NOX1 is essential for TNFα-induced intestinal epithelial ROS secretion and inhibits M cell signatures

SUPPLEMENTARY MATERIALS

SUPPLEMENTARY METHOD

Mice specimens

Eleven-week-old to Fifteen-week-old male mice of Nox1 knockout mice (Strain No. 018787, bred to C57B6 for 8 generations; Jackson Laboratory, Bar Harbor, ME) were used, with C57B6 male mice (Strain No. 000664; Jackson Laboratory, Bar Harbor, ME) used as wild type controls for colonoid line development and DSS injury model. All murine experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee at Mount Sinai school of medicine and were performed according to the Mount Sinai guidelines for the ethical treatment of animals.

ROS assays

Baseline or stimulated ROS generation by colonoid cells was measured using L-012-enhanced chemiluminescence (Wako Chemicals, USA) as previously described[1] with the following modifications. Four days before the ROS assay, mice colonoid cells were passaged, and $2x10^5$ colonoids/ well were seeded in opaque white 96-well plates (Falcon). To minimize chemoluminance background, spheroids were embedded in phenol red free Matrigel (Corning, Cat#356237) and cultured in phenol red free 50% WRN+10uM Y-27632. One day after cell seeding, spheroid cells went through the following treatments. For the basal level of ROS measurement under variable Wnt or Notch signaling, cells were treated for 48 hours with Wnt signaling gradients: 50%, 25%, 10%, or 5% WRN; Notch signaling gradients: 2.5μM, 5μM, 10μM or 20μM DAPT. For stimulated ROS, spheroid cells were incubated with TNFα (10ng/ml or 200 ng/ml) or RANKL (10ng/ml or 200 ng/ml). Treated colonoid cells were washed with phosphate-buffered saline, followed by 100 μM of L-012 for 25 minutes at 37C. Chemiluminescence was

measured at one second integration times in GLOMAX (Promega) in Supplementary Figure 1 or SYNERGY HTX (BioTek) in Figure 1A.

Real-time quantitative PCR

RNA was isolated using Trizol-LS (Invitrogen), and cDNA was synthesized with SuperScript III First-Strand Synthesis SuperMix (Invitrogen). qPCRs were prepared with SYBR Green PCR MasterMix (Applied Biosystems). All reactions were run for 40 cycles in duplicates and analyzed using the ViiA7 (Applied Biosystems). Relative gene expression was normalized to Hprt1. Primer sequences are listed below:

Primer	**Primer sequence (5 ' to 3 ‘)**
Muc2--Forward	GGTCAGCACCCCACACTAC
Muc2--Reverse	TCGGTGTTCAGAGCAGGAC
Reg4--Forward	GCCCCGAAGTCCTGAGCG
Reg4--Reverse	ATTGCTTCTTTTGTGGGTC
Car1--Forward	TATGGAAGCGAAAATGGTC
Car1--Reverse	CCACCTTTCAGAACAGATTG
Hprt1--Forward	GTCCCAGCGTCGTGATTAG
Hprt1--Reverse	AGCCCCCCTTGAGCACAC
GP2--Forward	AAAGGATGGTGGGTTGTGAC
GP2-Reverse	TTCTCTGTGCTTCTGGTAGG

M cell monolayer studies

1.4×10^{5} of TryoLE (Gibco) dissociated spheroids were seeded on eight well chamber slides (iBidi) precoated with 1:40 Matrigel in 50% WRN with 10 µM of Y-27632. The following day, the original culture media were replaced with a differentiation medium with and without stimuli (TNFα or

RANKL). For M cell induction, cells were incubated with a differentiation medium (25% WRN /10 μM of Y-27632 plus 20 μM DAPT, Sigma) stimulated with 200 ng/ml RANKL (Biolegend) or 10 ng/ ml TNFα, (R&D Systems) for 3 days. The RANKL dose and timing were based on de Lau W. et al. [2]. For M cell rescue studies, cells were co-incubated with a differentiation medium (25% WRN/10 μM of Y-27632 plus 20 μM DAPT) with 300 μM of hydrogen peroxide (Sigma) per Patel et al.[3], or 30 μM of paraquat (Sigma) per Rodriguez-Colman et al[4] for 3 days.

