HHS Public Access

Author manuscript

J Immunol. Author manuscript; available in PMC 2023 November 01.

Published in final edited form as:

J Immunol. 2022 November 01; 209(9): 1768–1777. doi:10.4049/jimmunol.2200125.

Th17-derived cytokines synergistically enhance IL-17C production by the colonic epithelium

Stephanie M Swedik[*], **Abson Madola**[†], **Michelle A Cruz**[‡], **Braulio J Llorens-Bonilla**[*], **Alan D Levine**[*,‡,§]

[*]Department of Molecular Biology and Microbiology, Medicine, and Pediatrics, Case Western Reserve University, 10900 Euclid Avenue, Cleveland OH 44106, United States

[†]Department of Biology, Medicine, and Pediatrics, Case Western Reserve University, 10900 Euclid Avenue, Cleveland OH 44106, United States

[‡]Department of Pathology, Medicine, and Pediatrics, Case Western Reserve University, 10900 Euclid Avenue, Cleveland OH 44106, United States

[§]Departments of Pharmacology, Medicine, and Pediatrics, Case Western Reserve University, 10900 Euclid Avenue, Cleveland OH 44106, United States

Abstract

Tightly regulated communication between the gastrointestinal (GI) epithelium and immune cells in the underlying lamina propria is critical for immune homeostasis and inflammation. Interleukin (IL)-17C, produced by epithelial cells after exposure to inflammatory stimuli, facilitates cell-to-cell communication by promoting inflammatory responses in Th17 cells. In this study, we demonstrate Th17 derived cytokines TNF-α, IL-17A, and IL-22, synergistically enhance IL-17C expression in both human transformed colonic epithelial cell lines and primary non-IBD colonic epithelial spheroids. This synergistic expression requires activation of the transcription factor NF-κB downstream of TNF-α stimulus, evidenced by the reduction of IL-17C expression in the presence of an IκBα inhibitor. IL-17A and IL-22 enhance IL-17C expression through the activation of the transcription factor AP-1 in a p38 MAPK dependent manner. Colonic spheroids derived from uninvolved epithelial of UC patients stimulated with TNF-α, IL-17A, and IL-22 show muted responses compared to non-IBD spheroids, and inflamed spheroids yielded more IL-17C expression in the presence of TNF-α, and no response to IL-22 stimulation. Altogether, a role for IL-17C in activating Th17 cells combined with our findings of Th17-derived cytokine driven synergy in the expression of IL-17C identifies a novel inflammatory amplification loop in the GI tract between epithelial cells and Th17 cells.

Correspondence: Alan D Levine, Case Western Reserve University, School of Medicine (W217C), 10900 Euclid Avenue, Cleveland OH 44106-4960, alan.levine@case.edu, Phone: 1 216 368 0342, Fax: 1 216 368 3055.

AUTHOR CONTRIBUTIONS

Conceptualization: SMS, ADL; Methodology: SMS, AM, MAC, BL; Validation: SMS; Formal analysis: SMS; Investigation: SMS, AM; Resources: SMS, ADL; Writing- original draft: SMS, ADL; Writing -review & editing: SMS, AM, MAC, ADL; Supervision: ADL; Project administration: ADL; Funding acquisition: ADL.

CONFLICT OF INTEREST

The authors do not have any financial conflicts of interest to report.

INTRODUCTION

The gastrointestinal (GI) epithelium is the largest surface area in the body and forms a barrier between the host and the hostile environment of the intestinal lumen. Within the lumen is housed the highest concentration of microbes in the human body, equal in number to the totality of human cells (1), which are adjacent to a one cell-layer thick epithelium that overlays the largest population of immune cells (2). As the barrier to the outside world, this colonic epithelium must maintain its integrity and mount a vigorous immune response against pathogens. When the epithelial layer comes in direct contact with pathogenic organisms, cells will enhance mucus production, upregulate tight junction protein expression, and secrete chemokines and cytokines to recruit and activate immune cells to sites of inflammation or infection (3, 4).

Upon colonic infection, Toll-like Receptors are activated, and the epithelial cell layer releases a combination of antimicrobial peptides, chemokines, and cytokines to initiate an inflammatory response (5). One of the cytokines expressed by epithelial cells is IL-17C (6–8). In the colon, the IL-17C receptors, IL-17RA and IL-17RE, are expressed on epithelial cells (6, 9, 10), suggesting autocrine functionality. These receptors are also expressed on T-helper 17 (Th17) cells (11, 12), indicating communication between epithelial cells and this important T cell subtype commonly present in the lamina propria. Th17 cells are differentiated from naïve CD4+ T cells after exposure to Transforming Growth Factor-Beta (TGF-β), Interleukin 6 (IL-6), IL-21, and IL-23, and upon activation produce IL-17A, IL-17F, IL-21, IL-22, Tumor necrosis factor-α (TNF-α), among other cytokines (13–15).

The trademark cytokine released from Th17 cells is IL-17A, secreted as a homodimer or a heterodimer with IL-17F. IL-17A engages its receptor IL-17RA/RC, activating transcription factors NF-κB, AP-1, and C/EBP via an Act1/TRAF 6 pathway (16–19). In healthy individuals, IL-17A is associated with the clearance of bacterial and fungal infections by enhancing communication between the innate and adaptive immune systems, consistent with the known function of Th17 cells. Aberrant expression of IL-17A leads to a variety of diseases including autoimmunity, cancer, and counterintuitively, attenuated host defense against pathogens (20).

IL-22, another cytokine produced by Th17 cells, enhances the barrier function of epithelial cells (15, 21). Binding of IL-22 to the IL-22 receptor, composed of IL-22Rα1 and IL-10Rβ, recruits Jak1 and Tyk2, which facilitate STAT1, 3, and 5 activation, and to a lesser extent, MAPK pathway activation (22). Whereas IL-22 activity is protective in the colon, enhanced levels of IL-22 at other barrier surfaces (e.g., skin) exacerbates inflammation (21, 23–26), underscoring the importance for tight regulation of IL-22 expression and function to balance health and disease.

TNF-α is a potent activator of both cell survival and apoptosis and enhances proinflammatory mediator expression (27, 28). Ligation of TNF-α to the TNFR-1 receptor leads to the recruitment of TNFR associated death domain (TRADD) and TNFR associated factor 2 (TRAF2), which initiate signal transduction pathways leading to the activation of NF-κB and AP-1 (28). The link between TNF-α dysregulation and autoimmune and

autoinflammatory conditions has been thoroughly established, as this cytokine is a regularly used as a therapeutic target for diseases throughout the body (29, 30).

Recognizing that IL-17C is capable of activating Th17 cells during inflammatory conditions(11, 31), we characterized the ability of Th17 cells to induce the expression of IL-17C, using a selection of Th17 derived cytokines TNF-α, IL-17A, and IL-22, based on their distinct roles in intestinal health, connection to Inflammatory Bowel Disease (IBD), and the reported cooperativity between IL-17A and TNF-α (32–35), between IL-17A and IL-22 (36), and between IL-17A, TNF-α, and IL-22 (37, 38). In this study, we show that the Th-17 derived cytokines, TNF-α, IL-17A, and IL-22 synergize to stimulate the expression IL-17C in colon epithelial cells in an NFκB, p38, and AP-1-dependent manner. This novel finding suggests that IL-17C may contribute to inflammatory conditions in which Th17 cells are activated.

MATERIALS AND METHODS

Cell Culture

The human colorectal adenocarcinoma cell lines HT-29, DLD-1, and Caco-2 (purchased from American Type Culture Collection) were mycoplasma-free (tested monthly) and cultured in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal calf serum (FCS, Corning) at 37°C with 5% CO_2. Cells were seeded 2 d before performing experiments to allow for cell attachment and growth to reach 50–70% confluence in 12-well 35mm^2 culture plates for RT-qPCR and ELISA experiments, and 6-well, 96mm^2 culture plates for immunoblot experiments.

Human colonic epithelial spheroids were generously provided by Dr. Stappenbeck, under University Hospitals Cleveland Medical Center IRB protocol #01-06-11, and were cultured according to the methods described in VanDussen, KL et al. (39). All non-IBD and UC-derived spheroids proliferated at the same rate. Demographic information on the participants is shown in Table 1. Briefly, spheroids were thawed, washed in DMEM/F12, carefully suspended in Matrigel and plated in 24 or 48 well plates. The plate was flipped and Matrigel was allowed to harden for 15 min at 37°C to ensure that epithelial cells did not adhere to the surface plastic. Cells were grown in culture media (50% L-WRN media made in house: 50% DMEM/F12) supplemented with 20% FBS, EGF 500ng/ml, FGF 50ng/ml, TGFβIR inhibitor 10uM, and Rock inhibitor 10uM at 37°C with 5% CO2. Media was changed every 2–3 days and cells were passaged using 1mM EDTA followed by vigorous pipetting in TryplE at 37°C every 4th day depending on confluency.

