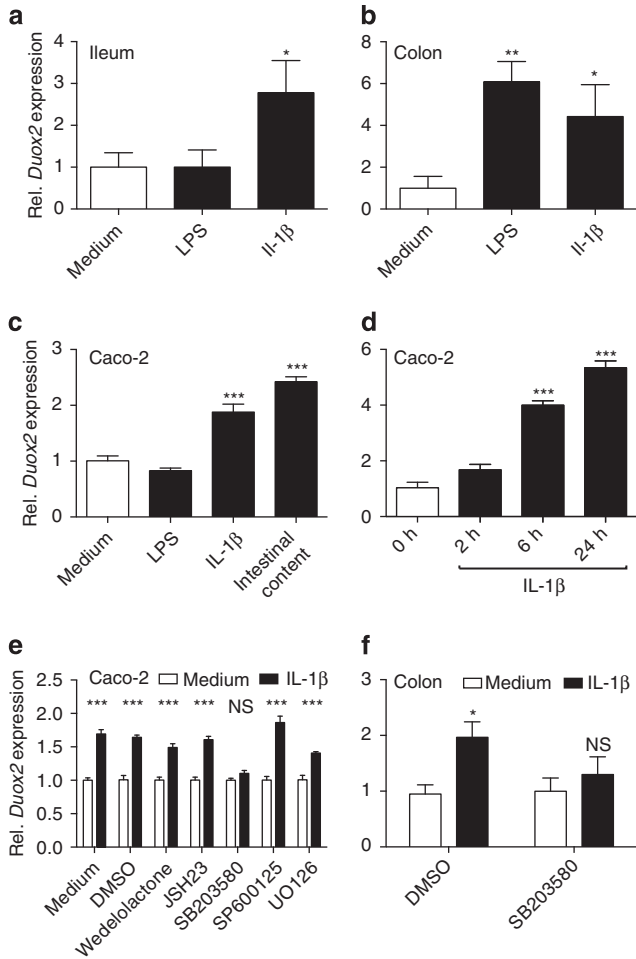
DISCUSSION

We investigated the expression of *Duox2* in the mouse intestinal tract and its regulation. Using LCM, we found that *Duox2* was predominantly expressed in the tip epithelium of the ileum and colon and not in mesenchymal cells. In line with previous findings,[23] *Duox2* expression was induced by a normal microbiota in both ileum and colon, but the expression kinetics differed in the two tissues: in the colon, *Duox2* expression was readily induced one day after colonization, whereas induction was delayed in the ileum, suggesting different regulatory pathways. In the ileum, microbial induction of *Duox2* expression involved NF-κB p50/p65 signaling through TRIF. In contrast, colonic *Duox2* expression induction was mediated through MyD88 and p38 MAPK (**Figure 6**).

DUOX2 levels are increased in several inflammatory states including chronic intestinal inflammation or infections with *H. pylori*;[15–17] however, it is unknown whether DUOX2 is driving inflammation or activated in a secondary response. Our finding that DSS treatment immediately induced *Duox2* expression argues for that DUOX2 may drive the inflammatory response as the induction occurred before any tissue damage.[24] This hypothesis is further supported by our finding that changes in *Duox2* expression during colonization

of GF mice preceded those of *Tnfa*, a general marker for the inflammatory tone.

Duox2 expression kinetics during colonization differed greatly between the ileum and colon, which might reflect the different signaling pathways involved in these tissues. The delayed response in ileum could be due to indirect signaling toward a transient inflammatory reaction caused by colonization. However, no preceding increases in inflammatory markers were detected. Instead, we speculate that epithelial cells of the small intestine are resistant to stimuli after leaving the crypt to avoid excessive immune reactions toward the microbiota, as they are not covered by a thick mucus layer that would inhibit contact to the resident bacteria. This hypothesis is supported by the finding that only basolateral stimuli are capable of activating Toll-like receptor 5 (TLR5) or TLR9 proinflammatory signaling in polarized intestinal epithelial cells, thus limiting responsiveness only to microbes that penetrate the epithelial barrier.[25,26] Another possible explanation could be that we used a cecal inoculum for colonization, which might be more adapted to the colon habitat and thus it could take longer for the microbiota to adapt to the ileum and induce proper signaling.
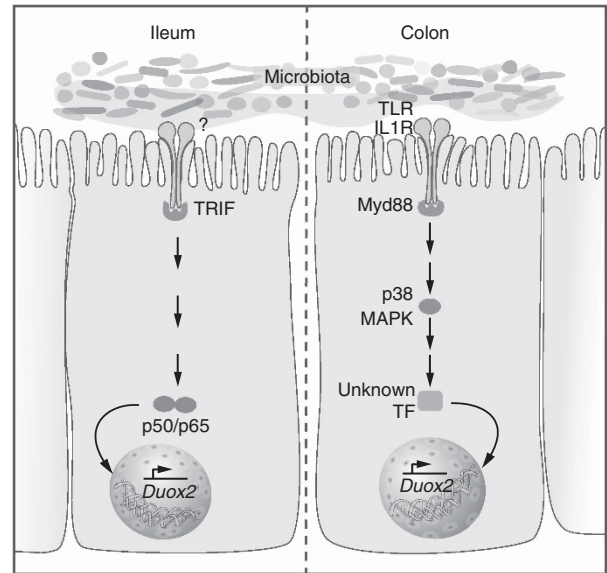
Duox2 expression levels were unaffected by colonization with 13 tested commensals including *Escherichia coli*, *Bifidobacterium longum*, or *Bacteroides thetaiotaomicron*. Thus, it seems unlikely that a single microbe-associated molecular pattern could be the stimulus. It is known that *Duox2* expression is stimulated by several pathogens including *Listeria monocytogenes* or *Helicobacter felis* or in patients with inflammatory bowel disease, which is associated with an increase of pathogens.[21,27] However, these bacteria are not present in our