Human colonoid line bulk RNA seq

Human colonoid lines were generated from colon biopsies of ulcerative colitis and healthy individuals according to the procedure described in the colonoid development and differentiation section. $2X10^5$ of colonoid cells were cultured in 50% WRN (stem cells) or 5% WRN+5μM DAPT (colonocyte) for 2 days. Colonoid RNA was isolated using Trizol-LS (Invitrogen). High-quality total RNA (RIN>8) was submitted to Genetic Resources Core Facility at Johns Hopkins University for library prep and sequencing. The sequencing library was generated using TruSeq stranded RNA seq library prep kit with Ribo-Zero depletion (Illumina) and sequenced on Hiseq 2500 platform at 100 bp pair-end read (Illumina). The analysis pipeline was adapted from the previously described[5]. Briefly, RNA-seq reads were mapped using TopHat2 (the human reference genome version 19). Following RNA-seq mapping, expression levels at the gene and isoform levels were determined. The expression was quantified using Cufflinks to calculate FPKM estimates and HTSeq to calculate raw read counts.

Dextran sulfate sodium (DSS) injury model

Nox1 wild type and Nox1 knockout mice were treated with or without 5% DSS in drinking water for 5 days. For the DSS treatment group, 7 mice per genotype were used in GP2 qPCR and basal lymphoplasmacytosis analysis. For water treated mice group, 4 mice per genotype were used in GP2 qPCR and basal lymphoplasmacytosis analysis. For GP2 qPCR , 2 cm of distal colon were harvested and cleaned with cold RNase free 1X PBS. Then the distal colons were minced into

size < 0.5 cm and submerged in 500 ul of RNA later solution (Invitrogen) at 4C overnight. Tissues were stored at -80C until RNA extraction was performed. Total RNA was isolated using Trizol-LS (Invitrogen) and cleaned up with $LiCl_2$ [6]. GP2 qPCR was done as described in the section on real-time quantitative PCR.

Basal lymphoplasmacytosis quantification

Proximal colons (excluding cecum) were harvested and rolled into a Swiss roll and fixed in 10% formalin for 1 day, followed by 70% ethanol. Tissues were submitted to Mount Sinai Biorepository core for standard histology processing. Briefly, tissue was embedded in parafilm, and 5 μm of sections were used for Hematoxylin and Eosin (H & E) staining, followed by 40X whole slide scanning via NanoZoomer S210 Digital slide scanner (Hamamatsu). H & E images were used for basal lymphoplasmacytosis quantification. The basal lymphoplasmacytosis region and entire colonic area were marked, and the area (μm^2) was obtained from HALO® Image Analysis Platform (Version -HALO v3.4.2986.151). The basal lymphoplasmacytosis regions were identified via the following four parameters. (1) the condensation of lymphocytes in the area is lesser than regular lymphoid aggregates in water treated group. (2) the histological staining of nuclear lymphocytes is much darker stained than the basal epithelium cells. (3) the crypt structure is still intact. (4) the area along the colon can be identified in 5X enlargement of 20X magnification-scanned image, in 200 um scale bars. The % of basal lymphoplasmacytosis area was expressed as dividing the sum of lymphoplasmacytosis area by the entire colonic area.

Colon UEA1 staining and quantification

The slides with 5 μm of colonic sections were deparaffinized with xylene and ethanol (2 time of 100% xylene; 1 time of 1:1 100% xylene:100% ethanol; 2 time of 100% ethanol; 1 time of 95%, 70% and 50%) and rinsed with 1X TBS. The slides were blocked and permeabilizated with 1% BSA 5% Hi-FBS, 0.1% Triton X-100 in 1X TBS for 1 hour at room temperature. Slides were incubated with UEA1-Rhodamine (1: 400 dilutions; VECSO #RL-1062) in blocking solution (1X TBS, 1% BSA, 5% HI-FBS) at room temperature in the dark for 90 minutes. Slides were washed

6 times of 1X TBS and mounted in DAPI-Fluoromount-G (Electron Microscopy Sciences). UEA1 and DAPI Images were obtained on the Zeiss LSM880 (Carl Zeiss) confocal laser scanning microscope using the 10X Plan-Apochromat objective with the bit depth at 12. All confocal images were exported as TIFF files, converted to 16 bit and analyzed on ImageJ software (NIH). Crypt structures were marked based on DAPI and UEA merge images, and the mean fluorenes intensity of UEA images was extracted. UEA intensity was normalized to the average water treated Nox1 wild-type mice group.