Cytokines

Recombinant human (rh) TNF-α, IL-17A, IL-22, and IL-17C were purchased from R&D Systems. Unless stated otherwise, IL-17A and IL-17C were used at a concentration of 100 ng/mL, IL-22 at 10 ng/mL, and TNF-α at 50 ng/mL.

Pharmacological Inhibitors

NF-κB inhibitor Bay 11–7082 (30 μM; Sigma-Aldrich), p38 MAPK inhibitor SB202190 (1 μM; Tocris bio-techne), ERK inhibitor FR 180204 (30 nM - 10 μM; Tocris bio-techne), JNK inhibitor TCS JNK 6o (30 nM - 10 μM; Tocris bio-techne), AP-1 Inhibitor SR 11302 (10μM - 100μM; Tocris bio-techne), and MEK inhibitor U0126 (100 μM; Promega). Actinomycin D (Sigma-Aldrich) was used at a concentration of 10 μg/mL.

Enzyme-linked Immunosorbent Assay

Cell culture conditioned media was collected and IL-17C expression was detected with the IL-17C Duo Set ELISA (R&D Systems), performed following the manufacturer's instructions.

RT-qPCR

RNA was isolated using the PureLink RNA Mini Kit (Thermo Fisher Scientific) and reverse transcribed (0.4 μg RNA) with the High-Capacity RNA-to-cDNA Kit (Thermo Fisher Scientific). qPCR was performed using Power-SYBR green reagents (Thermo Fisher Scientific). Primer sequences are listed in Table 2. Fold changes in IL-17C mRNA levels were calculated in Excel (Microsoft) using the standard $2^{-\Delta\Delta CT}$ method, normalized using glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Protein Isolation

Whole cell lysates were prepared by washing cells in 500 μL PBS and lysed in 75 μL 1x Cell Lysis Buffer (Cell Signaling Technology) supplemented with the Halt protease and phosphatase inhibitor at a 1:100 dilution (Thermo Fisher Scientific). After lysis samples were kept on ice for 15 min, followed by a clearing centrifugation of 12,000 × g for 15 min at 4°C. Protein concentrations in the supernatant were determined using the QuickStart Bradford Protein Assay Kit (Bio-Rad). Lysate volumes were adjusted to mix 20 μg of protein with 5x Laemmli buffer at a 5:1 ratio and boiled for 5 min before electrophoresis.

Nuclear Fractionation

Cells cultured in a 6-well plate were washed with PBS, collected into Eppendorf tubes using a cell scraper, transferred to ice, and lysed with Buffer A (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, with protease inhibitors) for 15 min. 0.6% NP-40 was added to the lysis buffer and the samples were vortexed, followed by a centrifugation spin of 12,000 × g for 30 s at 4°C. The pelleted nuclei were washed with additional Buffer A, and then lysed in Buffer B (20 mM Hepes, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, with protease inhibitors) for 30 min on ice, vortexing every 10 min. Samples were centrifugated at 12,000 × g for 10 min. The supernatant was collected, and protein was quantified using the QuickStart Bradford Protein Assay Kit.

Antibodies

Antibodies used were mouse anti-RelA (1:1,000 dilution of F-6, Santa Cruz), mouse anti-p50 (1:500 dilution of E-10, Santa Cruz), mouse anti-hnRNP-A1 (1:5,000 dilution of 4B10, Santa Cruz), mouse anti-GAPDH (1:1000 dilution of 6C5, Thermo Fisher Scientific),

rabbit anti-IL-22Rα1 (1:1,000 dilution of ab5984, Abcam), goat anti-IL-17RA (1:1000 dilution of AF177, R&D Systems), rabbit anti-p38 (1:5,000 dilution of D13E1, Cell Signaling Technology), rabbit anti-phospho-p38 (1:1,000 dilution of #9211, Cell Signaling Technology), rabbit anti-ERK 1/2 (1:10,000 dilution of #EPR17526, Abcam), rabbit anti-phospho-ERK 1/2 (1:500 dilution of 197G2, Abcam), rabbit anti-JNK1/2/3 (1:1,000 dilution of EPR167979, Abcam), rabbit anti-phospho-JNK1/2/3 (1:2,000 dilution of EPR5693, Abcam), rabbit anti-ATF-2 (1:1000 dilution of D4L2X, Cell Signaling Technology), and rabbit anti-phospho-ATF-2 (1:1000 dilution of A8J7P, Cell Signaling Technology). The secondary antibodies conjugated to horseradish peroxidase (HRP) were goat anti-rabbit IgG-HRP (Millipore), goat anti-mouse IgG-HRP (Thermo Fisher Scientific), rabbit anti-goat IgG-HRP (R&D Systems), and rabbit anti-sheep IgG-HRP (Abcam) used at a 1:10,000 dilution.

Surface staining antibodies used for flow cytometry were APC anti-human CD217 (Biolegend, W15177A), FITC anti-human IL-17RC (Miltenyi Biotec, REA571), PE anti-human CDw210b (IL-10Rβ, Miltenyi Biotec, REA848), and DyLight 405 anti-human IL-22Rα1 (R&D Systems, 305405).

SDS-PAGE and Immunoblot Analysis

Protein samples were separated using 10–12% polyacrylamide gel electrophoresis, as noted in the figure legends, followed by a transfer onto an ImmunoBlot PVDF membrane (Bio-Rad). Membranes were blocked in 5% dried milk in TBST (20mM Tris, 150mM NaCl, 0.1% Tween-20, pH 7.5) and incubated overnight at 4°C with primary detection antibodies. Membranes were washed 3 × 10min with TBST, then treated with secondary HRP-conjugated antibodies for 1 h at RT. Membranes were washed 3 x with TBST for 10 min, followed by 3 × 10min washes with TBS (20mM Tris, 150mM NaCl, pH 7.5). Protein was detected using WesternBright Sirius HRP Substrate (Advansta).

Flow Cytometric Analysis

HT-29 cells were stimulated with TNF-α for 3 h, then washed with PBS and incubated with Trypsin/EDTA solution for 5 min at 37°C. Cells were centrifuged at 300g for 3 min and resuspended in FACS buffer (Dulbecco's Phosphate Buffered Saline with 0.005% sodium azide and 0.1% Bovine Serum Albumin). To assure a single cell suspension, cells were passed through an 18-gauge needle and filtered through a 40 μm cell strainer. Cell concentrations were adjusted to 2×10^6 cells/mL and were incubated with antibodies for 30 min at 4°C before analysis on an LSRFortessa cytometer (BD Biosciences). Samples were analyzed and gated using FCS and SCS through FlowJo.

Reproducibility and Statistical Analysis

Each experiment was repeated a minimum of three times. Each data point represents distinct experimental biological replicates, averaged among 3 replicate wells and technical duplicates during ELISA or RT-qPCR analysis of those wells. Graphs were generated in Prism (GraphPad), and P values calculated using statistical tests as stated in the corresponding figure legends.

RESULTS

IL-17C is synergistically upregulated in the presence of Th-17 derived cytokines

To understand how IL-17C expression is regulated in the colon in response to Th17 cell activation, the human colonic adenocarcinoma cell line HT-29 was stimulated with sub-saturating concentrations of TNF-α, IL-17A, and IL-22. When the cytokines were added to culture individually, TNF-α alone induced the highest levels of IL-17C mRNA and protein expression (Figure 1a,b). Although stimulation with either IL-17A or IL-22 alone yielded modest increases in IL-17C mRNA expression (3.6 and 1.6 fold induction, respectively), we saw a marked amplification of IL-17C expression when pairing these cytokines with TNF-α (Figure 1a,b). When cells were stimulated with all three cytokines concurrently, we witnessed on average an incredible synergistic 11,000-fold increase in IL-17C mRNA expression and greater than 13 ng/mL in IL-17C protein expression (Figure 1a,b). Because TNF-α was a required stimulant for IL-17C expression, cells were stimulated with either TNF-α alone, pairwise with IL-17A or IL-22, or all three cytokines together, and IL-17C expression measured over time. Synergistic IL-17C production after triple cytokine stimulation was maximal at 5 h for mRNA expression and 24 h for protein expression (Figure 1c–f). All subsequent protein and mRNA analyses were completed at these timepoints unless otherwise stated. To demonstrate the universality of this synergistic response, we surveyed different colonic cell lines for their ability to express IL-17C in response to cytokine stimulation. The synergistic increase in IL-17C production in response to all three cytokines was observed in the additional cell lines tested (Caco-2, DLD-1 Supplemental Figure 1). HT-29 cells were selected as the model system due to their robust response and dynamic range of IL-17C production, allowing for studies on the mechanism of action.