Figure 6 Model for the differential microbial regulation of *Duox2* expression in the ileum and colon. In the ileum the microbial signal might be sensed by an unknown receptor and transmitted through TIR-domain-containing adaptor protein including interferon-β (TRIF) and p50/p65 initiating *Duox2* expression. In the colon the microbial signal might be recognized by a TLR or IL-1 receptor and transmitted through MyD88, p38 MAPK and a yet unknown non-p50/p65 transcription factor (TF) leading to *Duox2* expression.

Figure 5 p38 pathway is required for IL-1β-induced *Duox2* expression induction in the colon. *Duox2* expression after incubation with 100 ng ml^{-1} lipopolysaccharide (LPS) or 20 ng ml^{-1} interleukin-1β (IL-1β) (**a**) for 24 h in the ileum explant, (**b**) for 24 h in the colon explant. (**c**) *Duox2* expression after incubation with 100 ng ml^{-1} LPS, 20 ng ml^{-1} IL-1β, or 10% sterile filtered intestinal content for 6 h in Caco-2 cells. (**d**) *Duox2* expression in Caco-2 cells after incubation with 20 ng ml^{-1} IL-1β for 2, 6, or 24 h. (**e**) *Duox2* expression in Caco-2 cells treated with specific inhibitors of IL-1β downstream signaling cascades before stimulation with IL-1β. Inhibitors were wedelolactone (IκB kinase (IKK) inhibitor, 50 μM for 60 min), JSH-23 (NF-κB activation inhibitor, 10 μM for 60 min), SB203580 (p38 mitogen-activated protein kinase (MAPK) inhibitor, 10 μM for 60 min), SP600125 (c-Jun N-terminal kinase (JNK)-1/2/3 inhibitor, 50 μM for 45 min) and U0126 (MAP kinase kinase (MEK1/2) inhibitor, 10 μM for 2 h). All inhibitors were dissolved in dimethyl sulfoxide (DMSO). (**f**) *Duox2* expression in the colon explants treated with DMSO or SB203580 before stimulation with IL-1β for 24 h; $n = 3–5$ per group. Data show mean ± s.e.m.; *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. medium.

animal facility and thus cannot explain why our normal specific pathogen-free microbiota induces *Duox2* expression in the absence of inflammation. It is possible that only a limited number of bacteria (different from the ones we tested) might be able to induce *Duox2* expression. Alternatively, the bacterial density may be needed to be higher or specific metabolites may be required to induce *Duox2* expression.

Duox2 expression was differentially regulated in the ileum and colon. We noted that signaling in the ileum was partially mediated via NF-κB and TRIF, whereas Myd88 and p38 were

involved in the colon. There are several differences in these tissues that could account for the differential regulation. For example, the microbiota composition differs between the ileum and colon,[1] and therefore the microbiota signals inducing *Duox2* expression could differ in these tissues. Moreover, the cellular composition,[28] tissue architecture,[29] and functional metabolic properties[30] differ between the ileum and colon.

Both LPS and IL-1β activate MyD88 signaling pathways and induced *Duox2* expression in the colon explants. However, in Caco-2 cells IL-1β, but not LPS, was capable of inducing *Duox2*. This differential response in the Caco-2 cells might be because they are a cancer cell line and thus do not exactly recapitulate primary intestinal epithelial cells. The prototypical receptors for LPS and IL-1β are TLR4 and the IL-1 receptor, respectively.[31,32] Only TLR3 or TLR4 signal through TRIF, whereas all TLRs (except TLR3) and the IL-1 and IL-18 receptors signal through MyD88. Thus, our data suggest that in the ileum TLR3 or TLR4 and TRIF and in the colon TLRs and MyD88 may be involved in the regulation of *Duox2*. However, in $Myd88^{-/-}Trif^{-/-}$ mice, which completely lack pattern recognition receptor signaling, *Duox2* expression was not completely abolished, indicating the presence of another signaling mechanism. Our findings are supported by recent findings showing that in human pancreas cell lines TLR4 is required to induce *Duox2*[33] and that some NADPH oxidase family members interact physically with components of the TLR signaling cascade.[34–36] However, we cannot exclude that the gut microbiota induces *Duox2* expression indirectly

