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**NOX1-derived ROS drive the expression of Lipocalin-2 in colonic epithelial cells in inflammatory conditions.**

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**ABSTRACT** (199 words)

Inflammatory bowel disease (IBD) is characterized by severe and recurrent inflammation of the gastrointestinal tract, associated with altered patterns of cytokine synthesis, excessive reactive oxygen species (ROS) production and high levels of the innate immune protein, lipocalin-2 (LCN-2) in the mucosa. The major source of ROS in intestinal epithelial cells is the NADPH oxidase NOX1, which consists of the transmembrane proteins, NOX1 and p22<sup>PHOX</sup>, and the cytosolic proteins, NOXO1, NOXA1 and Rac1. Here, we investigated whether NOX1 activation and ROS production induced by key inflammatory cytokines in IBD causally affects LCN-2 production in colonic epithelial cells. We found that the combination of TNF $\alpha$  and IL-17 induced a dramatic upregulation of NOXO1 expression that was dependent on the activation of p38MAPK and JNK1/2, and resulted into an increase of NOX1 activity and ROS production. NOX1-derived ROS drive the expression of LCN-2 by controlling the expression of I $\kappa$ B $\zeta$ , a master inducer of LCN-2. Furthermore, LCN-2 production and colon damage were decreased in NOX1-deficient mice during TNBS-induced colitis. Finally, analyses of biopsies from patients with Crohn's disease showed increased JNK1/2 activation, and NOXO1 and LCN-2 expression. Therefore, NOX1 might play a key role in mucosal immunity and inflammation by controlling LCN-2 expression.

## INTRODUCTION

Inflammatory bowel disease (IBD) represents a group of chronic relapsing disorders, comprising Crohn's disease and ulcerative colitis, all characterized by severe, uncontrolled and recurrent inflammation of the gastrointestinal tract, arising from a dysregulated immune response to the intestinal microflora. The etiology and pathogenesis of the disease are multifactorial and may include predisposing genetic background, environmental factors, and gut microbial dysbiosis [1]. The aberrant immune responses bring about altered patterns of cytokine synthesis by immune cells from the lamina propria [2] and excessive reactive oxygen species (ROS) production in the inflamed mucosa [3-5].

Tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) and interleukin-17 (IL-17) are two dominant players in IBD, as evidenced by the discovery of the dramatic beneficial effect of TNF $\alpha$  blockade therapy in Crohn's disease [6], and the massive infiltration of Th17 cells, characterized by their ability to produce high levels of IL-17A and IL-17F in the inflamed mucosa of IBD patients [7]. These two pro-inflammatory cytokines control multiple cellular processes that contribute to chronic inflammation, such as the production of inflammatory mediators by immune cells and epithelial cells, and the recruitment and activation of the inflammatory cells mainly, neutrophils, macrophages and dendritic cells [2, 8].

One of the major sources of ROS in immune and epithelial cells is the nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOXs), a unique family of enzymes dedicated to the production of superoxide anion ( $O_2^{\cdot-}$ ) and hydrogen peroxide ( $H_2O_2$ ). This family encompasses seven members, i.e., NOX1-5 and DUOX1-2. NOX2 is expressed in phagocytes (neutrophils, monocytes, macrophages) and is well known to play a crucial role in host defense against microbial infections [9]. The intestinal mucosal epithelium also expresses NOX isoforms, with NOX1 abundantly expressed in the colon [10], and DUOX2 found all along the digestive tract [11]. NOX1 is an enzyme complex that consists of the

transmembrane proteins, NOX1 and p22<sup>PHOX</sup>, and the cytosolic proteins, NOX organizer 1 (NOXO1), NOX activator 1 (NOXA1) and Rac1. All components are required for the activity of the enzyme. The membrane p22<sup>PHOX</sup> subunit stabilizes NOX1, the catalytic core that gave its name to the whole complex, whereas NOXO1 and NOXA1 regulate NOX1 activity by organizing its assembly and promoting its catalytic activity, respectively [12].

A role for both NOX1 and DUOX2 has been reported in mucosal innate immune defense and homeostasis, exerting an effect on bacterial pathogenicity or favoring cell proliferation and wound healing [13-15]. However, a detrimental role has also been suggested, as studies showed that NOX1 is responsible for ileocolitis in mice and that DUOX2 is upregulated in active colitis [16, 17].

During IBD, a defect in the barrier function of the mucosa results in the translocation of commensal bacteria and microbial products from the lumen toward the intestinal epithelium. In addition to antimicrobial peptides such as  $\beta$ -defensins, REG3 $\gamma$  and REG3 $\beta$ , which are essential to protect against infection [18], the expression of lipocalin-2 (LCN-2) by intestinal epithelial cells also ensures mucosal defense by limiting bacterial growth [19]. LCN-2, also known as neutrophil gelatinase-associated protein (NGAL), is a 25 kDa glycoprotein originally identified and purified from human neutrophil specific granules [20]. It can act as a bacteriostatic agent that interferes with siderophore-mediated iron acquisition by bacteria [21, 22], and its expression has been shown to be induced by oxidative stress [23]. Interestingly, LCN-2 expression is strongly upregulated in colonic epithelium during IBD where oxidative stress is observed [2-5, 24]. Its protective role in intestinal inflammation and homeostasis has been demonstrated [25, 26]; however, its ability to bind MMP-9 (or gelatinase B) and to preserve MMP9 enzymatic activity may also favor the inflammatory processes [27].

Here, we investigated whether NOX1 activation and ROS production induced by key inflammatory cytokines in IBD causally affects LCN-2 production in colonic epithelial cells.

We found that ROS produced by NOX1 drive the expression of LCN-2 in human epithelial cells co-stimulated by TNF $\alpha$  and IL-17, and that the underlying mechanisms involve a p38MAPK-JNK/NOXO1/I $\kappa$ B $\zeta$  axis. In addition, LCN-2 production and colon damage were decreased in NOX1-deficient mice during TNBS-induced colitis. Finally, analyses of biopsies from patients with Crohn's disease showed increased JNK activation, and NOXO1 and LCN-2 expression. Therefore, NOX1 might play a key role in mucosal immunity and inflammation by controlling LCN-2 expression.

## RESULTS

### **IL-17 potentiates TNF $\alpha$ -induced NOXO1 expression and ROS production in the human T84 colonic epithelial cell line.**

During IBD, secretion of cytokines by mucosal immune cells such as dendritic cells, monocytes/macrophages, Th17 and Treg, is dramatically enhanced [2]. We analyzed the effect of several of these cytokines on the expression of NOX1 subunits in the T84 human colonic epithelial cell line. These included IL-23, TNF- $\alpha$  and IL-6, which are produced by dendritic cells, monocytes or macrophages, IL-17 and IL-22 produced by Th17, and TGF- $\beta$  produced by Tregs [2]. Only TNF- $\alpha$ , and to a lower extent IL-17 increased the expression of NOX1 subunits, with a predominant effect on NOXO1 (**Fig. 1A and S.1**). Quantification showed that the effect of IL-17 on NOXO1 expression was much weaker than that of TNF $\alpha$  (**Fig. 1B**). NOXA1 was only weakly modulated by TNF- $\alpha$  or IL-17 (**S.1A**), whereas NOX1 was upregulated only at the mRNA level (**S.1B**) and not at the protein level (**S.1C**). IL-6, IL-23, TGF- $\beta$  and IL-22 had no direct effect on NOXO1 (**Fig. 1A**) or NOXA1 expression (**S.1A**). As Th17 and their related cytokines have emerged as important mediators of IBD [2, 7], we investigated whether the hallmark Th17 cytokines, i.e., IL-17 and IL-22, which *per se*

have little or no effect, respectively, could modulate TNF $\alpha$ -induced expression of NOX1 subunits. TNF- $\alpha$  was used at a concentration of 5 ng/ml in order to optimally detect modulating effects. Interestingly, IL-17 markedly increased TNF $\alpha$ -induced NOXO1 expression in a concentration-dependent manner (**Fig. 1C**). By contrast, no such effect was observed with IL-22 (**Fig. 1D**). The potentiating effect of IL-17 on the TNF $\alpha$ -induced response was also predominant for NOXO1 (**Fig 1E and 1F**). Indeed, the expression of NOXA1 and p22<sup>PHOX</sup> was either not modulated or only weakly at the mRNA and protein levels by TNF $\alpha$  +IL-17. Interestingly, NOX1 expression was also upregulated by TNF $\alpha$  +IL-17 at the mRNA level, but not at the protein level (**Fig 1E-F**). mRNA expression of other NOX family members, i.e., NOX2, NOX3, NOX4, and NOX5, was not modulated by TNF $\alpha$ , IL-17, or TNF $\alpha$ +IL-17, but that of DUOX2 was slightly increased by TNF $\alpha$ +IL-17 (**S.2 A**); however, DUOX2 could not be detected at the protein level with or without stimulation (**S.2 B**). Surprisingly, a basal expression of NOX2 mRNA was detected in T84 cells, but was not modulated by cytokines (**S. 2A**), and did not result into NOX2 protein expression (**S.2 B**). Together, these data indicate that the co-stimulatory effect of TNF $\alpha$ +IL-17 is selective for NOX1 subunits, in particular NOXO1.

To determine if the synergistic effect of TNF $\alpha$  and IL-17 on NOXO1 expression was associated with a synergistic effect on ROS production, we used the luminol-amplified chemiluminescence and SOD-inhibitable reduction of cytochrome c assays to assess ROS production in the presence of phorbol myristate acetate (PMA) that allows optimal activation of the NOX1 complex and therefore a better visualization of its activity [28] (**Fig. 2A-B**). While IL-17 by itself did not trigger ROS production, it clearly potentiated TNF $\alpha$ -induced ROS production (**Fig. 2A and 2B**). Furthermore, diphenyleneiodonium (DPI), an inhibitor of NOXs, inhibited this ROS production (**Fig. 2B**). In the absence of PMA, a weak signal that tends towards the same results was observed with the chemiluminescence technique (data not

shown). ROS production was observed both with the luminol-amplified chemiluminescence technique, which detects both intra- and extracellular ROS, and with the SOD-inhibitable reduction of cytochrome c, which only detects extracellular ROS. This suggests that the NOX1 complex might be expressed both intracellularly and at the plasma membrane. Confocal microscopy performed on TNF- $\alpha$ +IL-17-stimulated T84 cells showed that NOXO1 was indeed found close to the plasma membrane, but also in the cytoplasm with a dotted pattern, suggesting localization to intracellular vesicles (not shown).

**The co-stimulatory effect of TNF $\alpha$  and IL-17 on NOXO1 expression and ROS production involves the p38MAPK and JNK1/2 pathways in colonic epithelial cells.**

As TNF $\alpha$  and IL-17 are known to induce the activation of ERK1/2, p38MAPK, and JNK1/2 [29, 30], we investigated whether these pathways were involved in NOXO1 expression in colonic epithelial cells co-stimulated by TNF $\alpha$ +IL-17. As expected, TNF $\alpha$  or IL-17 were each able to activate these signaling pathways, as evidenced by an increase in ERK1/2, p38MAPK, and JNK1/2 phosphorylation (S3A). Under the same conditions, AKT was barely activated (S3A). TNF $\alpha$  and IL-17 activated ERK1/2 and p38MAPK with the same efficiency, while IL-17 was less efficient than TNF $\alpha$  at activating JNK1/2 (S3A and S 3B). The combination of TNF- $\alpha$  and IL17 further increased the activation of p38MAPK and JNK1/2, but not that of ERK1/2 (Fig. 3A). To determine whether these pathways were involved in NOXO1 expression, cells were first treated with UO126 (10  $\mu$ M), SB203580 (25 $\mu$ M), SP600125 (20 $\mu$ M) or wortmannin (500 nM), inhibitors of the ERK1/2, p38MAPK, JNK1/2 and PI3K/AKT pathways, respectively then stimulated with TNF $\alpha$ , IL-17 or TNF $\alpha$ +IL-17. As shown in Fig. 3B, the expression of NOXO1 induced by TNF $\alpha$  and to a lower extent by IL-17, was inhibited by SB203580 (SB) or SP600125 (SP), but not by UO126 (UO) or wortmannin (Wort), indicating that the p38MAPK and JNK1/2 pathways regulated NOXO1

expression under these conditions. In contrast to SP and SB, UO126 induced an increase in NOXO1 expression. Likewise, the co-stimulatory effect of TNF $\alpha$ +IL-17 on NOXO1 expression was inhibited by SB203580 or SP600125, but not by UO126 or wortmannin, showing that the p38MAPK and JNK1/2 pathways also controlled NOXO1 expression in co-stimulatory conditions (**Fig. 3C and 3D**). UO126, SB203580, SP600125 and wortmannin had no effect on cell viability (**S 4A**). Interestingly, the ROS production induced by TNF $\alpha$  and TNF $\alpha$ +IL-17 was also inhibited by SB203580 or SP600125, and not by UO126 or wortmannin (**Fig. 3E**). The close association between NOXO1 expression level and the capability of cells to produce ROS provides evidence for the involvement of the NOX1 complex in ROS production in cells stimulated by TNF $\alpha$  and TNF $\alpha$ +IL-17.