Colon UEA1 and B220 staining and quantification

The slides with 5 µm of colonic sections were deparaffinized, antigen retrieval, blocked and permeabilizated. For Antigen retrieval, slides were treated with pH6 sodium citrate buffer (Abcam # ab93678) for 30 minutes in steamer follow by 30 minutes cool down at room temperature. Then slides were blocked and permeabilized for 1 hour at room temperature. The slides were incubated with B220 antibody (1:100 dilutions; Invitrogen # 14-0452-86) at 4C overnight follow by Alexa 488 anti-rat IgG (1:500 dilution; Invitrogen#A21208) and UEA1-Rhodamine (1:400 dilution; VECSO #RL-1062) at room temperature in the dark for 90 minutes. Slides were washed 6 times of 1X TBS and mounted in DAPI-Fluoromount-G (Electron Microscopy Sciences). UEA1, B220 and DAPI Images were obtained on the Zeiss LSM880 (Carl Zeiss) confocal laser scanning microscope using the 20X Plan-Apochromat objective with the bit depth at 12. All images were exported as TIFF files, converted to 16 bit and analyzed on ImageJ software (NIH). DAPI images were used to determine crypt numbers. B220 positive cell were selected, and B220 positive cell number and total area were analyzed on ImageJ software (NIH). B220 postive cell number and area were normalized to the crypt number per image.

ROS staining (NBT) of colonic crypts and organoids

Reactive oxygen species (ROS) productions in colonic crypts were visualized using p-nitroblue tetrazolium (NBT) chloride as previously described, turning into blue-colored NBT formazan when reacting with ROS[1]. Patients were recruited as a part of the Oxford Gastrointestinal Illness Biobank (REC 21/YH/0206). For *ex vivo* colonic crypt staining, fresh colonic biopsies were obtained from a healthy male patient without intestinal inflammation. Each biopsy was cut in half and incubated in RPMI (Gibco,) at 37 °C. One-half of each biopsy was treated with the NADPH oxidase inhibitor diphenyleneiodonium chloride (DPI; Tocris, Cat#0504; 10 µM) for 3 hours, whereas the other half was not treated. NBT chloride (100 µg/mL; Sigma-Aldrich Cat#484235) was added for 1 hour. Images from colonic crypts were obtained using brightfield microscopy on the Zeiss Axioskop 2 (Carl Zeiss) using the Plan-Neofluar 20x/0.50 objective. Crypts from a total of 4 biopsies were counted (901 crypts in total), and the relative fraction of positively stained crypts was calculated. Colon organoids were cultured from a healthy female control patient without intestinal inflammation. Organoids were incubated with TNFα only (200ng/ml; Gibco, Cat# PHC3011) or with a combination of TNFα (200ng/ml) and DPI (10 µM) for 3 hours. 2 hours after the start of the incubation, organoids were mechanically disrupted. NBT chloride (500 µg/mL) was added for 1 hour. Images from organoids were obtained using brightfield microscopy on the Leica Inspection microscope DM IL LED (Leica Microsystems GmbH) using the HI Plan 40x/0.50 PH2 objective.

The integrative M cell genes analysis

Included genes that are upregulated in M cells only based on Seurat DEG analysis [7–10] (FindMarkers, thresholds are > 0.25 fold change, min.pct = 0.1) with a p-value cut off <0.05. Genes were mapped using BioMart [11,12] to extract Ensemble IDs with non-zero expression in MSCCR samples (127 genes total, 111 included in MSCCR)[13]. These genes were used to calculate PCs across the 144 inflamed and 167 non-inflamed rectum samples, which were

then included in a logistic regression model to test for significance of each PC in separating inflamed vs. uninflamed samples (accounting for sex and race as well): glm(formula = finalMcell$Inflammation ~ as.factor(finalMcell$Sex) + as.factor(finalMcell$Race) + finalMcell$PC1,family="binomial"). Regression testing was run using R, and plots were made using ggplot2.

RNA Velocity Analysis

RNA velocity analysis was done as previously described[14]. Briefly, the aligned bam files from Cell Ranger were used as input for implementing Velocyto (v0.17) to get the counts of spliced and unspliced abundances in loom format. The loom files were then merged with the Seurat objects and analyzed using scVelo (v0.2.4) to get transcriptional dynamics for genes. The velocities were estimated using the default second-order moments, the stochastic model. The directionality on the cellular level can be inferred by resolving velocities on the gene level. A higher abundance of unspliced mRNA suggests upregulation for a gene, and conversely, downregulation is indicated by negative velocity. We generated scVelo scatter plot of DNMT1 of stem cell 1, stem cell 2 and stem cell 3 of UC epithelial cells using pl.scatter(). Uninflamed and inflamed samples were compared.