through a pathway that involves host production of IL-1 or IL-18, which also mediate signaling from their receptors through MyD88. Furthermore, we cannot rule out effects of non-epithelial cells in the signaling leading to increased *Duox2* expression but analyzing conditional intestinal-specific knockout mice could clarify this.

Microbiota-induced *Duox2* expression may at first glance seem useful for the host but undesirable for the bacteria as ROS are antibacterial. However, not all bacteria show the same susceptibility to ROS.[37,38] Thus, one hypothesis could be that those bacteria that induce *Duox2* expression might also be more resistant to ROS, thereby exploiting a host defense system to reduce competition by other more susceptible bacteria. Furthermore, it has been shown that commensal bacteria tone down proinflammatory NF-κB signaling through ROS.[39,40] However, the origin of these ROS is not clear. If these ROS originate from host DUOX2 induced by the microbiota, they could counteract their direct antibacterial effects and instead be beneficial for the microbes by toning down other inflammatory responses.

Taken together, here we found that in the mouse expression of *Duox2* is differentially induced by the microbiota and regulated via two different signaling pathways in the ileum and colon. These findings highlight the need for further studies delineating host microbial interactions in the gut.

METHODS

Mice. C57Bl6/J and Swiss-Webster mice, as well as mice lacking different components of innate immune signaling—$Myd88^{-/-}$, $Trif^{-/-}$, $MyD88^{-/-}Trif^{-/-}$, $p50^{-/-}$, and IEC $p65^{-/-}$ (VillinCre/p65$^{f/f}$)—were maintained under standard specific pathogen-free or GF conditions as described previously.[24] All mice were 10–15 weeks of age and sex and age matched for individual experiments. GF mice were colonized with a normal cecal microbiota by oral gavage and maintained for up to 14 days as described.[41] A similar protocol was used to colonize GF mice with single bacterial species *Escherichia coli*, *Bifidobacterium longum*, or *Bacteroides thetaiotaomicron* or with an assembly of 10 commensal bacteria (Petia Kovetcheva, personal communication). The mice colonized with the 10 commensal bacteria were bred in isolators for at least eight generations before use in an experiment. Mice were killed by cervical dislocation and the distal part of the small intestine (ileum) and the proximal part of the colon were removed and frozen in liquid nitrogen. Animal protocols were approved by the Gothenburg Animal Ethics Committee.

DSS treatment. Colonic inflammation was induced in 9–14-week-old mice by administration of 3% DSS (TdB TdB Consultancy, Uppsala, Sweden) for four days in drinking water as described previously.[42] Colitis severity was assessed as described and presented elsewhere.[24]

Laser capture microdissection. C57Bl6/J female mice were used at 12 weeks of age. LCM was performed as described previously.[43] Briefly, mice were killed by cervical dislocation and the intestine removed. The ileum and the proximal part of the colon were excised, flushed with phosphate-buffered saline, and finally flushed and embedded with OCT freezing medium. Eight-micrometer-thick cryosections were cut using a microtome (Leica, Nussloch, Germany), placed onto PEN membrane slides (Zeiss, Jena, Germany), and stained immediately. After dehydration in absolute and 70% ethanol, OCT was removed by dipping in RNase-free water before staining with 1% cresyl violet in 50% ethanol. Slides were partially destained by dipping in 70% and absolute ethanol before air-drying. Slides were stored in airtight

containers at $-80\,^{\circ}$C until LCM. LCM was performed using PALM Microbeam Microdissection microscope (Zeiss) and fractions were collected dry. The harvested fractions were immediately lysed in RLT (a guanidine-thiocyanate–containing lysis buffer) containing 1% β-mercaptoethanol and stored at $-80\,^{\circ}$C. RNA was isolated from the lysates using RNeasy Micro Kit (Qiagen, Hilden, Germany). The complete RNA volume was used for synthesis of cDNA before qPCR.

Preparation of sterile mouse intestinal content. Intestinal content from almost the complete intestine of one CONV-R donor mouse (from jejunum until distal colon) was mixed with 15 ml phosphate-buffered saline. After centrifugation, the supernatant was passed through a 0.2 μm filter.

Peroxidase activity. Peroxidase activity was measured as described previously.[44] Briefly, ileum or colon tissue was homogenized in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate). Fifty micrograms of protein was added to 100 μl TMB solution (Sigma, St Louis, MO). After incubation at room temperature for 30 min, absorbance at 650 nm was recorded.