**Co-stimulation of colonic epithelial cells with TNF $\alpha$  and IL-17 induced the expression of LCN-2 in a p38MAPK- and JNK1/2-dependent manner.**

As LCN-2 is strongly upregulated in colonic epithelium during IBD [24], and TNF $\alpha$  and IL-17 are known to regulate the expression of LCN-2 in T84 cells [31], we wanted to determine whether there was a relation between LCN-2 and NOXO1 expression. First, we showed that TNF $\alpha$ +IL-17 strongly upregulated the expression of LCN-2, whereas each cytokine alone had no significant effect (**Fig 4A-B**), and the time-course of NOXO1 expression coincided with the one of LCN-2 (**Fig 4C**). Second, IL-22, which had no synergistic effect with TNF- $\alpha$  on NOXO1 expression, did not affect the expression of LCN-2 in the presence of TNF- $\alpha$ , as opposed to IL-17 (**Fig. 4D**). Finally, as with NOXO1, expression of LCN-2 co-induced by TNF- $\alpha$ +IL-17 was inhibited by SP600125 and SB203580, inhibitors of the JNK 1/2 and p38MAPK pathways, respectively, but not by UO126, inhibitor of the ERK 1/2 pathway (**Fig. 4E**). Thus, in intestinal epithelial cells, both LCN-2 and NOXO1 expression are similarly

increased by co-stimulation with TNF $\alpha$  and IL-17 and the same pathways control this process.

**Expression of LCN-2 induced by co-stimulation of colonic epithelial cells with TNF- $\alpha$  and IL-17 is dependent on NADPH oxidase-derived ROS.**

As expression of NOXO1 and LCN-2 co-induced by TNF $\alpha$ +IL-17 was closely linked in colonic epithelial cells, we hypothesized that ROS produced by NOX1 might control the expression of LCN-2. To verify that NADPH oxidase-derived ROS are indeed involved in the regulation of LCN-2, T84 cells were stimulated or not with TNF $\alpha$ , IL-17 or TNF $\alpha$ +IL-17, and treated with N-acetylcysteine (NAC), a ROS scavenger, or DPI, an inhibitor of NOXs. As shown in **Figure 5A**, NAC and DPI clearly inhibited LCN-2 expression co-induced by TNF $\alpha$ +IL-17, and this effect was concentration-dependent, starting at 5 mM for NAC (**Fig 5B, left**) and 50 nM for DPI (**Fig 5B, right**). NAC and DPI had no effect on cell viability under our experimental conditions (**S. 4B**). In contrast to LCN-2, the induction of several cytokines and chemokines by TNF $\alpha$ +IL-17, such as GRO $\beta$ / $\gamma$ , CXCL1, IL-8 and RANTES, assessed with a semi-quantitative Human Cytokine Antibody Array, was not inhibited by DPI (**Fig. 5 C**). Quantitative measurement of IL-8 using the Single Analyte ELISA Kit confirmed the absence of effect by DPI on TNF $\alpha$ , IL-17 and TNF $\alpha$ +IL-17-induced secretion of IL-8 (**Fig. 5D**). Therefore, in colonic epithelial cells co-stimulated by TNF $\alpha$ +IL17, NADPH oxidase-derived ROS selectively drives the expression of LCN-2.

**NOX1-derived ROS drive the expression of Lipocalin-2 in colonic epithelial cell line co-stimulated by TNF $\alpha$  and IL-17.**

The above results strongly suggest that expression of LCN-2 induced by TNF $\alpha$ +IL-17 is controlled by NOX1-derived ROS *via* the increase of NOXO1 expression. To confirm this

hypothesis, expression of NOXO1 was specifically inhibited using a pool of 3 target-specific NOXO1 siRNAs in cells stimulated or not by TNF $\alpha$ , IL-17 or TNF $\alpha$ +IL-17. Under these conditions, NOXO1 expression was indeed repressed at the protein level by NOXO1 siRNAs, and not by the scrambled siRNAs (**Fig 6A, left and right**), as was the expression of LCN-2 (**Fig. 6B left and right**). Furthermore, NOXO1 siRNAs, but not scrambled siRNAs, significantly decreased ROS production in intestinal epithelial cells co-stimulated by TNF $\alpha$ +IL-17 (**Fig. 6C**).

As a functional NOX1 complex is constituted of NOX1, p22<sup>PHOX</sup>, NOXA1 and Rac1 in addition to NOXO1, we next checked whether NOX1 siRNAs could also inhibit TNF $\alpha$ +IL-17-induced expression of LCN-2. As shown in **Figure 6D (upper)**, a pool of 3 target-specific NOX1 siRNAs repressed the expression of the targeted gene, *NOX1*, at the mRNA level while scrambled siRNAs had only a marginal effect. Furthermore, NOX1 siRNAs specifically decreased the expression of LCN-2 (**Fig. 6D, lower**). Thus, the above data confirm that NOX1-derived ROS support the expression of LCN-2 in human colonic epithelial cell line co-stimulated by TNF $\alpha$  and IL-17.

### **Expression of I $\kappa$ B- $\zeta$ , the central regulator of LCN-2 induction, is controlled by NOX1-derived ROS in colonic epithelial cell line and in human colon organoids co-stimulated by TNF $\alpha$ and IL-17.**

It has been shown in a lung epithelial cell line that expression of LCN-2 requires the binding of both NF- $\kappa$ B (p50/p65) and Inhibitor of NF- $\kappa$ B (I $\kappa$ B)- $\zeta$  to the promoter region (**Fig. 7A**) [32]. Despite its name, I $\kappa$ B- $\zeta$ , a non-canonical member of the NF- $\kappa$ B transcription factor family can act as a positive regulator of NF- $\kappa$ B upon interaction with the NF- $\kappa$ B-p50 subunit [33]. We therefore determined whether NOX1-derived ROS could drive the expression of LCN-2 through the regulation of NF- $\kappa$ B (p50/p65) activation and/or I $\kappa$ B $\zeta$  expression in T84

cells. First, the activation status of NF- $\kappa$ B and the level of I $\kappa$ B- $\zeta$  expression were analyzed in resting, and TNF $\alpha$ , IL-17 or TNF $\alpha$ +IL-17-stimulated cells. Activation of NF- $\kappa$ B was evaluated by the phosphorylation of the NF $\kappa$ B-p65 subunit. As shown in **Fig. 7B**, phosphorylation of the NF $\kappa$ B-p65 subunit was enhanced as compared to resting cells in TNF $\alpha$ -stimulated T84 cells, whereas no induction of I $\kappa$ B $\zeta$  was observed. In IL-17-stimulated cells, phosphorylation of the NF $\kappa$ B-p65 subunit was also increased, and expression of I $\kappa$ B $\zeta$  was slightly induced (**Fig. 7B**). In contrast, both NF $\kappa$ B-p65 phosphorylation and I $\kappa$ B $\zeta$  expression were increased in TNF $\alpha$ +IL-17-stimulated cells (**Fig. 7B**). Of note, as found in other studies [24], the combination of TNF $\alpha$ +IL-17 was more efficient at inducing expression of I $\kappa$ B- $\zeta$  than IL-17 alone, a condition where LCN-2 mRNA was also the most highly expressed (**Fig. 7A**). Next, the potential role of NOX1-derived ROS on the regulation of NF- $\kappa$ B (p50/p65) activation and/or I $\kappa$ B- $\zeta$  expression was analyzed using the ROS scavenger NAC, the NADPH oxidase inhibitor DPI, and NOXO1 siRNA strategy. Interestingly, while NAC and DPI inhibited expression of I $\kappa$ B- $\zeta$  in a concentration-dependent manner in colonic epithelial cells stimulated by TNF $\alpha$ +IL-17 (**Fig. 7C**), they did not significantly alter NF $\kappa$ B-p65 phosphorylation (**Fig. 7D**), in agreement with the finding that DPI inhibited LCN-2 expression (**Fig. 5B**, right), but not the NF $\kappa$ B-dependent secretion of chemokines, in particular IL-8 (**Fig. 5 C, 5D**). Finally, NOXO1 siRNAs decreased the expression of I $\kappa$ B- $\zeta$  in T84 cells co-stimulated by TNF $\alpha$ +IL-17 as compared to control cells, while scrambled siRNAs did not alter the expression of I $\kappa$ B- $\zeta$  (**Fig. 7E**), thereby confirming that NOX1-derived ROS regulate the expression of I $\kappa$ B- $\zeta$ .

In order to determine if LCN-2 expression is similarly regulated in primary epithelial cells, we developed human colon organoids from biopsies of patients without IBD undergoing routine colonoscopy as described in the Supplementary Methods. **Figure 7F** (upper) shows the typical

development of organoids from colon crypts. Crypt budding occurs within 5 to 6 days after seeding, and multi-lobed structures appeared between Day 7 to 10. Expression of NOXO1, I $\kappa$ B $\zeta$  and LCN-2 were analyzed in colon organoids stimulated or not with TNF $\alpha$ , IL-17 or TNF $\alpha$ +IL-17. **Figure 7F** (lower left) shows that the combination of TNF $\alpha$ +IL-17 was the most efficient at inducing NOXO1, I $\kappa$ B $\zeta$  and LCN-2 expression in colon organoids, as in T84 cells. Furthermore, NAC and DPI also inhibited the expression of I $\kappa$ B $\zeta$  and LCN-2 in colon organoids stimulated with TNF $\alpha$ +IL-17 (**Fig.7**, lower right). These data suggest that the NOXO1/I $\kappa$ B $\zeta$  axis may also control LCN-2 expression in primary human epithelial cells.

### **Colon damage and LCN-2 production were decreased in NOX1-deficient mice during TNBS-induced colitis.**

In order to determine if NOX1-derived ROS could modulate LCN-2 expression and intestinal inflammation *in vivo*, we used a mouse model of colonic inflammation induced by 2,4,6-trinitrobenzene sulfonic acid (TNBS) in which macrophage-derived cytokines such as TNF $\alpha$ , and Th17 cytokines such as IL-17 have been shown to play a role in the underlying inflammatory response [34]. As shown in **Figure 8** and in agreement with the study by Yokota et al. [35], we found that NOX1-deficient mice were protected against colon damage as compared to wild type mice (**Fig. 8A**). This was evidenced by a decrease in the weight/colon length ratio (**Fig. 8B**) and a lower Wallace score (**Fig. 8 C**) that reflects the severity of the damage induced by TNBS (**Fig. 8A**). Histological examination of the colon confirmed these observations and showed extensive ulceration and abundant infiltration of inflammatory cells accompanied by loss of crypts in wild type mice treated with TNBS, whereas cell damage and infiltration of inflammatory cells were significantly attenuated in NOX1-deficient mice (**Fig. 8D**). In addition, NOX1 expression was increased in wild type mice during TNBS-induced colitis, while lack of NOX1 expression was confirmed by

western-blot in NOX1-deficient mice (**Fig 8E**). Interestingly, the LCN-2 levels in NOX1-deficient mice were clearly decreased in the colon during TNBS-induced colitis as shown by immunoblotting (**Fig 8F**) and ELISA quantification (**Fig 8G**) in comparison to wild type mice where a dramatic increase could be observed (**Fig 8 F, 8G**). These data suggest that NOX1 might contribute to LCN-2 production and colon damage *in vivo*.