The presence of IL-17C in culture does not enhance or inhibit its own expression

Due to the dramatically increased levels of IL-17C protein in cytokine-stimulated culture and recognizing that the IL-17C receptor subunits IL-17RA and IL-17RE are both expressed on colonic epithelial cells (6, 9, 10), we tested whether the robust secretion of IL-17C was promoting additional production of IL-17C expression. IL-17RA is a shared receptor subunit between IL-17A and IL-17C, thus inhibiting IL-17RA would also inhibit IL-17A-induced expression of IL-17C. In the absence of a commercially available neutralizing antibody for IL-17RE, epithelial cells were instead treated with IL-17A, IL-22, and TNF-α with or without the addition of IL-17C (Figure 1g). The presence of excess IL-17C did not lead to either a decrease or increase of IL-17C mRNA expression, indicating that autocrine activity of IL-17C does not directly influence its own expression.

TNF-α does not prime colonic epithelial cells to respond to IL-17A or IL-22

The time course of IL-17C mRNA expression (Fig. 1c,d) reveals that TNF-α stimulated IL-17C mRNA levels peak at 2 h and decline to baseline thereafter. It appears that the synergy initiated by IL-17A and IL-22 is achieved by a sharp increase in IL-17C mRNA synthesis that expands dramatically for the next 3 h. These results suggest the possibility that within the first 3 h TNF-α primes colonic epithelial cells to respond to IL-17A and IL-22. To test this hypothesis, the expression of the IL-17A receptor chains IL-17RA and IL-17RC,

and IL-22 receptor chains IL-10Rβ and IL-22Rα1, were measured via flow cytometry (Figure 2a) and immunoblot (Supplemental Figure 2). Basal expression of all four receptor subunits was not altered by cytokine stimulation.

An alternate approach to evaluate the possibility that TNF-α primes intestinal epithelial cells for an IL-17A and IL-22 response is to delay the addition of IL-17A and IL-22 until 3 h. The delayed addition of IL-17A and IL-22 resulted in significantly decreased levels of IL-17C mRNA synthesis in comparison to the simultaneous addition of all three cytokines at culture initiation (Figure 2b). Delaying cytokine addition was comparable to that of the pairwise treatment of the other two cytokines, suggesting IL-17A and IL-22 signaling pathways are activated immediately and the first three hours of culture are critical to achieve synergy.

IL-17A does not stabilize the mRNA transcripts of IL-17C

Studies have demonstrated that IL-17A can not only enhance expression of inflammatory genes via transcription but can also stabilize the mRNA transcripts of certain genes through an Act1-mediated mechanism (34, 40). To determine if mRNA transcripts for IL-17C were stabilized by IL-17A, cells were stimulated with TNF-α or TNF-α and IL-17A for 1 h, followed by treatment with Actinomycin D. mRNA expression levels of IL-17C were assessed at 30, 60, 90, and 120 min to calculate half-life. Using CXCL1 transcripts as a positive control for IL-17A-mediated increased mRNA half-life, it was determined that IL-17C mRNA transcripts are not stabilized in the presence of IL-17A stimulus (Supplemental Figure 3). This result confirms the findings of Friedrich et al. (35).

Transcription factor NF-κB downstream of TNF-α is necessary for IL-17C expression

Previous studies have shown that TNF-α regulates IL-17C production through activation of the transcription factor NF-κB (35, 41, 42). To determine the contribution of NF-κB activation for the synergistic expression of IL-17C, NF-κB translocation into the nucleus was inhibited with Bay 11 7082, as demonstrated via immunoblot (Figure 3a). Using an optimized, non-toxic concentration of the inhibitor, IL-17C expression, induced by all cytokine treatment combinations, was completely inhibited at the mRNA and protein levels (Figure 3b,c). This finding indicates that IL-17C production is dependent on TNF-α-induced NF-κB translocation and that IL-17A and IL-22 mediated synergy does not occur in the absence of NF-κB signaling.

IL-17A or IL-22 do not enhance canonical NF-κB translocation

In addition to TNF-α-mediated activation of NF-κB, IL-17A has also been shown to activate this transcription factor (18), suggesting the synergy dependent on IL-17A may be due to enhanced translocation of the canonical subunits of NF-κB RelA/p65 and p50. Intestinal epithelial cells were stimulated with TNF-α and IL-17A alone or in combination and the nuclear translocation of NF-κB followed by immunoblot. Cytosolic and nuclear fractionation of the stimulated cells showed that TNF-α activated both RelA and p50 nuclear translocation, while IL-17A alone did not (Figure 4). In combination, there was no appreciable increase of RelA or p50 activity, indicating that IL-17A mediated synergistic production of IL-17C is not regulated through NF-κB. In addition, IL-22 did not activate RelA and p50 translocation alone and in combination with TNF-α (Supplemental Figure 4).

Inhibition of MEK, JNK, and ERK 1/2 does not affect IL-17C expression

Alternate signaling pathways activated downstream of TNF-α, IL-17A, and IL-22 were investigated for their role in regulating IL-17C expression. We first investigated if the MAPKs, ERK 1/2 and JNK, regulate synergistic production of IL-17C, due to their known function in TNF-α, IL-17A, and IL-22 signaling. Neither a selective pharmacological inhibitor of ERK 1/2 nor JNK decreased IL-17C production in the presence of all three cytokines (data not shown). In light of the regulation of the MAPK family by MEK, we chose to block MEK1/2, a kinase upstream of ERK and JNK. Neither IL-17C protein nor mRNA expression decreased in the presence of the MEK inhibitor (Figure 5a,b). In fact, in cells treated pairwise with TNF-α and IL-22, there was a slight increase in IL-17C expression in the presence of the MEK inhibitor. Immunoblot analysis of both phosphorylated and total amounts of the three MAPKs showed that at the lowest concentration of the MEK inhibitor, there was striking inhibition of ERK and p38, not JNK, phosphorylation (Figure 5c).

Critical role for MAPK p38 in the synergistic expression of IL-17C in cells treated with TNF-α, IL-17A, and IL-22

The known cross-regulation observed among the MAPK pathways (43) suggests that the third MAPK p38, which activates the transcription factor AP-1 and is triggered downstream of TNF-α and IL-17A and to a lesser extent IL-22. Intestinal epithelial cells were pretreated with the p38 inhibitor SB202190 followed by stimulation with TNF-α, IL-17A, and IL-22. The effectiveness of this inhibitor was demonstrated by its ability to block ATF-2 phosphorylation (80% decrease of phospho-ATF-2 in the presence of SB202190, TNF-α, IL-17A, and IL-22 compared to cells stimulated without inhibitor). Addition of the p38 inhibitor significantly decreased the degree of IL-17C synergistic expression in the presence of TNF-α, IL-17A, and IL-22 stimulation (Figure 6a,b). Stimulation with TNF-α alone showed no change in IL-17C mRNA or protein expression in the presence of the p38 inhibitor, indicating that TNF-α mediated activation of p38 does not contribute to IL-17C regulation (Figure 6a,b). While the decrease in IL-17C production when cells were treated pairwise with TNF-α and IL-17A or TNF-α and IL-22 in the presence of the inhibitor did not reach significance, the reduction in IL-17C expression is noteworthy (Figure 6a,b).

AP-1 plays an essential role in synergistic expression of IL-17C

To determine if MAPK p38 regulates IL-17C expression through the transcription factor AP-1, pharmacological and RNA silencing inhibition of AP-1 was performed using the inhibitor SR 11302. AP-1 (activating protein-1) is a collective term referring to dimeric transcription factors composed of cJun, cFos or ATF (activating transcription factor) subunits that bind to a common DNA site, the AP-1-binding site. Similar to the results obtained with the p38 inhibitor, decreased expression of IL-17C was seen in the pairwise treatments with TNF-α and IL-17A and with TNF-α and IL-22 with SR11302, and significant downregulation was observed in the triple cytokine treated cells (Figure 6c). Together, these findings underscore a role for the MAPK p38 in IL-17C-induced synergy and indicate that p38 is likely inducing the expression of IL-17C through the transcription factor AP-1.