Expression analysis using q PCR. Duodeum, jejunum, ileum, or colon tissue (30 mg) was homogenized with TissueLyser (Qiagen, Hilden, Germany), and total RNA was isolated with the RNeasy Mini Kit (Qiagen). The High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) was used to synthesize 20 μl cDNA templates from 500 ng purified RNA with random hexamer primers, and the products were diluted seven times before use in subsequent reactions. SYBR Green Master Mix buffer (1 × ; Bio-Rad Laboratories) was used for q PCR at final reaction volumes of 25 μl. Gene-specific results were normalized to the ribosomal protein L32 mRNA (primer sequences can be found in **Supplementary Table S1**). Assays were performed in a CFX96 Real-Time System (Bio-Rad Laboratories). The reactions were analyzed with the ΔΔCT analysis method.

Ex vivo stimulation. Mice were killed by cervical dislocation and a 2 cm colon segment was collected. Intestinal contents were removed by flushing with phosphate-buffered saline and the segment was divided into four equal parts. Each part was placed in 24-well cell culture plates containing the growth medium (Dulbecco's modified Eagle's medium with glutamine and pyruvate, 4.5 g l^{-1} glucose, 10% fetal calf serum, 100 U ml^{-1} penicillin, and 100 mg ml^{-1} streptomycin) and either LPS (100 ng ml^{-1}) or IL-1β (20 ng ml^{-1}). The plates were then incubated overnight at 37 $^{\circ}$C with 5% CO$_2$. The content of each well was transferred to Eppendorf tubes and centrifuged briefly. The tissue pellet was used for the analysis of mRNA expression.

Caco-2 cell culture. Human colon carcinoma cells (Caco-2; clone HTB-37) were purchased from ATCC (Manassas, VA) and maintained according to the supplier's instructions. Briefly, cells were grown in Eagle's minimum essential medium supplemented with 20% fetal bovine serum, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1.5 g l^{-1} sodium bicarbonate, 1.0 mM sodium pyruvate, and 1% penicillin–streptomycin antibiotic solution. Cells were passaged at 80% confluency and seeded into 24-well plates for experiments. Cells were either incubated with LPS (100 ng ml^{-1}) for 6 h or with IL-1β (20 ng ml^{-1}) for 2, 6, or 24 h or mouse intestinal content (10%) for 6 h. For inhibitor experiments, cells were pretreated with indicated specific inhibitors of different IL-1β downstream signaling cascades before stimulation with 20 ng ml^{-1} IL-1β for 6 h. The inhibitors were wedelolactone (IKK inhibitor, 50 μM for 60 min), JSH-23 (NF-κB activation inhibitor, 10 μM for 60 min), SB203580 (p38 MAPK inhibitor, 10 μM for 60 min), SP600125 (JNK-1/2/3 inhibitor, 50 μM for 45 min), and U0126 (MEK1/2 inhibitor, 10 μM for 2 h). All inhibitors were dissolved in dimethyl sulfoxide. Medium was removed and the cells were lysed using RLT containing 1% β-mercaptoethanol directly in the wells. Lysates were then used for analysis of mRNA expression.

Luciferase reporter system. Luciferase reporter constructs were generated using a modified pGL4.17 reporter vector (Promega, Madison, WI) and 1 or 3 kb of the *Duox2* promoter. Putative NF-κB binding sites were predicted using ConSite[45] and mutated using GENEART Site-Directed Mutagenesis System (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Primer sequences can be found in **Supplementary Table S1**. Sequences of the reporter constructs have been submitted to GenBank (accession numbers KJ408274–KJ408277). Caco-2 cells were stably transfected with the different *Duox2*::luciferase reporter constructs using Lipofectamine (Invitrogen) and successive G418 (Invitrogen; 0.4 mg ml^{-1}) selection. In all, 500,000 cells per well were seeded into a 24-well plate and grown overnight to confluency. Cells were incubated with 20 ng ml^{-1} IL-1β for 6 h and lysed in Glo Lysis buffer (Promega). After mixing the lysate with Steady-Glo luciferase reagent (Promega), luminescence was measured using GloMax 20/20 luminometer (Turner BioSystems, Sunnyvale, CA).

Statistical analysis. Data are presented as mean ± s.e.m. Statistical differences between groups of two were analyzed with a Student's *t*-test, comparisons of three or more groups with one independent variable (e.g., genotype) were analyzed by one-way analysis of variance with *ad hoc* Bonferroni *post hoc* tests using Prism 5 (GraphPad Software, La Jolla, CA).

Supplementary Material is linked to the online version of the paper at http://www.nature.com/mi

DISCLOSURE
FB is founder and owns equity in Metabogen AB. The remaining author declared no conflict of interest.

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