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SUPPLEMENTARY FIGURE LEGEND

Supplementary Figure 1. Colonoids ROS production with decreasing Notch (left) and Wnt (right) culture conditions stratified by Nox1 genotype. ROS production using GLOMAX measured 3 days after differentiation. N=6-7 independent measurements.

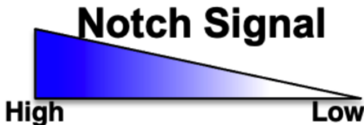
Supplementary Figure 2. Unbiased cluster-specific markers for UC epithelium and colonoid scRNAseq data sets. A. Heatmap to show top 4 upregulated genes in each cluster from UC epithelium scRNAseq. Expression is log2 normalized. **B.** Heatmap to show top 4 upregulated genes in each cluster from scRNAseq of untreated and TNFα-stimulated Nox1WT and Nox1KO cultured colonoids. Expression is log2 normalized.

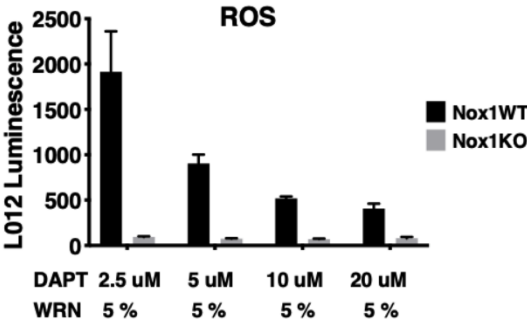
Supplementary Figure 3. Ccl20 is upregulated upon TNFα stimulation in colonids system. Violin plot of Ccl20 expression in colonoids. ****, $P < 0.0001$ (two-sided Wilcoxon signed-rank test).

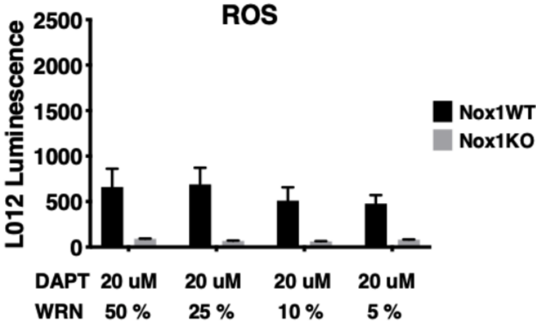
Supplementary Figure 4. Human UC scRNA seq demonstrates NOX1 is strictly expressed in epithelial cells while TNFα, TNFα receptors, RANKL and RANK are mainly expressed in immune and mesenchymal cells. A. Feature plot of expression distribution of TNFα receptor (TNFRSF1A and TNFRSF1B), **B.** NOX1, TNFα, RANKL (TNFSF11), and RANK (TNFRSF11A) from scRNAseq of human ulcerative colitis. N = 4 patients, selected for having inflamed rectum and uninflamed sigmoid

Supplementary Figure 5. B220 positive cells is located to the bases of crypt. Representative immunofluorescence images of B220 (left panel), UEA1 (middle panel) and B220/ UEA1 (right panel). Scale bars 50 µm.