Synergy of IL-17C expression induced by TNF-α, IL-17A, and IL-22 is seen in colonic epithelial stem cell spheroids

To confirm that the synergy between TNF-α, IL-17A, and IL-22 is physiologically relevant, colonic epithelial stem cell spheroids derived from non-IBD affected donors were stimulated with combinations of the Th17-derived cytokines. Similar to the transformed cell line data, TNF-α was the primary stimulus for IL-17C production in these cells, and both IL-17A and IL-22 pairwise with TNF-α demonstrated synergistic increases in expression (Figure 7a). The addition of all three Th17-derived cytokines stimulated a 1,500-fold increase in IL-17C mRNA expression in primary spheroid culture. These findings in the primary spheroids not only support synergistic Th17 cell mediated expression of IL-17C but suggest that the mechanisms uncovered in the transformed cell lines likely play a role in IL-17C regulation in vivo.

Primary colonic epithelial spheroids derived from patients with Ulcerative Colitis respond differently to Th17 derived cytokine stimulation than spheroids from non-IBD participants

In agreement with the results seen with non-IBD colonic spheroids, colonic epithelial stem cell spheroids derived from Ulcerative Colitis (UC) patients from uninvolved areas responded to the cytokines singly and in combination with an identical pattern, although the response was notably muted as seen by lower expression of IL-17C mRNA across all treatment conditions (Figure 7b). In contrast, the spheroids derived from inflamed regions of UC colon showed stronger IL-17C mRNA expression in the presence of TNF-α stimulation alone compared to that of both non-IBD and uninvolved spheroids. However, the pairwise and overall synergy among all three cytokines was not observed, as inflamed UC spheroids do not respond to IL-22 stimulation (Figure 7b). To evaluate if the difference in response between non-IBD and inflamed UC derived spheroids was due to a change in cytokine receptor expression, levels of IL-17RA, IL-17RC, IL-10Rβ, and IL-22Rα1 expression were determined via flow cytometry. Results show no differences in their expression between non-IBD and UC patient-derived spheroids (Figure 7c). These results highlight novel differences in the response to Th17 derived cytokines in the context of UC, suggesting that disease progression may be due to changes both in immune cell activity and the reciprocal responses of the inflamed epithelium.

DISCUSSION

In this study we demonstrated that a combination of cytokines, a signature of Th17 cells (13–15), regulates expression of IL-17C in the colon, inducing IL-17C mRNA and protein levels by greater than 11,000-fold in an intestinal epithelial cell line and 1,500-fold in a primary culture of human colonic spheroids, clearly indicating a strong synergy among the three cytokines tested. The induction parameters and kinetics of IL-17C mRNA expression indicated that TNF-α was the primary stimulant of IL-17C transcription, with mRNA levels maximal. Strikingly, the addition of IL-17A and/or IL-22 enhanced IL-17C mRNA expression only after TNF-α induction reached its maximum, suggesting that the observed synergy is due to a maintenance and expansion of ongoing transcription, as opposed to increased transcription at the initiation of gene expression. This prolonged activation of IL-17C expression beyond that which TNF-α could achieve alone suggested initially that

TNF-α was priming the epithelial cells to respond better to IL-17A and IL-22 stimuli. However, our findings indicate that this was not the mechanism of action, as the receptor dimers for both IL-17A and IL-22 are expressed basally by the cells, and stimulation with TNF-α did not affect these expression levels. While it is formally possible that TNF-α may induce the expression of downstream signaling molecules within the IL-17A and IL-22 transduction pathways, this is unlikely since synergistic expression of IL-17C was lost when addition of either IL-17A or IL-22 was delayed.

It was previously reported that IL-17C expression is induced by the combination of TNF-α and IL-17A (35), yet the mechanism for this induction was unknown as was the dramatic 11,000-fold synergy seen with the inclusion of IL-22. To define the mechanism of synergy, we initially focused on the transcription factor NF-κB, which is activated downstream of TNF-α and IL-17A (17, 28). Due to the nature of the IκBα inhibitor used (44), the activity of all canonical and non-canonical NF-κB subunits were inhibited, although we did indeed limit our biochemical analysis to translocation of the canonical subunits Rel-A and p50. Since inhibition of IL-17C synergy was complete in the presence of the IκBα inhibitor, it is not likely that IL-17A or IL-22 will increase the activation or translocation of alternate NF-κB subunits, Rel-B, c-Rel, or p52, complementing our observation of their not affecting activation of p65 and p50.

The contribution of the MAP kinase pathway to synergy proved to be far more complex. Neither ERK 1/2 nor JNK inhibition affected IL-17C expression (data not shown), while the p38 MAPK inhibitor and the AP-1 inhibitor both reduced levels of synergy in cells stimulated with TNF-α, IL-17A, and IL-22. Of note, inhibiting these pathways did not block pairwise induction of IL-17C (i.e., IL-17A and TNF-α or IL-22 and TNF-α), suggesting cooperation between dual IL-17A and IL-22 signaling pathways. While difficult to address in the current model, we propose that both IL-17A and IL-22 activate p38 MAPK more robustly when together in culture. When we targeted a kinase upstream of p38 MAPK with the MEK inhibitor U0126, phosphorylation of both p38 and ERK 1/2 was inhibited, yet, surprisingly, the MEK inhibitor did not block IL-17C expression. While we acknowledge that the p38 and MEK inhibitors are mechanistically different (45) lack of inhibition by U0126 was unexpected. One consideration is U0126 also decreases expression of the Dual Specificity Phosphatase 4 (DUSP4) (46), which dephosphorylates ERK, and to a lesser extent, JNK (47). Recognizing that the MAPK signaling cascade is a network of cross-communication and not linear (43), U0126 may also be inadvertently promoting JNK activity via downregulation of DUSP4, which, in turn, compensates for the loss of p38 and ERK signaling. This hypothesis, admittedly untested, is supported by our finding that at high concentrations of U0126, phosphorylation of JNK is increased.

Our proposal to consider the possible role of DUSP4 in this model is also supported by the observation that downregulation of DUSP4 in cells stimulated with TNF-α is NF-κB dependent (46). There is an 80% decrease of expression of DUSP4 in human lung microvascular endothelial cells (HMVECs) stimulated with TNF-α at 2 h, a decrease that is maintained for 48 h (46). In intestinal epithelial cells stimulated with TNF-α, IL-17A, and IL-22, there is a sharp increase in IL-17C mRNA expression between 2 and 3 h of stimulation, possibly due to a TNF-α / NF-κB-mediated decrease in DUSP4, leading to

enhanced activation of the MAPK pathways activated downstream of both IL-17A and IL-22. This crosstalk between the NF-κB and MAPK signaling pathways may also account for the complete ablation of IL-17C expression in the presence of the NF-κB inhibitor.

In addition to the mechanistic studies performed in transformed cell lines, the use of colonic stem cell spheroids provides additional insight into cytokine regulation of IL-17C expression in non-IBD individuals and patients with UC. The data derived from non-IBD colonic spheroids demonstrated that the regulation of IL-17C expression seen downstream of TNF-α, IL-17A, and IL-22 stimulation is not unique to transformed cell lines. This also suggests that the mechanisms behind IL-17C regulation are relevant in humans. Compared to non-IBD colonic spheroids, those derived from the uninvolved colon of UC patients showed a similar pattern of cytokine-induced IL-17C expression, although IL-17C expression levels were only about 15% of the non-IBD control spheroids. We propose that the muted response to Th17 derived cytokines by the UC patient spheroids may in part be due to their sourcing from a chronic inflammatory environment. In stark contrast, IL-17C expression in colonic spheroids from inflamed regions of UC patients showed a distinct pattern altogether. Inflamed UC spheroids responded more robustly to TNF-α stimulation than did non-IBD cells, which is consistent with TNF-α exacerbating UC inflammation (48). This observation suggests that in addition to the increased levels of TNF-α in UC tissue, the inflamed epithelium in the UC colon is also more responsive to TNF-α exposure. In addition, the inflamed UC spheroids demonstrated little to no response to IL-22 stimulation. To investigate these differences, the expression of the receptors for both IL-22 and IL-17A were analyzed, and there were no significant differences in expression of these receptors in UC spheroids *vs*. non-UC spheroids. In healthy individuals, IL-22 expression in the gut is important for functions such as mucosal healing, improved barrier function, and expression of anti-microbials (24, 49). The lack of activation of IL-17C expression in the presence of IL-22 in UC patients underscores the loss of IL-22 mediated protection and implicates IL-17C as being a part of this inflammatory axis. As one might predict when studying human disease with cells in primary culture, the spread of the response is broad; however, the conclusions drawn, after analyzing the data in aggregate or individual by individual, hold true.