**The activation of JNK1/2 and the expression of NOXO1 and LCN-2 are enhanced in biopsies from patients with Crohn's disease.**

High levels of cytokines, including TNF- $\alpha$  and IL-17 [2, 24], are found in patients with IBD, and previous studies have shown that the expression of LCN-2 is increased in the epithelium from these patients. As our data showed that combination of the pro-inflammatory TNF $\alpha$  and IL-17 cytokines could increase the expression of LCN-2 *via* a p38MAPK-JNK/NOXO1/IkB $\zeta$  axis in colonic epithelial cells, we sought to determine whether activation of JNK1/2 and expression of NOXO1 were also upregulated in biopsies from patients with Crohn's disease (CD). Patients did not receive any medication at the time of biopsy collection, except for one who received sulfasalazine. Clinical status was assessed by the Crohn's Disease Activity Index (CDAI) [36] on the day of endoscopic examination (**Supplemental Table I**). Tissue samples from CD patients were taken from inflamed and adjacent non-inflamed areas of the colon, except for one patient whose sample was taken from the rectum. Tissue samples from control patients without IBD undergoing routine colonoscopy were also taken from the colon. Histological examination of colon sections from CD patients stained with hematoxylin-eosin shows an ulcerated mucosa with cryptitis lesions and polymorphous infiltrate containing neutrophils and mononuclear cells in the inflamed area, and an architectural rearrangement with bifid crypts and mononuclear cells in quiescent non-inflamed area (**Fig. 9A**). As shown in **Fig. 9 B-C**, expression of LCN-2 in tissue homogenates was increased in both inflamed

(Infl.) and adjacent non-inflamed (Adj.) areas of the colon in patients with CD, whereas only a weak LCN-2 expression was seen in control colons (Ctl). Interestingly, the phosphorylation of JNK1/2 was also increased in the inflamed and adjacent non-inflamed areas of the colon as compared to control patients, and was associated with increased NOXO1 expression, which was detected as the full length form (41 kDa), but also and mostly as a 34-38 kDa degraded form (**Fig. 9B-C**). In contrast, expression of DUOX2, the other NOX homolog expressed in the intestines, did not appear to be significantly increased when analyzed by western-blot (**S. 5A**). However, immunohistochemistry showed a very weak labelling with the anti-DUOX2 antibody at the tip of normal epithelium, which was increased in both inflamed and non-inflamed areas of CD patients (**S. 5B**, arrows). Interestingly, labelling was also observed in the crypts of the epithelium in the inflamed area (**S. 5B**, arrows).

## DISCUSSION

Intestinal epithelial cells are central to host defense against enteric pathogens, and the initiation and regulation of mucosal inflammation. The present study demonstrates that the bacteriostatic protein LCN-2 is regulated by NOX1 in colonic epithelial cells co-stimulated by TNF- $\alpha$  and IL-17. The underlying mechanisms involve an increase in NOXO1 expression that is controlled by the p38MAPK and JNK1/2 pathways. Consequently, NOX1 activity was increased and the resulting ROS induced the expression of I $\kappa$ B- $\zeta$ , allowing the expression of LCN-2 in conjunction with activated NF- $\kappa$ B (p50/p65) (**Fig. 10**). Interestingly, NOX1-deficient mice showed a decrease in colon damage and LCN-2 production during TNBS-induced colitis, suggesting the contribution of NOX1 to these processes *in vivo*. In addition, we found that phospho-JNK, and expression of NOXO1 and LCN-2 were increased in biopsies from patients with Crohn's disease as compared to control biopsies. Therefore,

NOX1 expressed in intestinal epithelial cells might play a key role in mucosal immunity and inflammation by controlling LCN-2 expression.

TNF- $\alpha$  and IL-17 are two key mediators in IBD; however, while TNF- $\alpha$  is a strong inducer of inflammation, IL-17 is not, despite having a strong impact on inflammation *in vivo* [37]. This may result from its ability to have a synergistic effect with other cytokines. In this study, we found that IL-17 potentiates TNF $\alpha$ -induced NOXO1 expression and consequently ROS production by NOX1, while having little effect on its own. Understanding the mechanisms underlying this synergistic effect is essential to understand the etiology of IBD and to develop novel pharmacological approaches for these diseases. Here, we found that the IL-17 and TNF- $\alpha$  signaling pathways converge at the level of the MAPK as both p38MAPK and JNK1/2 activation were increased by the cytokine combination (**Fig. 3A**). In addition, stabilization of mRNA may be another mechanism by which IL-17 can synergize with TNF- $\alpha$ , as it has been shown that IL-17 can promote mRNA stabilization by inducing the phosphorylation of mRNA destabilizing proteins such as tristetrapolin and blocking its ability to recruit the degradation machinery [38]. While TNF $\alpha$ +IL-17 increased NOXO1 and NOX1 mRNAs, only NOXO1 expression was increased at the protein level. This may be due to an inefficient translation of the NOX1 mRNA or a short half-life of the protein. It has been shown that steady-state transcript abundance only partially predicts protein abundance, and that contribution of translation and protein degradation can play an important role, perhaps even dominant, in the regulation of protein expression levels [39]. DUOX2, the other NOX homolog found in intestinal epithelial cells [11] was not modulated by TNF $\alpha$  or IL-17 alone, and only weakly by the combination of the two cytokines at the mRNA level, and without protein expression increase. The lack of effect on DUOX2 expression by TNF $\alpha$  is in accordance with a previous study showing that TNF $\alpha$  was not able induce DUOX2 expression in ileal enteroids [40]. It has been reported that DUOX2 expression is mainly

found in differentiated, post-confluent colon epithelial cells cultures [11]. Our study used exponentially growing non-confluent T84 cells, a condition that do not favor DUOX2 expression; therefore, we cannot totally exclude the possibility that DUOX2 could contribute to LCN-2 regulation in highly differentiated epithelial cells found at the apex of the epithelium. Using immunohistochemistry, we indeed found that DUOX2 expression increased at the apex of inflamed and non-inflamed epitheliums in CD patients as compared to that of normal epithelium. This regulation might occur downstream of NOX1, as it was recently shown that NOX1 could regulate the expression of DUOX2 in the intestinal epithelium [41].

Although several studies have reported a role for NOX1 in mucosal innate immunity [13, 42, 43, 44], it is still not entirely clear how ROS produced by NOX1 confer host protection against intestinal microbial pathogens. Contrary to the rapid and robust production of ROS induced by NOX2 activation, the production of ROS by NOX1 is persistent and low, and is therefore unlikely to exert a direct microbicidal effect. One suggested mechanism by which ROS generated by epithelial NOXs may be involved in enteric bacterial defense is by altering bacterial capsule formation and virulence through the disruption of bacterial signaling [13]. Our study uncovers a new mechanism by which NOX1 may participate to innate immunity, as we demonstrated that NOX1-derived ROS drive the expression of LCN-2 by controlling the expression of I $\kappa$ B $\zeta$ , a required master regulator of LCN-2 expression [32, 33]. I $\kappa$ B $\zeta$  is a non-canonical member of the NF- $\kappa$ B transcription factor family, which shows structural and functional similarities with the I $\kappa$ B family proteins. I $\kappa$ B $\zeta$  is barely detectable in resting cells, but is strongly induced upon stimulation of the innate immune system. It binds to the NF- $\kappa$ B-p50 subunit in the nucleus and regulates its transcriptional activity both positively and negatively depending on the genes [32]. How ROS generated by NOX1 induce the expression of I $\kappa$ B $\zeta$  remains to be determined. However, our findings are in agreement with the observation that oxidative stress is associated with upregulation of I $\kappa$ B $\zeta$  [45]. Contrary to

I $\kappa$ B $\zeta$ , activation of NF- $\kappa$ B was not dependent on ROS in colonic epithelial cells, as neither NAC nor DPI inhibited the phosphorylation of NF $\kappa$ B-p65. In accordance with this, we found that chemokine synthesis, in particular IL-8, which expression is under the control of NF- $\kappa$ B, was not inhibited by DPI in colonic epithelial cells co-stimulated by TNF $\alpha$  and IL-17.

LCN-2 was initially identified as a component of the neutrophil specific granules (hence its other name, NGAL for neutrophil associated gelatinase lipocalin), and was demonstrated to be a potent bacteriostatic agent [22]. This property results from its ability to bind siderophores, small chemical structures secreted by pathogenic bacteria that allow them to acquire the iron that is essential for their growth. Mice deficient in LCN-2 are more susceptible to bacteria that depend on siderophore-based iron uptake [21]. Therefore, by inducing the expression of LCN-2 in colonic epithelial cells, NOX1 could limit gut bacteria burden, thus ensuring intestinal homeostasis under physiological conditions. The increase of NOX1 and LCN-2 expression in intestinal tissue from patients with IBD might represent a compensatory mechanism of host defense against pathogens that have translocated because of the defective barrier function of the mucosa associated with IBD. However, the ability of LCN-2 to bind MMP-9 (aka gelatinase B) and to preserve its enzymatic activity may also render LCN-2 overexpression detrimental by favoring tissue injury through MMP-9 activity [27] and MMP-9 stabilization, thus exacerbating inflammation and leading to cancerous processes. It is interesting to note that MMP9 is expressed in resting T84 cells and that its expression increased in TNF $\alpha$  and/or IL-17-stimulated cells (data not shown), arguing for this possibility. The fact that NOX1 deficiency is protective and associated with a decrease of LCN-2 production in the TNBS-induced colitis mouse model supports the detrimental effect of the NOX1-LCN-2 axis during pathological conditions such as intestinal inflammation. Interestingly, the increase of LCN-2 in colon biopsies from patients with Crohn's disease was associated with enhanced expression of NOX1 and its upstream regulating pathway,

JNK1/2, in inflamed as well as non-inflamed areas of the colon. Of note, the pro-inflammatory cytokines TNF- $\alpha$  and IL-17 have also been found to be increased in both areas of the mucosa in IBD patients [46, 47]. LCN-2 present in the inflamed intestinal mucosa could originate from epithelial cells or from immune cells, in particular neutrophils that store high amount of LCN-2 in their granules. However, a recent study using bone marrow-chimeric mice demonstrated that non-immune cells, especially intestinal epithelial cells, contributed to the majority of systemic LCN-2 in inflamed states [26]. In colon biopsies of patients with Crohn's disease, the specific NOXO1 antibody [28] detected the protein as its full-length form (41 kDa), but also mostly as its 34-38 kDa degraded form. Interestingly, it has been demonstrated that NOX1 activity could be regulated by proteasomal degradation of NOXO1 in colon cancer cells [48].

In conclusion, our study revealed that production of ROS by NOX1 drives the expression of the bacteriostatic protein, LCN-2, in colonic epithelial cells co-stimulated with TNF- $\alpha$  and IL-17, and that the underlying mechanisms involve a p38MAPK-JNK/NOXO1/I $\kappa$ B $\zeta$  axis (**Fig. 10**). In addition, LCN-2 production and colon damage were decreased in NOX1-deficient mice during TNBS-induced colitis. Finally, phospho-JNK, NOXO1 and LCN2 expressions were increased in biopsies of patients with Crohn's disease as compared to control biopsies. Therefore, NOX1 might play a key role in mucosal immunity and inflammation by controlling LCN-2 expression and inhibitors of NOX1 could be promising anti-inflammatory agents in pathological conditions where NOX1 activation and LCN-2 production are dysregulated such as IBD.

## **METHODS**

**Reagents and antibodies-** Details for reagents and antibodies can be found in the Supplementary Methods.

**Animals** - Mice deficient in Nox1<sup>Y/-</sup> were from The Jackson Laboratory (Sacramento, CA). Animal studies were performed in accordance with the European Community Guidelines. All protocols were approved by the Bichat Ethics Committee for Animal Research (APAFIS authorization number 11901). Induction of TNBS-induced colitis in mice was detailed in Supplementary Methods.

**Cell culture, colon organoids and treatment with cytokines and inhibitors** - T84 colon epithelial cells from ATCC-LGC (Molsheim, France) were cultured in 1:1 Dulbecco's Modified Eagle Medium (DMEM)/Ham's F-12 Nutrient Mixture (F-12) as detailed in the Supplementary Methods. They were seeded in 6-well plates to be 70% confluent on the experimental day. Colon organoids were established from biopsies of control patients without IBD obtained after approval by the Bichat Hospital Institutional Review Board as detailed in the Supplementary Methods. Cells and colon organoids were treated with cytokines alone or in combination at the indicated concentrations for 24 hr at 37°C. In some assays, UO126 (10 µM), SB203580 (25 µM), SP600125 (20 µM), wortmannin (500 nM), NAC and DPI at various concentrations were added just prior cytokine treatment.