Supplementary Figure 1



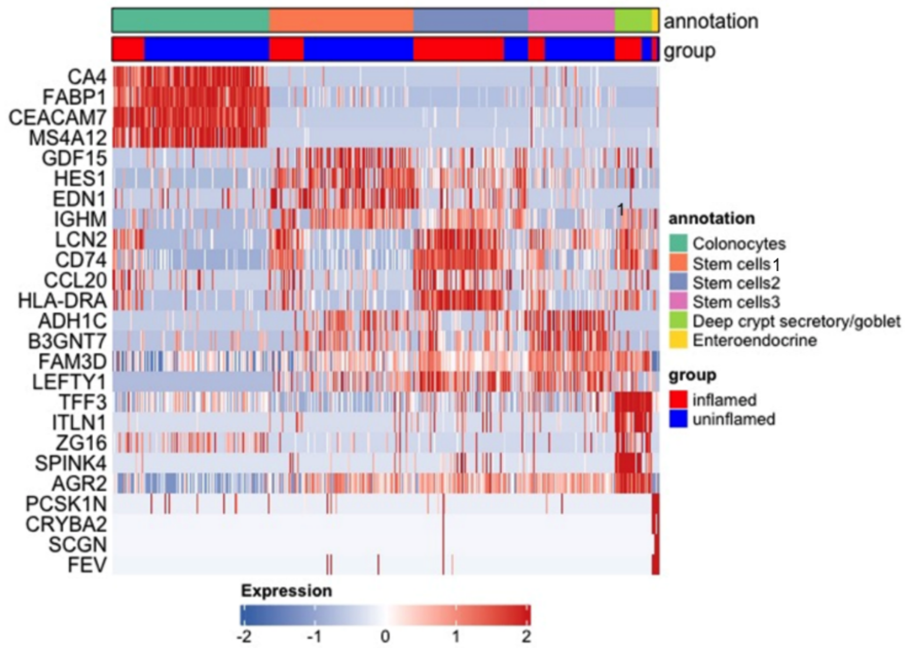




Supplementary Figure 2

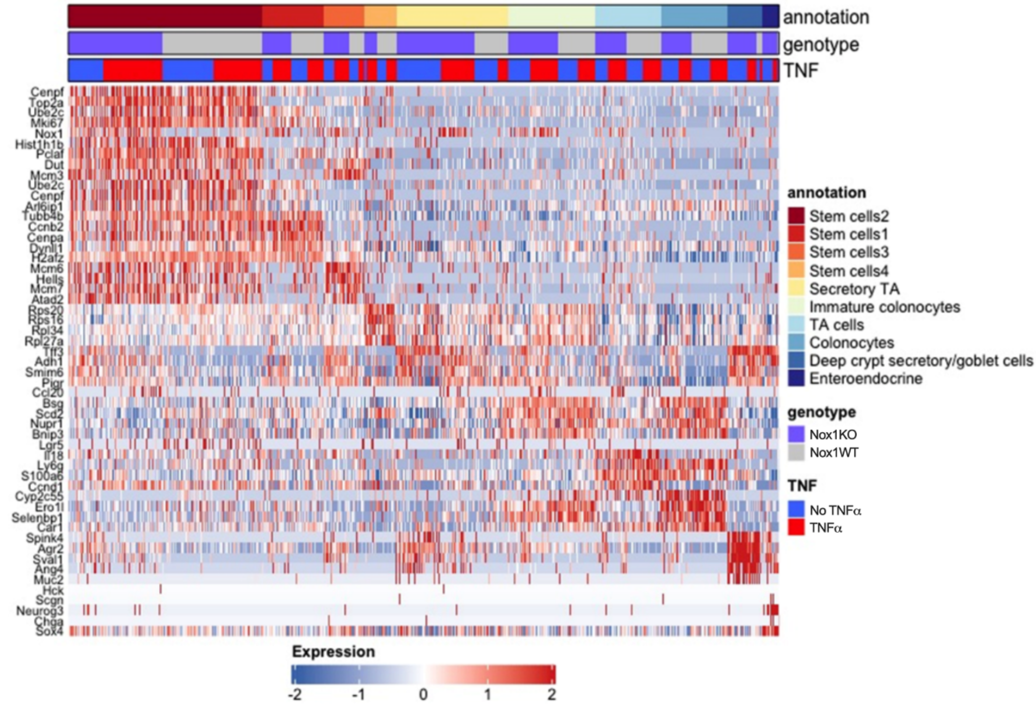
A

UC epithelium