IL-17C is associated with a number of autoimmune and autoinflammatory diseases (8). As inflammatory Th17 cell function is also linked to IBD (14), we propose that IL-17C may play an important role in IBD pathogenesis, as this synergy is absent in UC patient derived spheroids. Future studies of the effects of Th17 derived cytokines on the epithelium should include a more comprehensive analysis of the gene expression of inflammatory mediators and an in vivo characterization of the regulation of IL-17C gene expression in IBD. Understanding the mechanism behind IL-17C regulation in the context of disease will expand our options for selecting novel therapeutic targets.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

The authors wish to thank G. Dubyak, B. Min, and D. Anthony for advice in experimental design. The authors would also like to acknowledge the laboratory of Dr. Thaddeus Stappenbeck (Cleveland Clinic Foundation) for access and training on the primary human rectal organoid samples.

Grant Support:

Research was supported by grants from the National Institute of Health: National Institute of Drug Abuse (NIDA) [DP1 DA-037997 to ADL] and National institute of Allergy, and Infectious Disease (NIAID) CWRU/UH Center for AIDS Research (CFAR) [P30 AI-036219]. SMS, AM, MAC, and BJLB were supported by the National Institute of the General Medical Sciences (NIGMS) of the NIH, the National Heart, Lung, and Blood Institute, NIDA, and NIAID under award numbers T32 GM008056, R25 HL103152, T32 GM007250, and R01 DA043253-S1, respectively.

REFERENCES

1. Jones ML, Ganopolsky JG, Martoni CJ, Labbe A, and Prakash S. 2014. Emerging science of the human microbiome. Gut Microbes 5: 446–457. [PubMed: 25013912]
2. Geuking MB, Koller Y, Rupp S, and McCoy KD. 2014. The interplay between the gut microbiota and the immune system. Gut Microbes 5: 411–418. [PubMed: 24922519]
3. Gunther C, Josenhans C, and Wehkamp J. 2016. Crosstalk between microbiota, pathogens and the innate immune responses. Int J Med Microbiol 306: 257–265. [PubMed: 26996809]
4. Kim D, Zeng MY, and Nunez G. 2017. The interplay between host immune cells and gut microbiota in chronic inflammatory diseases. Exp Mol Med 49: e339. [PubMed: 28546562]
5. Hug H, Mohajeri MH, and La Fata G. 2018. Toll-Like Receptors: Regulators of the Immune Response in the Human Gut. Nutrients 10.
6. Ramirez-Carrozzi V, Sambandam A, Luis E, Lin Z, Jeet S, Lesch J, Hackney J, Kim J, Zhou M, Lai J, Modrusan Z, Sai T, Lee W, Xu M, Caplazi P, Diehl L, de Voss J, Balazs M, Gonzalez L Jr., Singh H, Ouyang W, and Pappu R. 2011. IL-17C regulates the innate immune function of epithelial cells in an autocrine manner. Nat Immunol 12: 1159–1166. [PubMed: 21993848]
7. Im E, Jung J, and Rhee SH. 2012. Toll-like receptor 5 engagement induces interleukin-17C expression in intestinal epithelial cells. J Interferon Cytokine Res 32: 583–591. [PubMed: 22994872]
8. Swedik S, Madola A, and Levine A. 2021. IL-17C in human mucosal immunity: More than just a middle child. Cytokine 146: 155641. [PubMed: 34293699]
9. Kusagaya H, Fujisawa T, Yamanaka K, Mori K, Hashimoto D, Enomoto N, Inui N, Nakamura Y, Wu R, Maekawa M, Suda T, and Chida K. 2014. Toll-like receptor-mediated airway IL-17C enhances epithelial host defense in an autocrine/paracrine manner. Am J Respir Cell Mol Biol 50: 30–39. [PubMed: 23944933]
10. Reynolds JM, Martinez GJ, Nallaparaju KC, Chang SH, Wang YH, and Dong C. 2012. Cutting edge: regulation of intestinal inflammation and barrier function by IL-17C. J Immunol 189: 4226–4230. [PubMed: 23024280]
11. Chang SH, Reynolds JM, Pappu BP, Chen G, Martinez GJ, and Dong C. 2011. Interleukin-17C promotes Th17 cell responses and autoimmune disease via interleukin-17 receptor E. Immunity 35: 611–621. [PubMed: 21982598]
12. Huang J, Yuan Q, Zhu H, Yin L, Hong S, Dong Z, Jin W, and Dong C. 2017. IL-17C/IL-17RE Augments T Cell Function in Autoimmune Hepatitis. J Immunol 198: 669–680. [PubMed: 27956525]
13. Diller ML, Kudchadkar RR, Delman KA, Lawson DH, and Ford ML. 2016. Balancing Inflammation: The Link between Th17 and Regulatory T Cells. Mediators Inflamm 2016: 6309219. [PubMed: 27413254]
14. Hundorfean G, Neurath MF, and Mudter J. 2012. Functional relevance of T helper 17 (Th17) cells and the IL-17 cytokine family in inflammatory bowel disease. Inflamm Bowel Dis 18: 180–186. [PubMed: 21381156]

15. Marshall EA, Ng KW, Kung SH, Conway EM, Martinez VD, Halvorsen EC, Rowbotham DA, Vucic EA, Plumb AW, Becker-Santos DD, Enfield KS, Kennett JY, Bennewith KL, Lockwood WW, Lam S, English JC, Abraham N, and Lam WL. 2016. Emerging roles of T helper 17 and regulatory T cells in lung cancer progression and metastasis. Mol Cancer 15: 67. [PubMed: 27784305]

16. Liu C, Qian W, Qian Y, Giltiay NV, Lu Y, Swaidani S, Misra S, Deng L, Chen ZJ, and Li X. 2009. Act1, a U-box E3 ubiquitin ligase for IL-17 signaling. Sci Signal 2: ra63.

17. Song X, He X, Li X, and Qian Y. 2016. The roles and functional mechanisms of interleukin-17 family cytokines in mucosal immunity. Cellular & molecular immunology 13: 418–431. [PubMed: 27018218]

18. Song X, and Qian Y. 2013. IL-17 family cytokines mediated signaling in the pathogenesis of inflammatory diseases. Cell Signal 25: 2335–2347. [PubMed: 23917206]

19. Zhang B, Liu C, Qian W, Han Y, Li X, and Deng J. 2014. Structure of the unique SEFIR domain from human interleukin 17 receptor A reveals a composite ligand-binding site containing a conserved alpha-helix for Act1 binding and IL-17 signaling. Acta Crystallogr D Biol Crystallogr 70: 1476–1483. [PubMed: 24816115]

20. Baranovski BM, Freixo-Lima GS, Lewis EC, and Rider P. 2015. T Helper Subsets, Peripheral Plasticity, and the Acute Phase Protein, alpha1-Antitrypsin. Biomed Res Int 2015: 184574. [PubMed: 26583093]

21. Rutz S, Eidenschenk C, and Ouyang W. 2013. IL-22, not simply a Th17 cytokine. Immunol Rev 252: 116–132. [PubMed: 23405899]

22. Lejeune D, Dumoutier L, Constantinescu S, Kruijer W, Schuringa JJ, and Renauld JC. 2002. Interleukin-22 (IL-22) activates the JAK/STAT, ERK, JNK, and p38 MAP kinase pathways in a rat hepatoma cell line. Pathways that are shared with and distinct from IL-10. J Biol Chem 277: 33676–33682. [PubMed: 12087100]

23. Eyerich K, Dimartino V, and Cavani A. 2017. IL-17 and IL-22 in immunity: Driving protection and pathology. Eur J Immunol 47: 607–614. [PubMed: 28295238]

24. Sugimoto K, Ogawa A, Mizoguchi E, Shimomura Y, Andoh A, Bhan AK, Blumberg RS, Xavier RJ, and Mizoguchi A. 2008. IL-22 ameliorates intestinal inflammation in a mouse model of ulcerative colitis. The Journal of clinical investigation 118: 534–544. [PubMed: 18172556]