**NOXO1 and NOX1 silencing by siRNA** - To achieve NOX1 or NOXO1 silencing, cells were transfected with a pool of 3 target-specific 19-25 nt NOX1 siRNA or NOXO1 siRNA (Santa-Cruz Biotechnology, Heidelberg, Germany) using T84 Cell Avalanche™ Transfection

Reagent (EZ Biosystems, College Park, MD, USA). A detailed protocol can be found in the Supplementary Methods.

**Patients and tissue samples** - Human tissues were obtained after approval by the Bichat Hospital Institutional Review Board. Informed consent for the experimental use of the tissues was obtained during the patient hospitalization. Biopsies were obtained from patients subjected to colonoscopy either for confirmation of Crohn disease (CD) diagnosis or for routine control as detailed in the Supplementary Methods.

**Measurement of ROS production** – ROS production was measured by the luminol-amplified chemiluminescence in the presence of horseradish peroxidase (HRPO) in a Spark multimode microplate reader (TECAN, Spark<sup>R</sup>), or by the superoxide dismutase-inhibitable reduction of cytochrome c at 550 nm in a dual beam recording Uvikon 860 spectrophotometer thermostated at 37°C as detailed in the Supplementary Methods. PMA (500 ng/ml) was added in the assays for optimization of NOX1 detection activity [28].

**Cytokine measurement** - Cytokines were measured in the supernatant of cell cultures using the semi-quantitative Human Cytokine Antibody Array C1 from RayBiotech (Norcross, GA, USA) according to the manufacturer's instructions. Quantitative measurement of IL-8 was also performed using the Single Analyte ELISA Kit from Qiagen (Courtaboeuf, France) according to the manufacturer's instructions. A detailed protocol can be found in the Supplementary Methods.

**Protein extraction and Western blot analysis** - Colon biopsies were homogenized using rotor-stator while T84 cells were lysed in lysis buffer on ice. Protein concentration was

determined by the Bradford method [47]. Samples were analyzed for protein expression and phosphorylation with target specific antibodies using standard SDS-PAGE and immunoblotting techniques. Details can be found in the Supplementary Methods.

**RT-PCR** - Total RNA was extracted from T84 cells using the RNeasy Mini Kit from Qiagen (Courtaboeuf, France), and treated with RNase-free DNase in order to remove potential genomic DNA contaminants according to manufacturer's protocol. RNA concentrations were measured with the DeNovix DS 11 Series Microvolume Spectrophotometer. RT-PCR was performed with the Qiagen One-Step RT-PCR Kit with gene-specific primers as detailed in Supplementary Methods.

**Cytotoxicity assay** - The viability of T84 cells in the presence of inhibitors (UO126, SB203580, SP600125, wortmannin, DPI, NAC) was assessed using the Cyto-X Cell Cytotoxicity Kit (Cell Applications Inc., San Diego, CA) according to the manufacturer's instructions as detailed in Supplementary Methods.

**Statistical analysis** - Results are expressed as the mean  $\pm$  SEM of at least three independent experiments. One-way ANOVA analysis of variance with the Tukey test for multiple comparisons was employed using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA). The threshold of significance was fixed at  $p < 0.05$ .

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### **Author Contributions**

NM, MBK, DL, YK, AT, MM, CP, PL, AS, designed, performed experiments and analysed the data. VM, MHN, JCM, JEB analysed the data and contributed to the critical reading of the manuscript. ALP recruited the patients, contributed to the discussion and to the critical reading of the manuscript. PMCD supervised the project, designed experiments and wrote the manuscript. All the authors have read the manuscript and agreed with the data.

### **Disclosure**

The authors declare no competing conflict of interest.

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## FIGURE LEGENDS

### **Figure 1: Screening of the effects of several key pro-inflammatory cytokines found in IBD reveals the synergistic activity of TNF- $\alpha$ and IL-17 on the expression of NOXO1 in T84 colonic epithelial cells.**

**A-B)** T84 colonic epithelial cells were treated for 24 hr at 37°C with TNF $\alpha$ , IL-17, IL-6, IL-23, TGF $\beta$  or IL-22 at the indicated concentrations, then washed, and lysed. Total protein was assessed, and samples were subjected to SDS-PAGE (11 %) and analyzed by western blotting using specific rabbit anti-NOXO1 polyclonal antibodies. The blots were reprobed with mouse anti- $\beta$ -actin antibody as loading control. Recombinant NOXO1 (Rec. O1) was used as a positive control. The ratio of NOXO1 to the total amount of  $\beta$ -actin (**B**) was quantified using Image J 1.43u software (Wayne Rasband, National Institute of Health, USA). Values are expressed as means  $\pm$  SEM of 3 independent experiments. \*:  $p < 0.05$  as compared to the control (unstimulated cells); **C)** T84 cells were treated with a combination of TNF $\alpha$  (5 ng/ml) + IL-17 (25, 50 or 100 ng/ml) or with each cytokine alone (TNF $\alpha$  at 5 ng/ml, IL-17 at 100 ng/ml) for 24 hr at 37°C, and samples were analysed as described in A). Results are expressed as means  $\pm$  SEM of 3 independent experiments. \* $p < 0.05$  \*\* $p < 0.01$  and \*\*\* $p < 0.001$ ; **D)** Colonic epithelial cells were treated with the combination of TNF $\alpha$  (5 ng/ml) + IL-22 (25, 50 or 100 ng/ml) or each cytokine alone (TNF $\alpha$  at 5 ng/ml, IL-22 at 100 ng/ml) for 24 hr at 37°C, and samples were analysed as described in A). Results are expressed as means  $\pm$  SEM of 3 independent experiments. ns = non significant; **E)** Colonic epithelial cells were treated with TNF $\alpha$  (5 ng/ml), IL-17 (50 ng/ml), or TNF $\alpha$  (5 ng/ml) + IL-17 (50 ng/ml). Total RNA was then extracted and RT-PCR was performed as described in “Methods” using NOX1, NOXA1, NOXO1 or p22<sup>PHOX</sup> gene specific primers. Human GADPH served as internal control; **F)** T84 cells were treated with TNF $\alpha$  (5 ng/ml), IL-17 (50 ng/ml), or TNF $\alpha$  (5 ng/ml) + IL-17 (50 ng/ml). Samples were analyzed by western blotting using specific rabbit anti-NOX1, anti-NOXO1, anti-NOXA1 and anti-p22<sup>PHOX</sup> polyclonal antibodies.  $\beta$ -actin served as loading control as above.

### **Figure 2: IL-17 potentiates TNF- $\alpha$ -induced ROS production in colonic epithelial cell.**

T84 cells were treated by TNF $\alpha$  (5 ng/ml), IL-17 (50 ng/ml) or TNF $\alpha$  (5 ng/ml) + IL-17 (50 ng/ml) for 24 hr at 37°C. ROS production was then measured as described in “Methods” by **A)** luminol-amplified chemiluminescence in cells in 100  $\mu$ l of Hank’s balanced salt solution

(HBSS) containing 10  $\mu$ M luminol, 2.5 U/ml HRPO (HRPO) and 500 ng/ml of PMA for optimization of NOX1 detection activity or **B**) by the superoxide dismutase-inhibitable reduction of cytochrome c at 550 nm as an end point in 0.5 ml of HBSS in the presence of PMA (500 ng/ml), with (white) or without (black) DPI (1  $\mu$ M). Results are expressed as means  $\pm$  SEM of at least 3 independent experiments. \* $p$ <0.05 as compared to untreated cells; # $p$ <0.05 as compared to TNF $\alpha$ -treated cells.

**Figure 3: The co-stimulatory effect of TNF $\alpha$  and IL-17 on NOXO1 expression and ROS production involves p38MAPK and JNK1/2 pathways in colonic epithelial cells.**

**A)** T84 colonic epithelial cells were stimulated with TNF $\alpha$  (5 ng/ml), IL-17 (50 ng/ml) or TNF $\alpha$  (5 ng/ml)+IL-17 (50 ng/ml) for 30 min at 37°C activation of ERK1/2, p38MAPK, or JNK1/2 was assessed by western-blot using phospho-ERK1/2, phospho-p38MAPK or phospho-JNK1/2, respectively. The blots were probed again with rabbit anti-ERK1/2, anti-p38MAK or anti-JNK1 as loading controls. Representative of 3 independent experiments; **B)** T84 colonic epithelial cells were stimulated with TNF $\alpha$  (5 ng/ml) or IL-17 (50 ng/ml) alone for 24 hr at 37°C in the presence or absence of UO126 (10  $\mu$ M), SB203580 (25 $\mu$ M), SP600125 (20 $\mu$ M) or wortmannin (500 nM) and expression of NOXO1 was analyzed by western blotting using anti-NOXO1 antibody. Results are expressed as means  $\pm$  SEM of at least 3 independent experiments; \* $p$ <0.05 as compared to control. R: resting; C: control (no inhibitor); **C-D)** T84 colonic epithelial cells were stimulated with TNF $\alpha$  (5 ng/ml), IL-17 (50 ng/ml) or TNF $\alpha$  (5 ng/ml)+IL-17 (50 ng/ml) for 24 hr at 37°C in the presence or absence of UO126 (10  $\mu$ M), SB203580 (25 $\mu$ M), SP600125 (20 $\mu$ M) or wortmannin (500 nM); and the expression of NOXO1 was analyzed by western blotting using anti-NOXO1 antibody. The ratio of NOXO1 to the total amount of  $\beta$ -actin (**D**) was quantified using the Image J 1.43u software (Wayne Rasband, National Institute of Health, USA). Results are expressed as means  $\pm$  SEM of 3 independent experiments. \* $p$  < 0.05 as compared to unstimulated cells; † $p$  < 0.05 as compared to TNF $\alpha$ -stimulated cells; # $p$ <0.05 and #### $p$ <0.001 as compared to cells non-treated with inhibitors (CTL) under the same conditions of stimulation; **E)** T84 cells were stimulated with TNF $\alpha$  (5 ng/ml), IL-17 (50 ng/ml) or TNF $\alpha$  (5 ng/ml)+IL-17 (50 ng/ml) for 24 hr at 37°C in the presence or absence of UO126 (10  $\mu$ M), SB203580 (25 $\mu$ M), SP600125 (20 $\mu$ M) or wortmannin (500 nM), and ROS production was then measured as described in “Methods” by the superoxide dismutase-inhibitable reduction of cytochrome c at 550 nm in 0.5 ml of HBSS in the presence of PMA (500 ng/ml) as an end point. Results are expressed

as means  $\pm$  SEM of 3 independent experiments. \* $p < 0.05$  as compared to unstimulated cells, † $p < 0.05$  as compared to TNF $\alpha$ -stimulated cells, # $p < 0.05$  as compared to cells non-treated with inhibitors (CTL) under the same conditions of stimulation.

**Figure 4: TNF $\alpha$  and IL-17 induce the expression of the bacteriostatic protein, lipocalin-2 (LCN-2), in a p38MAPK- and JNK1/2-dependent manner in colonic epithelial cells.**

**A-D)** Except where indicated, T84 colonic epithelial cells were stimulated with TNF $\alpha$  (5 ng/ml), IL-17 (50 ng/ml), IL-22 (50 ng/ml), TNF $\alpha$  (5 ng/ml)+IL-17 (50 ng/ml) or TNF $\alpha$  (5 ng/ml)+IL-22 (50 ng/ml) for 24 hr at 37°C, and the expression of LCN-2 was then analysed by western blotting using an anti-LCN-2 antibody. The blots were probed again with mouse anti- $\beta$ -actin antibody as loading control. The ratio of LCN-2 to the total amount of  $\beta$ -actin (**B**) was quantified using the Image J software. Results are expressed as means  $\pm$  SEM of 3 independent experiments \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; **E**) T84 cells were stimulated with TNF $\alpha$  (5 ng/ml), IL-17 (50 ng/ml) or TNF $\alpha$  (5 ng/ml)+IL-17 (50 ng/ml) for 24 hr at 37°C in the presence or absence of SP600125 (20 $\mu$ M), UO126 (10  $\mu$ M), or SB203580 (25 $\mu$ M), and the expression of LCN-2 was then analysed by western blotting using an anti-LCN-2 antibody. The blots were probed again with a mouse anti- $\beta$ -actin antibody as loading control.