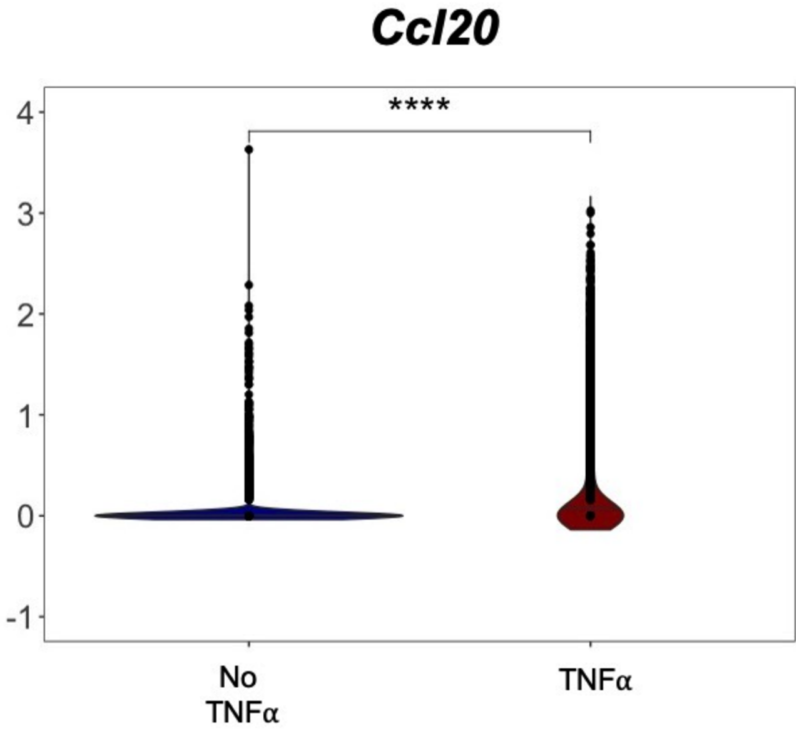


B

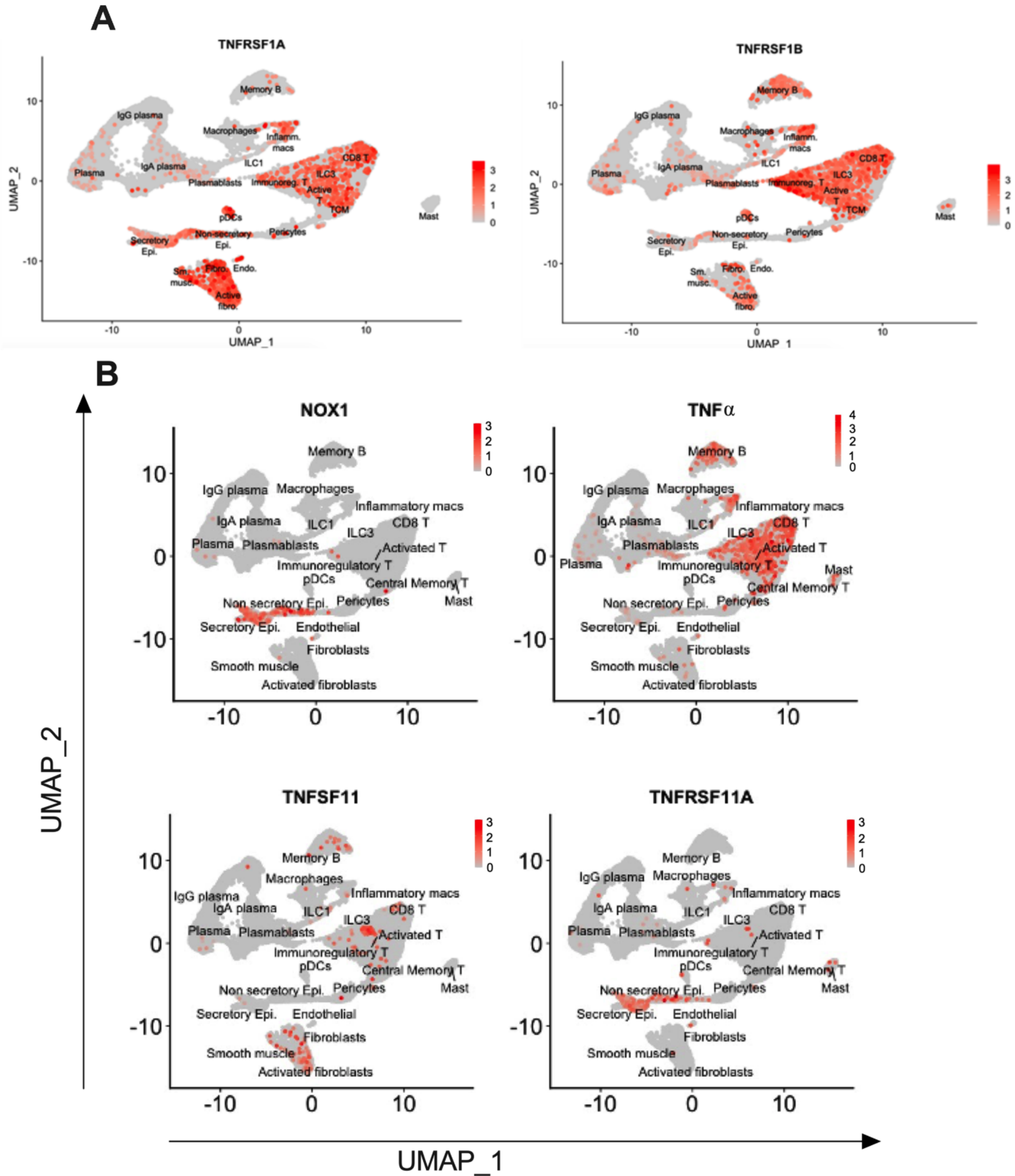
Colonoid



Supplementary Figure 3



Supplementary Figure 4



Supplementary Figure 5