25. Zenewicz LA, Yancopoulos GD, Valenzuela DM, Murphy AJ, Stevens S, and Flavell RA. 2008. Innate and adaptive interleukin-22 protects mice from inflammatory bowel disease. Immunity 29: 947–957. [PubMed: 19100701]

26. Zheng Y, Danilenko DM, Valdez P, Kasman I, Eastham-Anderson J, Wu J, and Ouyang W. 2007. Interleukin-22, a T(H)17 cytokine, mediates IL-23-induced dermal inflammation and acanthosis. Nature 445: 648–651. [PubMed: 17187052]

27. Fouser LA, Wright JF, Dunussi-Joannopoulos K, and Collins M. 2008. Th17 cytokines and their emerging roles in inflammation and autoimmunity. Immunol Rev 226: 87–102. [PubMed: 19161418]

28. van Horssen R, Ten Hagen TL, and Eggermont AM. 2006. TNF-alpha in cancer treatment: molecular insights, antitumor effects, and clinical utility. Oncologist 11: 397–408. [PubMed: 16614236]

29. Felquer ML, and Soriano ER. 2015. New treatment paradigms in psoriatic arthritis: an update on new therapeutics approved by the U.S. Food and Drug Administration. Curr Opin Rheumatol 27: 99–106. [PubMed: 25633241]

30. Jinesh S 2015. Pharmaceutical aspects of anti-inflammatory TNF-blocking drugs. Inflammopharmacology 23: 71–77. [PubMed: 25687751]

31. Krohn S, Nies JF, Kapffer S, Schmidt T, Riedel JH, Kaffke A, Peters A, Borchers A, Steinmetz OM, Krebs CF, Turner JE, Brix SR, Paust HJ, Stahl RAK, and Panzer U. 2018. IL-17C/IL-17 Receptor E Signaling in CD4(+) T Cells Promotes TH17 Cell-Driven Glomerular Inflammation. J Am Soc Nephrol 29: 1210–1222. [PubMed: 29483158]

32. Shinjo T, Iwashita M, Yamashita A, Sano T, Tsuruta M, Matsunaga H, Sanui T, Asano T, and Nishimura F. 2016. IL-17A synergistically enhances TNFalpha-induced IL-6 and CCL20

production in 3T3-L1 adipocytes. Biochem Biophys Res Commun 477: 241–246. [PubMed: 27311858]

33. Honda K, Wada H, Nakamura M, Nakamoto K, Inui T, Sada M, Koide T, Takata S, Yokoyama T, Saraya T, Kurai D, Ishii H, Goto H, and Takizawa H. 2016. IL-17A synergistically stimulates TNF-alpha-induced IL-8 production in human airway epithelial cells: A potential role in amplifying airway inflammation. Exp Lung Res 42: 205–216. [PubMed: 27269887]
34. Hartupee J, Liu C, Novotny M, Li X, and Hamilton T. 2007. IL-17 enhances chemokine gene expression through mRNA stabilization. J Immunol 179: 4135–4141. [PubMed: 17785852]
35. Friedrich M, Diegelmann J, Schauber J, Auernhammer CJ, and Brand S. 2015. Intestinal neuroendocrine cells and goblet cells are mediators of IL-17A-amplified epithelial IL-17C production in human inflammatory bowel disease. Mucosal immunology 8: 943–958. [PubMed: 25492478]
36. Liang SC, Tan XY, Luxenberg DP, Karim R, Dunussi-Joannopoulos K, Collins M, and Fouser LA. 2006. Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. J Exp Med 203: 2271–2279. [PubMed: 16982811]
37. Guilloteau K, Paris I, Pedretti N, Boniface K, Juchaux F, Huguier V, Guillet G, Bernard FX, Lecron JC, and Morel F. 2010. Skin Inflammation Induced by the Synergistic Action of IL-17A, IL-22, Oncostatin M, IL-1{alpha}, and TNF-{alpha} Recapitulates Some Features of Psoriasis. J Immunol 184: 5263–5270. [PubMed: 20335534]
38. Rabeony H, Petit-Paris I, Garnier J, Barrault C, Pedretti N, Guilloteau K, Jegou JF, Guillet G, Huguier V, Lecron JC, Bernard FX, and Morel F. 2014. Inhibition of keratinocyte differentiation by the synergistic effect of IL-17A, IL-22, IL-1alpha, TNFalpha and oncostatin M. PloS one 9: e101937. [PubMed: 25010647]
39. VanDussen KL, Marinshaw JM, Shaikh N, Miyoshi H, Moon C, Tarr PI, Ciorba MA, and Stappenbeck TS. 2015. Development of an enhanced human gastrointestinal epithelial culture system to facilitate patient-based assays. Gut 64: 911–920. [PubMed: 25007816]
40. Herjan T, Hong L, Bubenik J, Bulek K, Qian W, Liu C, Li X, Chen X, Yang H, Ouyang S, Zhou H, Zhao J, Vasu K, Cockman E, Aronica M, Asosingh K, Licatalosi DD, Qin J, Fox PL, Hamilton TA, Driscoll D, and Li X. 2018. IL-17-receptor-associated adaptor Act1 directly stabilizes mRNAs to mediate IL-17 inflammatory signaling. Nat Immunol 19: 354–365. [PubMed: 29563620]
41. Johansen C, Riis JL, Gedebjerg A, Kragballe K, and Iversen L. 2011. Tumor necrosis factor alpha-mediated induction of interleukin 17C in human keratinocytes is controlled by nuclear factor kappaB. J Biol Chem 286: 25487–25494. [PubMed: 21628458]
42. Yamanaka K, Fujisawa T, Kusagaya H, Mori K, Niwa M, Furuhashi K, Kono M, Hamada E, Suda T, and Maekawa M. 2018. IL-13 regulates IL-17C expression by suppressing NF-kappaB-mediated transcriptional activation in airway epithelial cells. Biochem Biophys Res Commun 495: 1534–1540. [PubMed: 29203240]
43. Fey D, Croucher DR, Kolch W, and Kholodenko BN. 2012. Crosstalk and signaling switches in mitogen-activated protein kinase cascades. Front Physiol 3: 355. [PubMed: 23060802]
44. Lee J, Rhee MH, Kim E, and Cho JY. 2012. BAY 11–7082 is a broad-spectrum inhibitor with anti-inflammatory activity against multiple targets. Mediators Inflamm 2012: 416036. [PubMed: 22745523]
45. Davies SP, Reddy H, Caivano M, and Cohen P. 2000. Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem J 351: 95–105. [PubMed: 10998351]
46. Kao DD, Oldebeken SR, Rai A, Lubos E, Leopold JA, Loscalzo J, and Handy DE. 2013. Tumor necrosis factor-alpha-mediated suppression of dual-specificity phosphatase 4: crosstalk between NFkappaB and MAPK regulates endothelial cell survival. Mol Cell Biochem 382: 153–162. [PubMed: 23812841]
47. Chen P, Hutter D, Yang X, Gorospe M, Davis RJ, and Liu Y. 2001. Discordance between the binding affinity of mitogen-activated protein kinase subfamily members for MAP kinase phosphatase-2 and their ability to activate the phosphatase catalytically. J Biol Chem 276: 29440–29449. [PubMed: 11387337]
48. Sands BE, and Kaplan GG. 2007. The role of TNFalpha in ulcerative colitis. J Clin Pharmacol 47: 930–941. [PubMed: 17567930]

49. Mizoguchi A, Yano A, Himuro H, Ezaki Y, Sadanaga T, and Mizoguchi E. 2018. Clinical importance of IL-22 cascade in IBD. J Gastroenterol 53: 465–474. [PubMed: 29075900]

KEY POINTS

- TNF-α induces IL-17C in colonic epithelial cells via an NF-κB mediated pathway.
- IL-17A and IL-22 synergistically increase IL-17C expression through p38 MAPK.
- Primary colonic spheroids from UC vs non-UC patients express IL-17C differently