**Figure 5: Expression of LCN-2, but not of chemokines, is inhibited by NAC and DPI in colonic epithelial cells co-stimulated with TNF $\alpha$  and IL-17.**

**A)** T84 cells were stimulated with TNF $\alpha$  (5 ng/ml), IL-17 (50 ng/ml) or TNF $\alpha$  (5 ng/ml)+IL-17 (50 ng/ml) for 24 hr at 37°C in the presence or absence of NAC (20 mM) or DPI (1  $\mu$ M). Expression of LCN-2 was then analysed by western-blot using an anti-LCN-2 antibody. The blots were probed again with a mouse anti- $\beta$ -actin antibody as loading control; **B**) T84 cells were stimulated with TNF $\alpha$  (5 ng/ml)+IL-17 (50 ng/ml) for 24 hrs at 37°C and the concentration-dependent effect of NAC and DPI on the expression of LCN-2 was analysed; **C**) T84 cells were stimulated or not (CTL) with TNF $\alpha$  (5 ng/ml)+IL-17 (50 ng/ml) for 24 hr at 37°C in the presence or absence of DPI (1  $\mu$ M). Cytokines were measured in the supernatant of cell cultures according to the manufacturer's instructions by using the semi-quantitative Human Cytokine Antibody Array from RayBiotech. The map of the cytokine array is shown on the left panel and the cytokines that were increased after TNF $\alpha$  and IL-17 treatment are shown in grey on the array map. Results are representative of 2 independent

experiments; **D**) Quantitative measurement of IL-8 was performed using the Single Analyte ELISA Kit from Qiagen according to the manufacturer's instructions after stimulation of cells with TNF $\alpha$  (5 ng/ml), IL-17 (50 ng/ml) or TNF $\alpha$  (5 ng/ml)+IL-17 (50 ng/ml) for 24 hr at 37°C in the presence or absence of DPI (1  $\mu$ M). Results are representative of 2 independent experiments performed in triplicate and expressed as means  $\pm$  SEM . \* $p$  < 0.05; \*\* $p$ <0.01 as compared to unstimulated cells.

**Figure 6: Expression of LCN-2 induced by co-stimulation of colonic epithelial cells with TNF $\alpha$  and IL-17 is inhibited by NOXO1 and NOX1 siRNAs.**

Colonic epithelial cells were stimulated with TNF $\alpha$  (5 ng/ml), IL-17 (50 ng/ml) or TNF $\alpha$  (5 ng/ml)+IL-17 (50 ng/ml), and transfected with a pool of 3 target-specific 19-25 nt NOXO1 siRNAs or scrambled siRNA as indicated in "Methods". Expression of NOXO1 (**A**) or LCN-2 (**B**) was analyzed 24 hr after transfection by western blotting (left). The ratio of NOXO1 and LCN-2 to the total amount of  $\beta$ -actin was quantified using Image J (right). Values are expressed as means  $\pm$  SEM of 3 independent experiments. \* $p$ < 0.05 as compared to control cells not treated with siRNAs (CTL); # $p$ <0.05 as compared to scrambled siRNA (si-Scr) treated cells; **C**) T84 cells stimulated with TNF $\alpha$  (5 ng/ml), IL-17 (50 ng/ml) or TNF $\alpha$  (5 ng/ml)+IL-17 (50 ng/ml) were transfected with a pool of 3 target-specific 19-25 nt NOXO1 siRNAs or scrambled siRNA as indicated in "Methods". Cells were washed 24 hr after transfection, and ROS production was measured by superoxide dismutase-inhibitable reduction of cytochrome c at 550 nm in 0.5 ml of HBSS in the presence of PMA (500 ng/ml ) as an end point. Values are expressed as means  $\pm$  SEM of 3 independent experiments. \* $p$ <0.05 as compared to scrambled siRNA (black) treated cells; **D**) T84 cells stimulated with TNF $\alpha$  (5 ng/ml)+IL-17 (50 ng/ml) were transfected with a pool of 3 target-specific 19-25 nt NOX1 siRNAs or scrambled siRNA. Expression of NOX1 was analyzed 24 hr later by RT-PCR (upper), and expression of LCN-2 was analyzed by western blotting (lower).  $\beta$ -Actin was used as loading control and human GADPH served as an internal control.

**Figure 7: Expression of I $\kappa$ B- $\zeta$  , the central regulator of LCN-2 induction, is controlled by NOX1-derived ROS in colonic epithelial cell and in human colon organoids co-stimulated by TNF $\alpha$  and IL-17.**

**A)** Diagram of the transcriptional regulation of LCN-2; **B)** T84 colonic epithelial cells were stimulated with TNF $\alpha$  (5 ng/ml), IL-17 (50 ng/ml) or TNF $\alpha$  (5 ng/ml)+IL-17 (50 ng/ml) for

24hr at 37 °C. Activation of NF- $\kappa$ B was assessed with a phospho-NF- $\kappa$ B-p65 antibody and expression of I $\kappa$ B $\zeta$  was analyzed with an I $\kappa$ B $\zeta$  antibody. The blots were reprobed with anti-P65 or  $\beta$ -actin as loading controls; **C**) T84 cells were stimulated with TNF $\alpha$  (5 ng/ml)+IL-17 (50 ng/ml) in the presence or absence of different concentrations of NAC or DPI at 37 °C for 24 hr, and expression of I $\kappa$ B $\zeta$  was analysed; **D**) T84 cells were stimulated with TNF $\alpha$  (5 ng/ml)+IL-17 (50 ng/ml) in the presence or absence of different concentrations of NAC or DPI at 37 °C for 24 hr, and activation of NF- $\kappa$ B was assessed with a phospho-NF- $\kappa$ B-p65 antibody; **E**) T84 cells stimulated with TNF $\alpha$  (5 ng/ml)+IL-17 (50 ng/ml) were transfected with a pool of 3 target-specific 19-25 nt NOXO1 siRNAs or scramble siRNA as indicated in “Methods”. Expression of I $\kappa$ B $\zeta$  was analyzed 24 hr later with an I $\kappa$ B $\zeta$  antibody (upper). The ratio of I $\kappa$ B $\zeta$  to the total amount of  $\beta$ -actin was quantified using Image J (lower). Results are expressed as means  $\pm$  SEM of 3 independent experiments. \* $p$ < 0.05 as compared to control cells not treated with siRNAs (CTL); # $p$ <0.05 as compared to scramble siRNA (si-Scr) treated cells; **F**) Colon organoids were established from colon biopsies of control patients as detailed in the Supplementary Methods. At Day 8, organoids were stimulated with TNF $\alpha$  (5 ng/ml), IL-17 (50 ng/ml) or TNF $\alpha$  (5 ng/ml)+IL-17 (50 ng/ml) for 24 hr at 37 °C in the presence or absence of NAC (20 mM) or DPI (0.2  $\mu$ M). Expression of NOXO1, I $\kappa$ B- $\zeta$ , LCN-2 and  $\beta$ -actin were analyzed by western-blot with specific antibodies. Scale bar: 100  $\mu$ m.

**Figure 8: Colon damage and LCN-2 production were decreased in NOX1-deficient mice during TNBS-induced colitis.** Colitis was induced by intra-rectal injection of TNBS as detailed in Supplementary Methods. **A**) Representative photograph of the colon 24 hr after injection of TNBS or vehicle (CTL); **B-C**) Macroscopic changes following TNBS treatment are represented by colon weight/length ratio (B) and Wallace score (C). Results are expressed as the mean  $\pm$  SEM from 6 to 8 animals; \*\*  $p$ < 0.01; \*\*\*  $p$ < 0.001 as compared to control; #  $p$ < 0.05 as compared to WT mice; **D**) Histologic images of the colon 24hr after TNBS-induced colitis (x200). Scale bar: 50  $\mu$ m; **E**) Western-blot of NOX1 in colon homogenates of WT or NOX1-deficient mice. Four mice are shown (M1 to M4) for each group: WT control (CTL), WT treated with TNBS (TNBS), NOX1-deficient CTL, NOX1-deficient treated with TNBS; **F**) Levels of LCN-2 were assessed by western-blot of mouse colon homogenates from WT or NOX1-deficient mice. Four mice are shown (M1 to M4) for each group defined as above; **G**) Levels of LCN-2 were examined in mouse colon homogenates from WT or NOX1-

deficient mice using the Lipocalin-2 Quantikine ELISA kit from R&D Systems. Data are expressed as the mean  $\pm$  SEM from 6 to 8 animals; \*\*\*  $p < 0.001$  as compared to control; #  $p < 0.05$  as compared to WT mice.

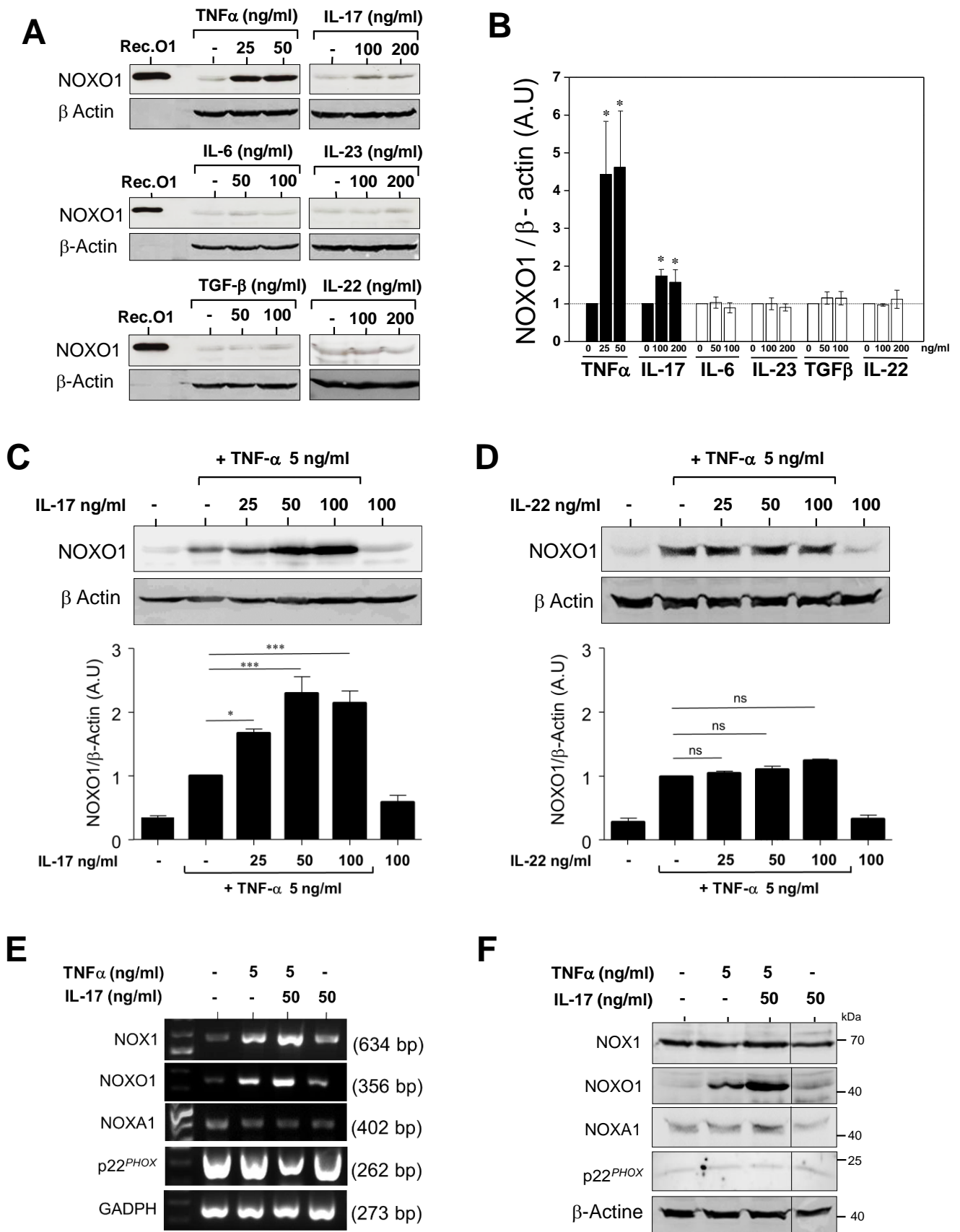
**Figure 9: Activation of JNK1/2, and expression of NOXO1 and LCN-2 are enhanced in biopsies from patients with Crohn's disease (CD).**