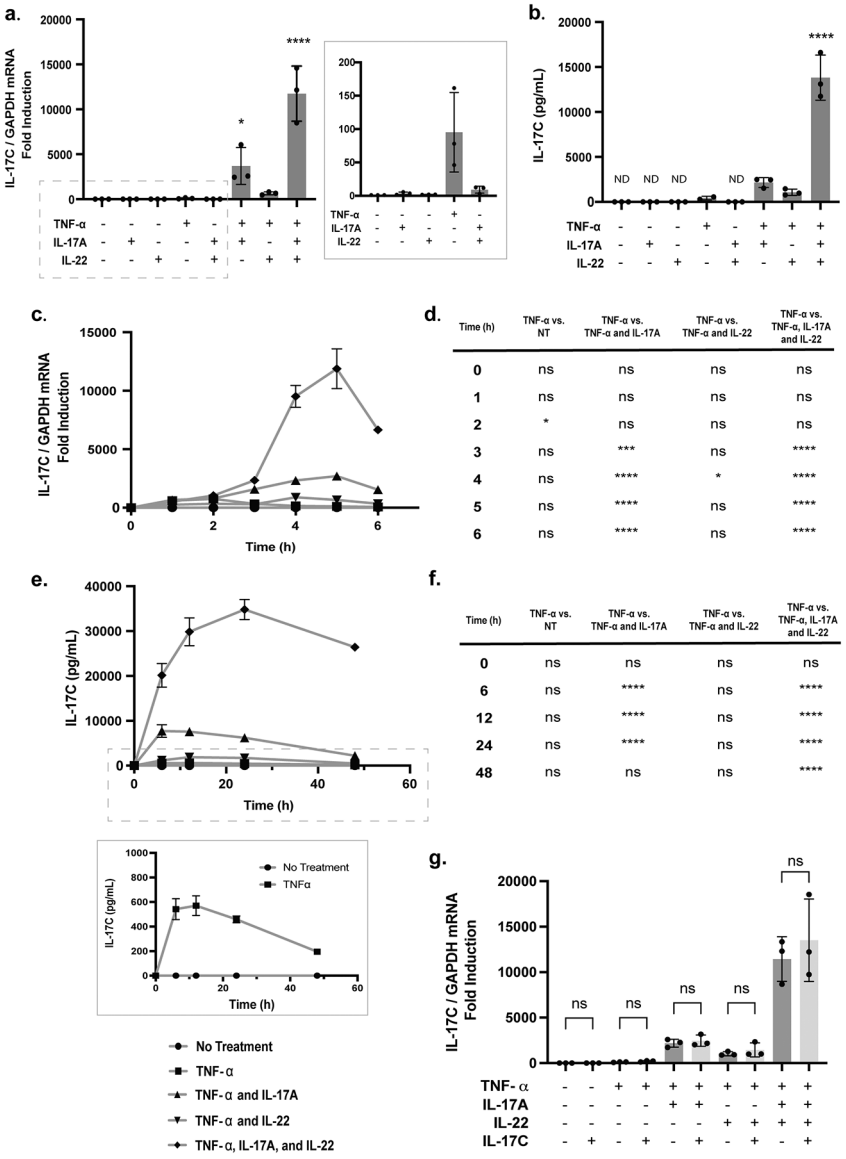

Figure 1. Th-17 derived cytokines TNF-α, IL-17A, and IL-22 synergistically stimulate the expression of IL-17C mRNA and protein in colonic epithelial cells.

HT-29 cells were treated with TNF-α (50 ng/mL), IL-17A (100 ng/mL), and IL-22 (10 ng/mL). RNA was extracted from cells after 5 h (**a**) or over a period of 6 h (**c**) and analyzed via RT-qPCR, where IL-17C fold induction ($2^{-\Delta\Delta CT}$) was controlled against GAPDH mRNA levels. Note that the panel in the gray box to the right of Fig. 1a is an expanded view of the first five treatment conditions. Conditioned media was harvested after 24 h (**b**) or over a period of 48 h (**e**) and tested for IL-17C protein via ELISA. The panel in the gray box below Fig. 1e is an expanded view of the no treatment and TNF-α stimulated conditions. HT-29 cells were treated with the given combinations of TNF-α (50 ng/mL), IL-17A (100 ng/mL), IL-22 (10 ng/mL), and IL-17C (100 ng/mL) for 5 h. RNA was extracted as described and fold induction was controlled against GAPDH (**g**). Statistical analysis was completed using a one-way ANOVA (a, b, and g) or two-way ANOVA (**c/d** and **e/f**) with Dunnett's multiple

comparisons test against TNF-α treatment alone. *, p<0.05; ***, p=0.0001; ****, p<0.0001. Each data point represents three separate experiments of biological replicates.

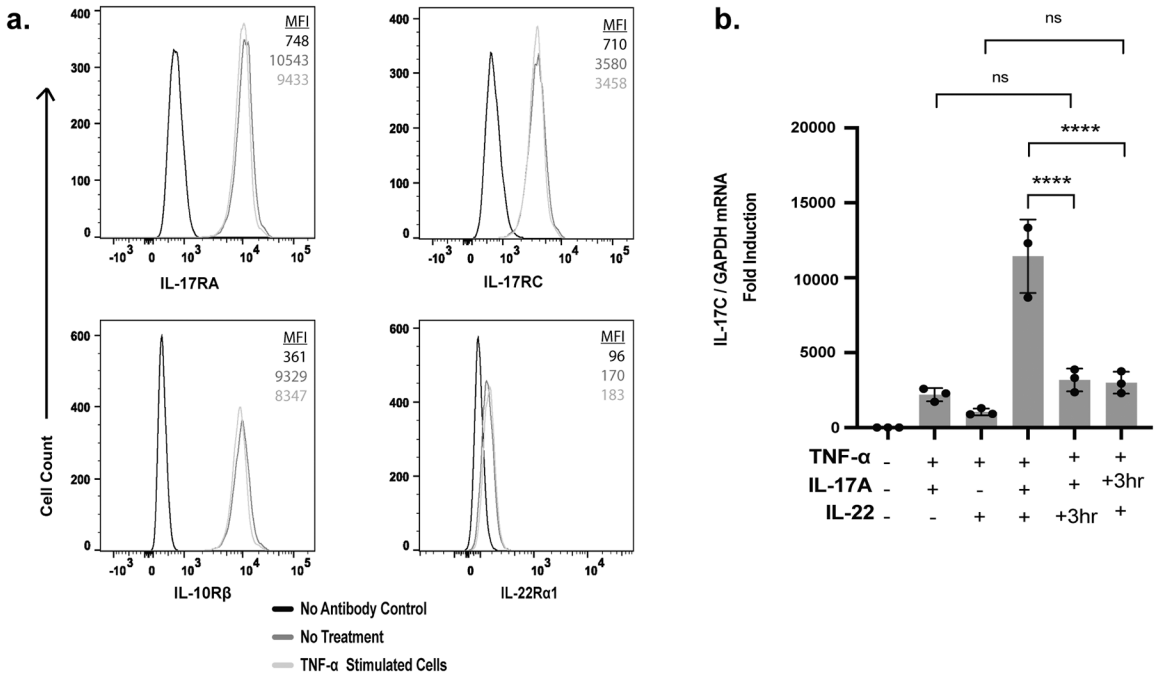

Figure 2. TNF-α stimulation does not prime cells to respond to IL-17A or IL-22.

Cells were stimulated with the stated combinations of cytokines for 3 h, and cells were prepared for flow cytometry analysis detecting IL-17RA, IL-17RC, IL-10Rβ, and IL-22Rα1 (**a**). Cells were stimulated with the stated combinations of cytokines for 5 h, or IL-17A or IL-22 were added 3 h after stimulation with TNF-α, as noted. RNA was extracted and analyzed by RT-qPCR, where IL-17C fold induction ($2^{-\Delta\Delta CT}$) was controlled against GAPDH mRNA levels (**b**). Statistical analysis was completed using a one-way ANOVA with Dunnett's multiple comparisons test as shown. ****, $p<0.0001$. Each data point represents three separate experiments of biological replicates.

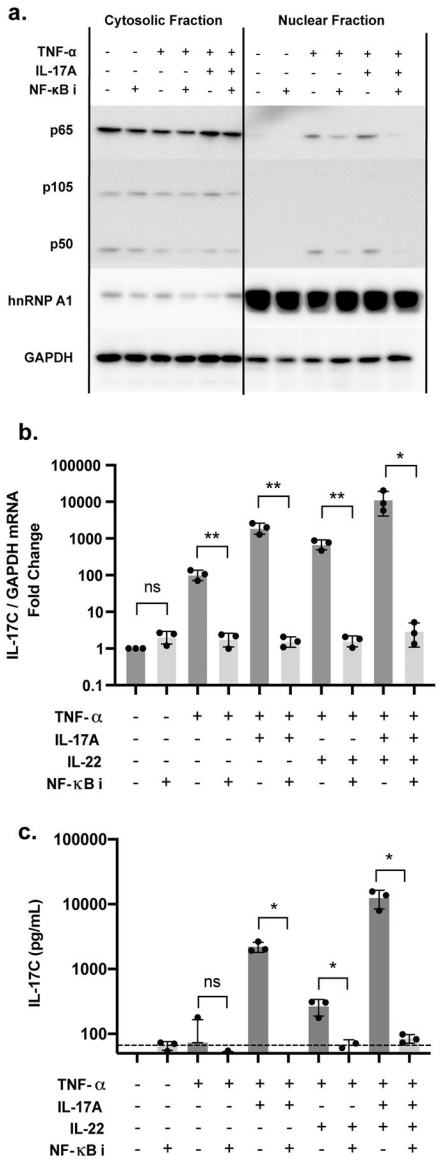

Figure 3. NF-κB activation is necessary for IL-17C expression.