Biopsies from eight patients with active Crohn's disease (median age 33 years, range 15-62, 40% female) and from 8 control subjects without IBD (median age 56, range 41-64, 50 % female) were analyzed. Tissue samples from CD patients were taken from the inflamed (Infl) and adjacent non-inflamed (Adj.) areas of the colon, except for one that was taken from the rectum. At least four specimens were sampled from the same area. Tissue sample from control subjects were taken from the colon; **A)** Histological examination of colon sections from CD patient stained with hematoxylin-eosin as compared to normal colon (control); **B)** Tissue samples were homogenized in homogenization buffer using a rotor-stator, proteins were measured, and samples were analyzed by western blotting with anti-phospho-JNK1/2, anti-NOXO1 and anti-LCN-2 antibodies.  $\beta$ -Actin was used as loading control; **C)** Ratio of phospho-JNK1/2, NOXO1 and LCN-2 to the total amount of  $\beta$ -actin were quantified using Image J. When multiple proteins were specifically recognized by the antibody (JNK and NOXO1), all the bands were captured for quantification. Results are expressed as means  $\pm$  SEM of the 8 samples. \* $p < 0.05$ ; \*\* $p < 0.01$  and \*\*\* $p < 0.001$  as compared to the control biopsies (CTL).

**Figure 10: Diagram of the regulation of lipocalin-2 expression by NOX1-derived ROS in colonic epithelial cell co-stimulated with TNF $\alpha$  and IL-17.**

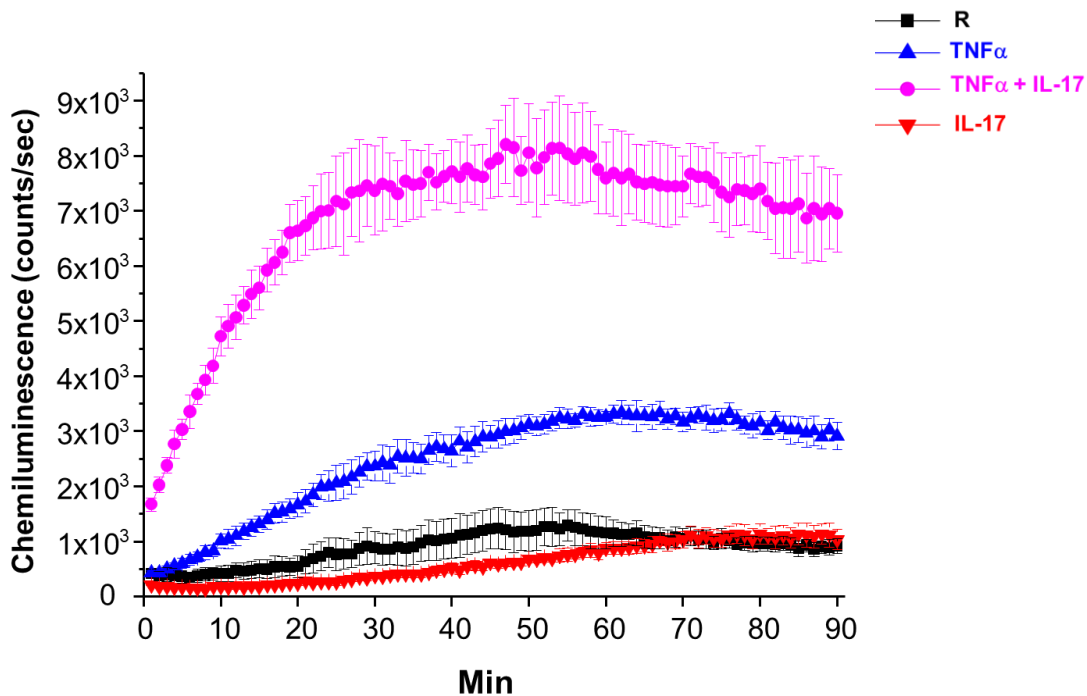
In colonic epithelial cell line, TNF $\alpha$ +IL-17 synergistically enhance the expression of NOXO1 *via* the activation of the p38MAPK and JNK1/2 pathways. Consequently, NOX1 activity was increased and the resulting ROS induced the expression of I $\kappa$ B- $\zeta$ . Concomitantly, TNF $\alpha$  or IL-17 induced the activation of NF $\kappa$ B in an ROS-independent manner. The synthesis of LCN-2, which requires the binding of both NF $\kappa$ B and I $\kappa$ B- $\zeta$  upstream of its promoter, could then be induced.

**Figure 1** (Makhezer et al.)

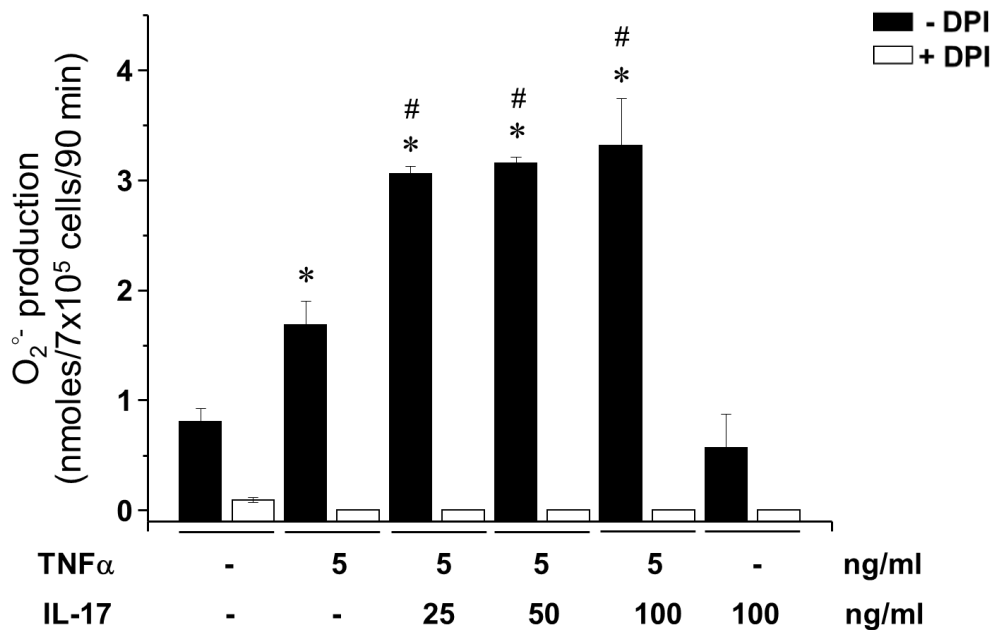


**Figure 2** (Makhezer et al.)

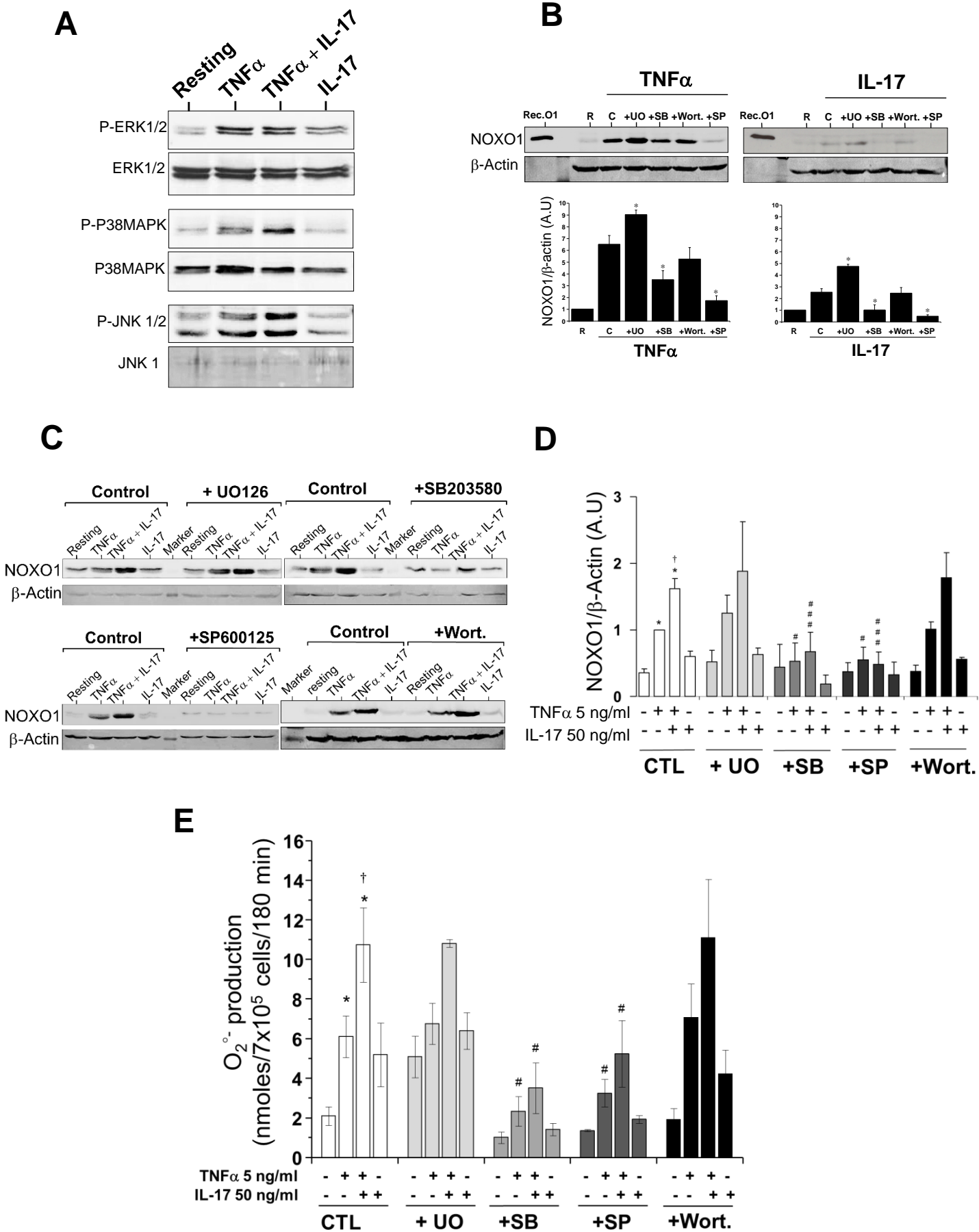
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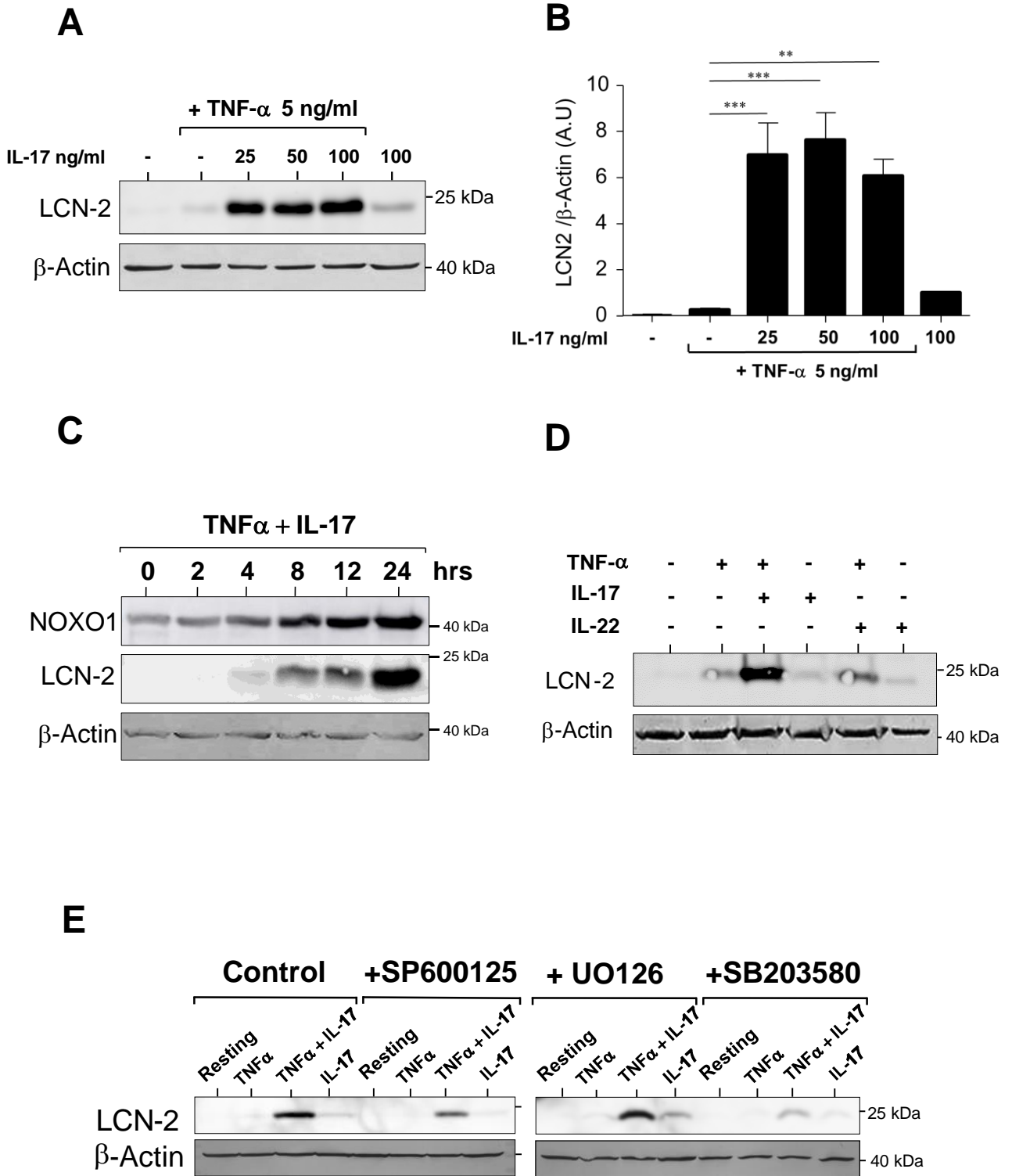
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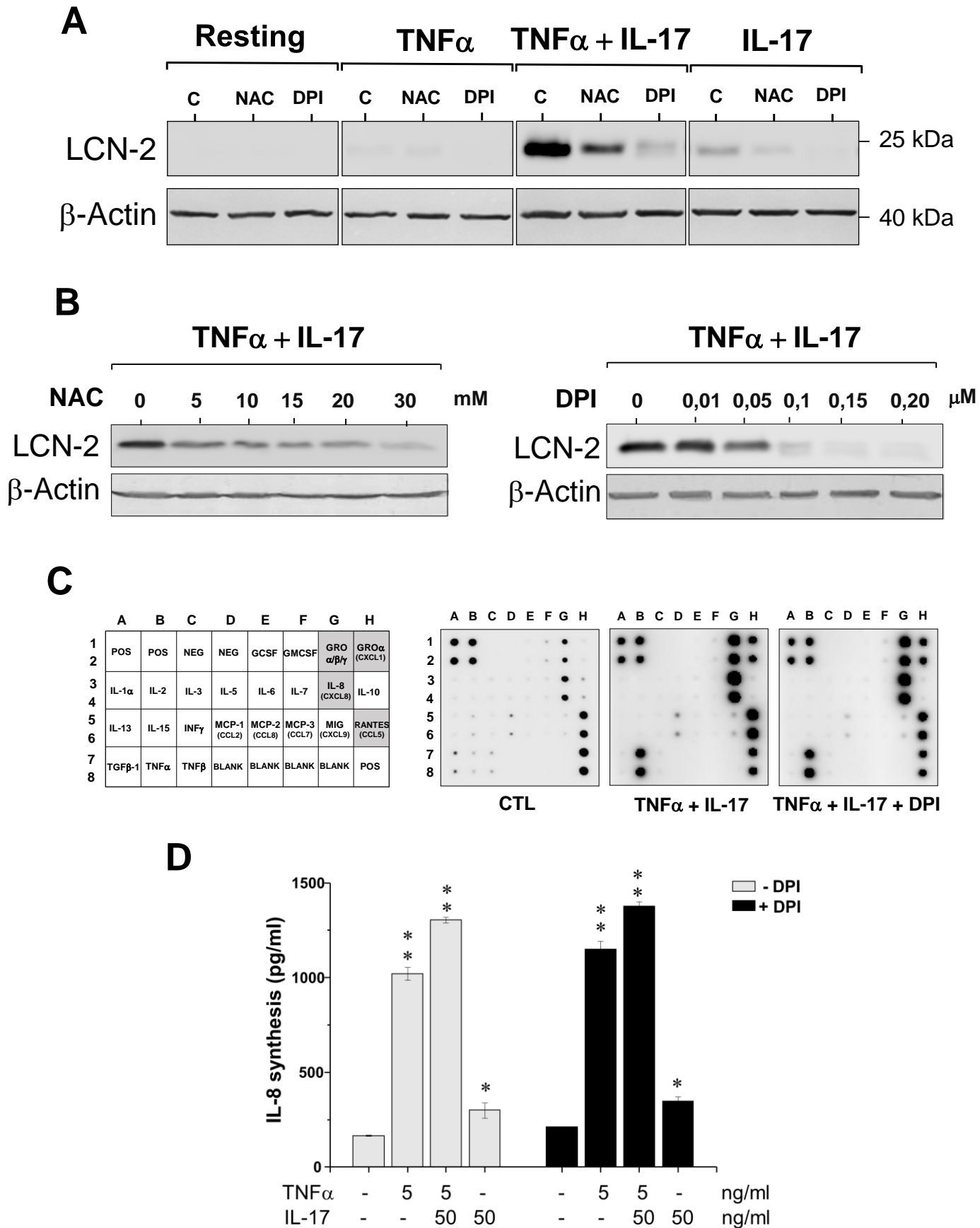
**Figure 3** (Makhezer et al.)



**Figure 4** (Makhezer et al. )

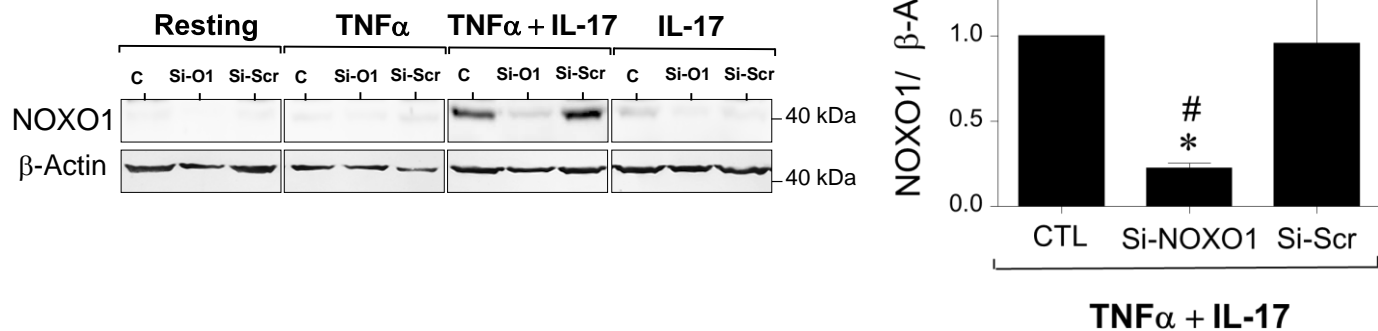


**Figure 5** (Makhezer et al.)

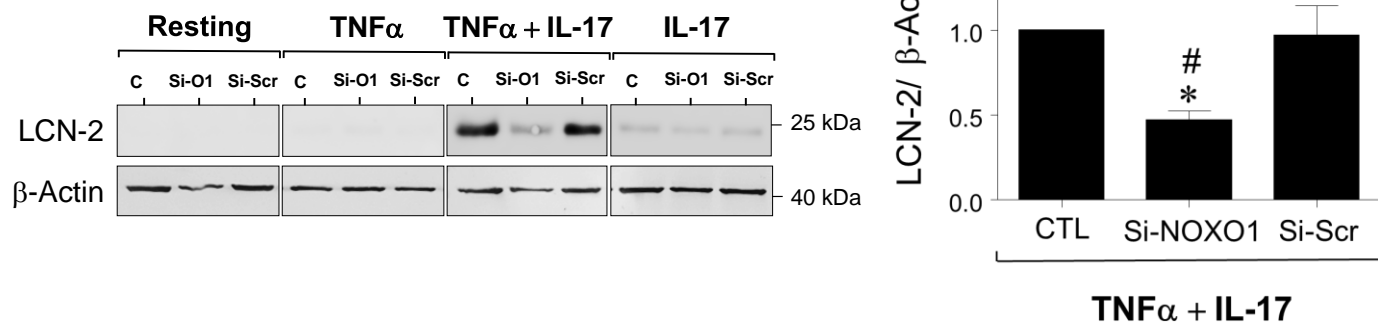


**Figure 6** (Makhezer et al.)

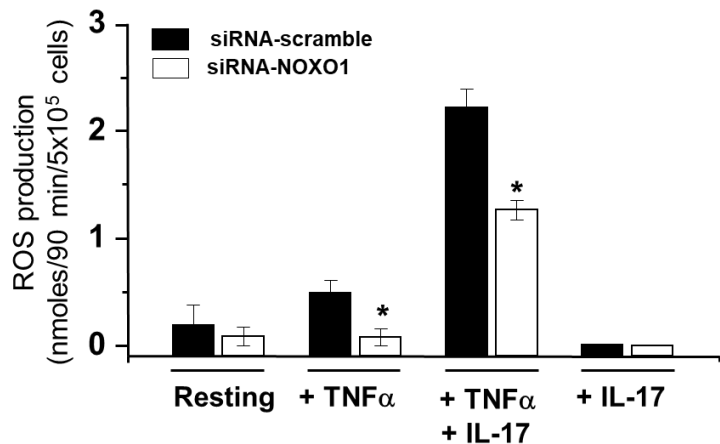
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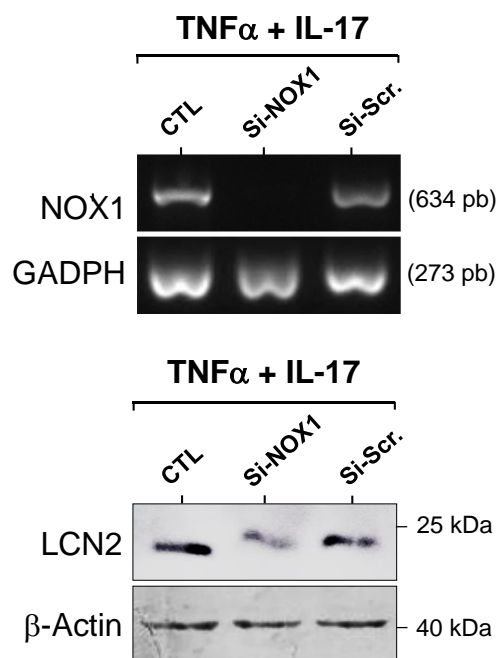
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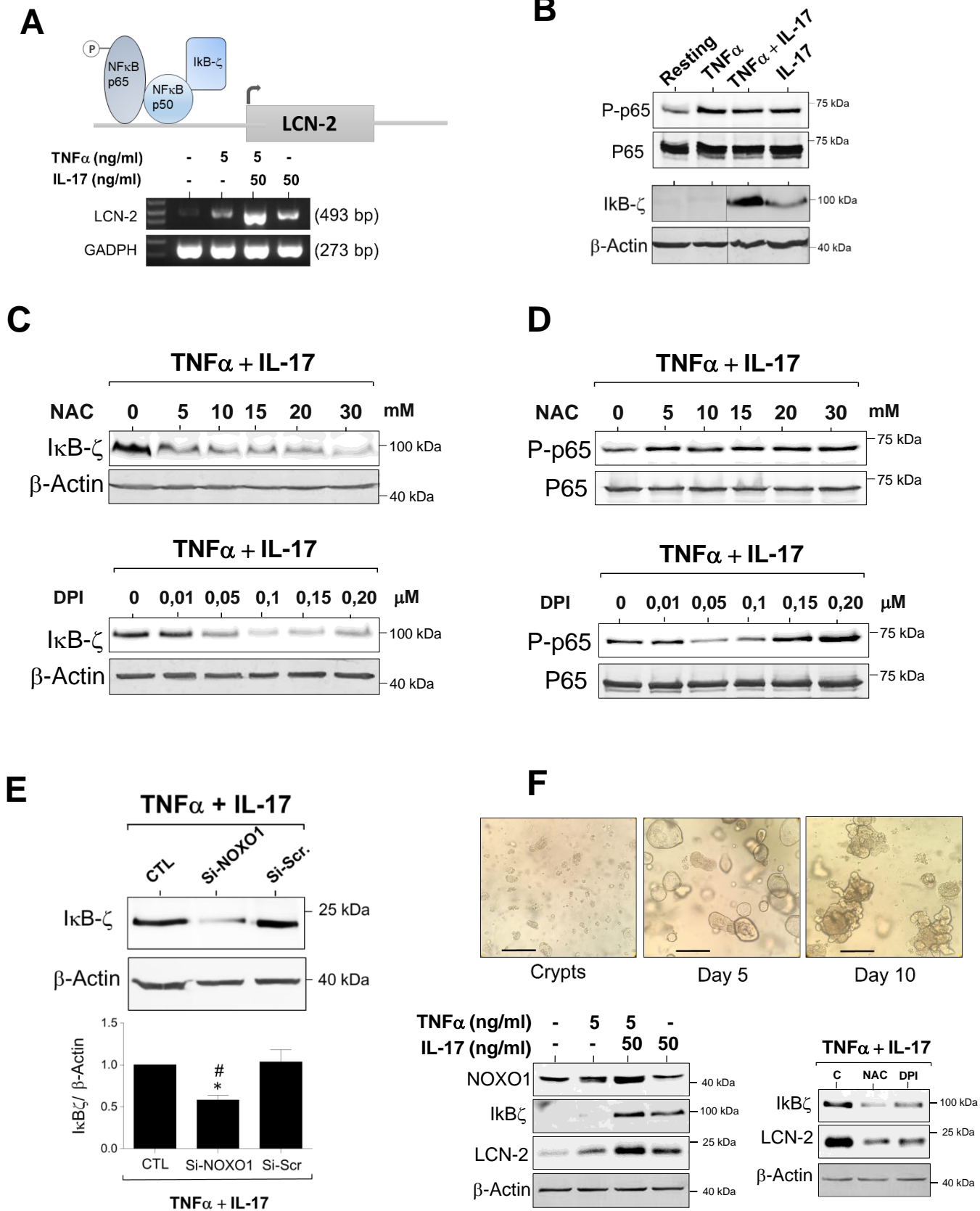
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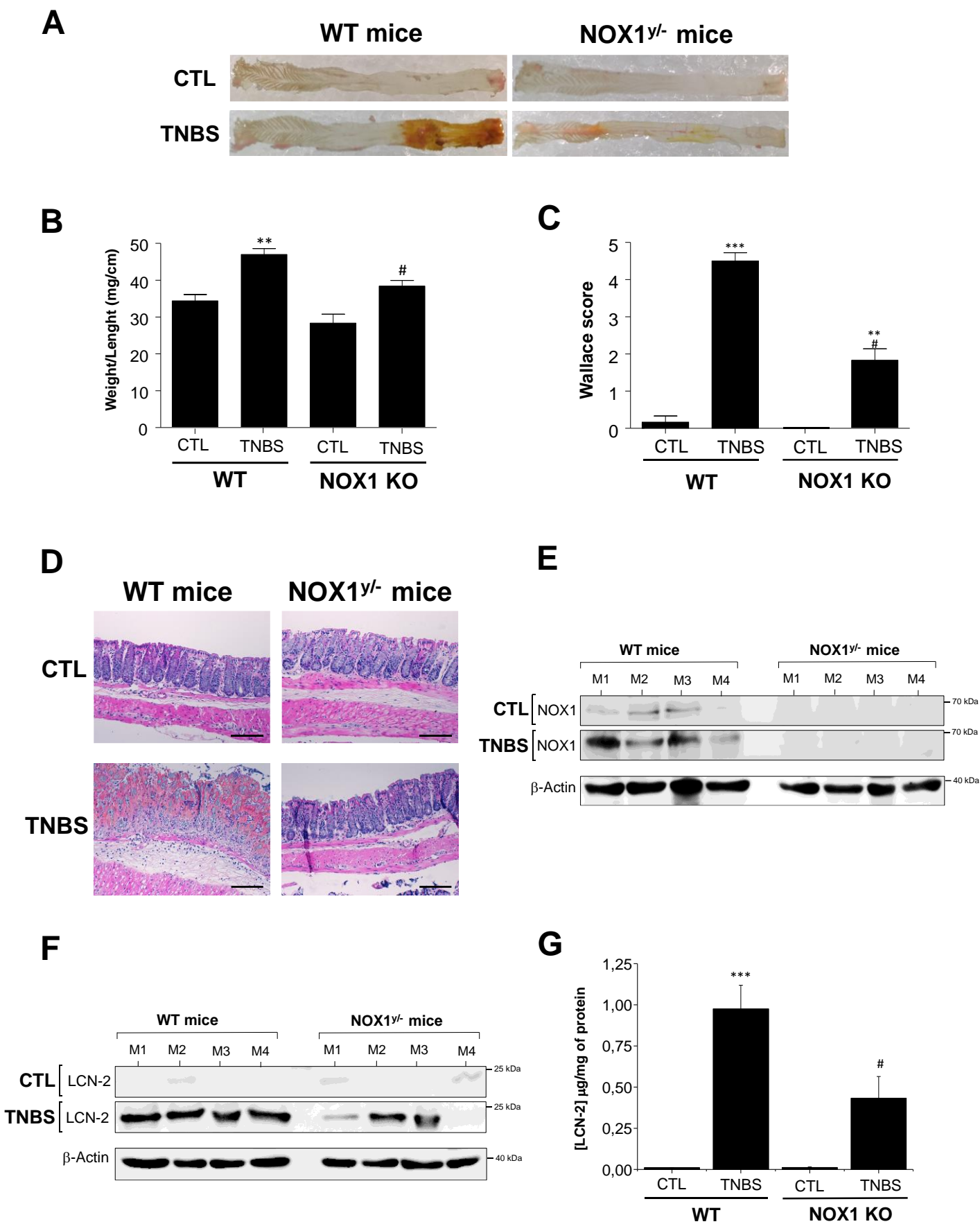
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# Figure 7 (Makhezer et al.)

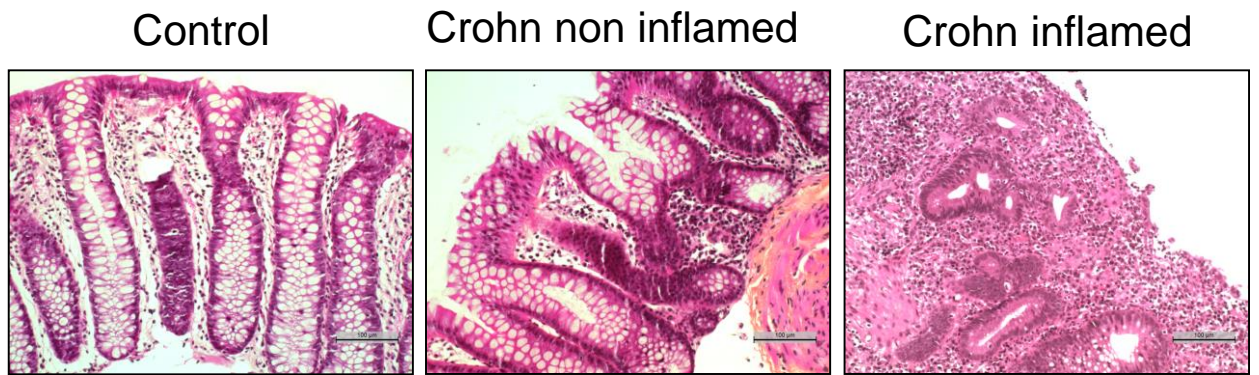


**Figure 8** (Makhezer et al.)

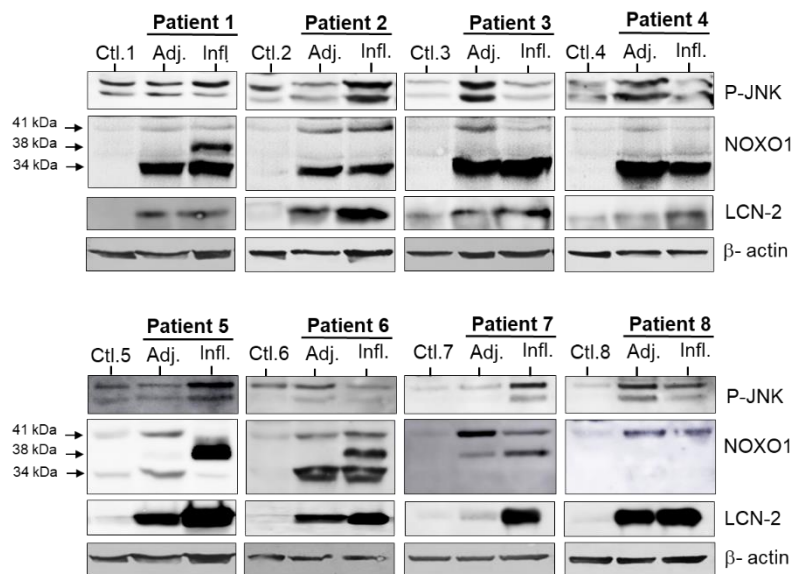


**Figure 9** (Makhezer et al.)

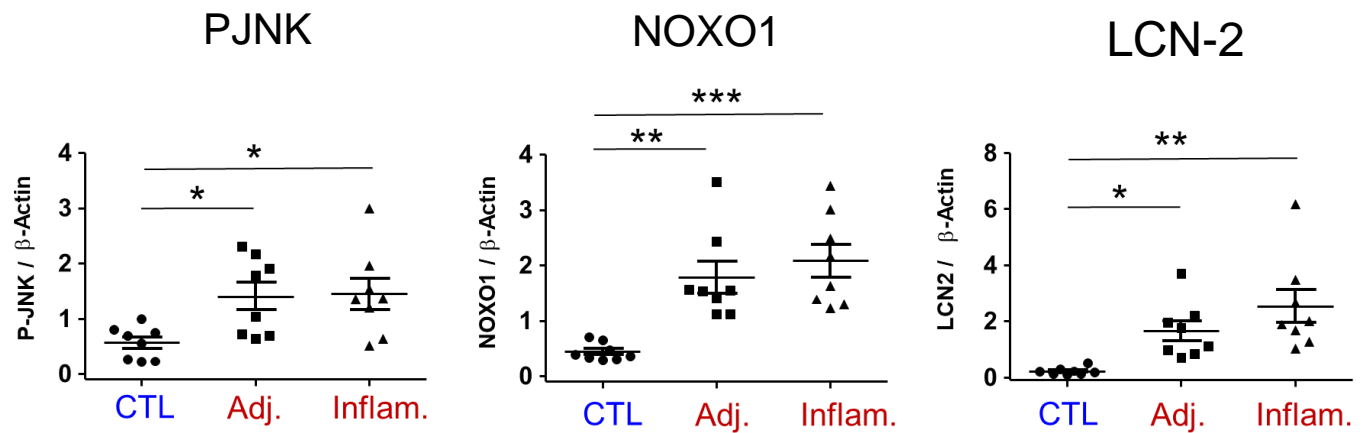
**A**



**B**



**C**



**Figure 10** (Makhezer et al.)