Cells were pretreated with NF-κB inhibitor Bay 11–7082 (30 μM) or DMSO for 1 h, followed by addition of TNF-α, IL-17A, and IL-22 for 30 min. Immunoblot analysis was performed on nuclear and cytosolic fractions detecting p65, p50/105, hnRNP A1, and GAPDH (**a**). RNA (**b**) and conditioned media (**c**) were collected at 5 and 24 h, respectively, and analyzed as described in legend to Fig. 1. The horizontal dotted line in (**c**) denotes the limit of detection for the ELISA. Statistical analyses were completed using Welch's two tailed t tests. *, $p<0.05$; **, $p<0.01$. Each data point represents three separate experiments of biological replicates.

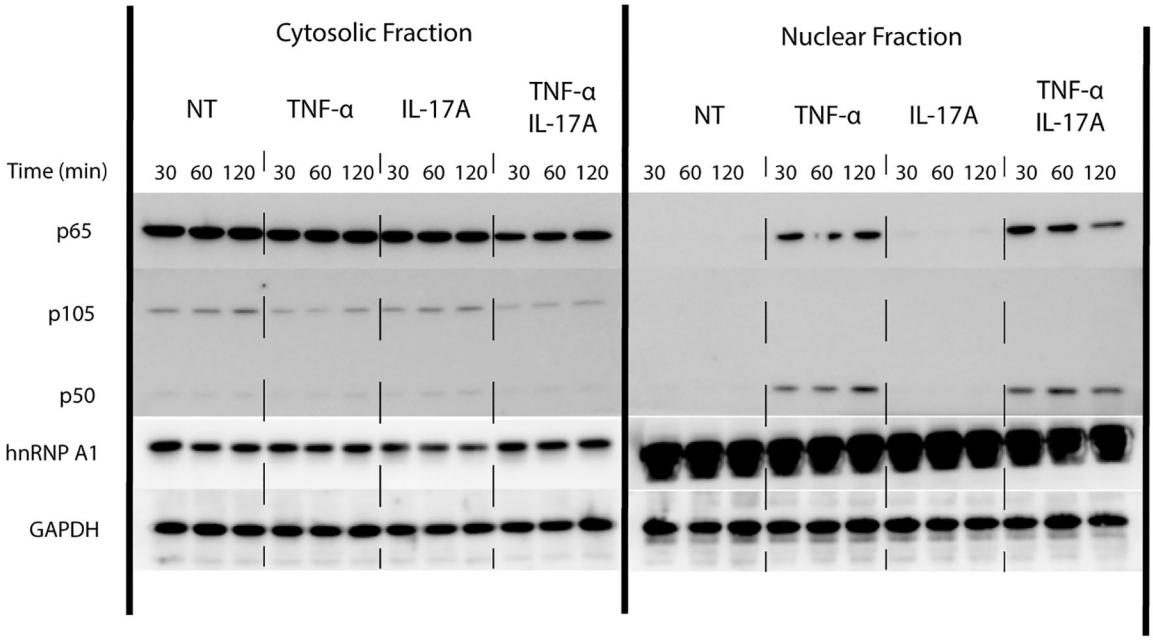

Figure 4: IL-17A does not increase the nuclear translocation of NF-κB subunits p65 or p50 above that of TNF-α alone.

Cells were treated with TNF-α and/or IL-17A for 30, 60, or 120 min. 20 μg of cell lysate were fractionated on a 12% polyacrylamide gel. Immunoblot analysis was performed on nuclear and cytosolic fractions detecting p65, p50/105, hnRNP A1, and GAPDH. Representative blot of 5 independent experiments.

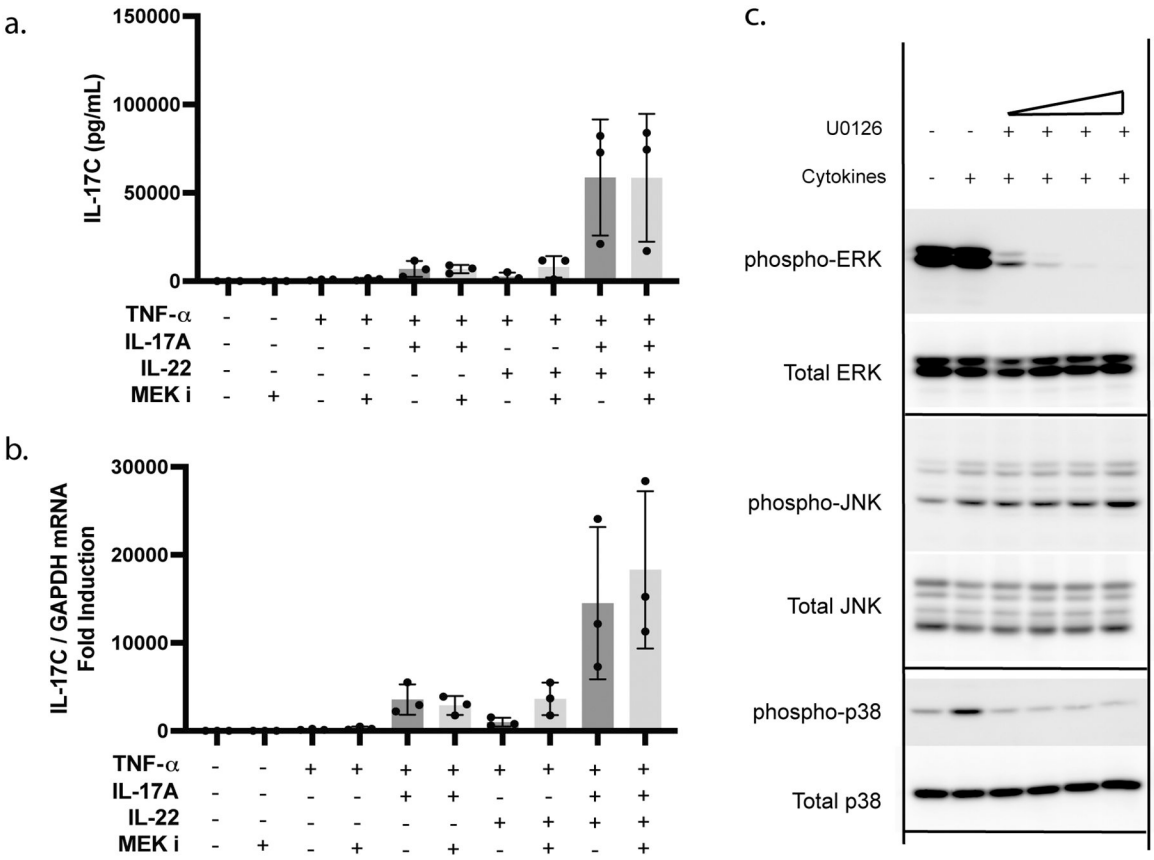

Figure 5: Synergistic IL-17C stimulation is not dependent on the MEK-ERK pathway.

Cells were pretreated with MEK inhibitor U0126 (100 μM) or DMSO for 1 h, followed by cytokine treatment. IL-17C protein at 24 h (**a**) and mRNA at 5 h (**b**) were quantified as previously described in the legend to Fig 1. Cell lysates were collected after a 1 h pre-treatment with U0126 at concentrations ranging from 3 μM – 300 μM and 15 min of TNF-α, IL-17A, and IL-22 stimulation. 20 μg of protein were separated on a 10% polyacrylamide gel, which was probed for phospho- and total ERK, JNK, and p38 (**c**). Statistical analysis in panels a and b performed using a one-way ANOVA demonstrated no significant differences between cells with or without U0126 pretreatment. Each data point represents three separate experiments of biological replicates.

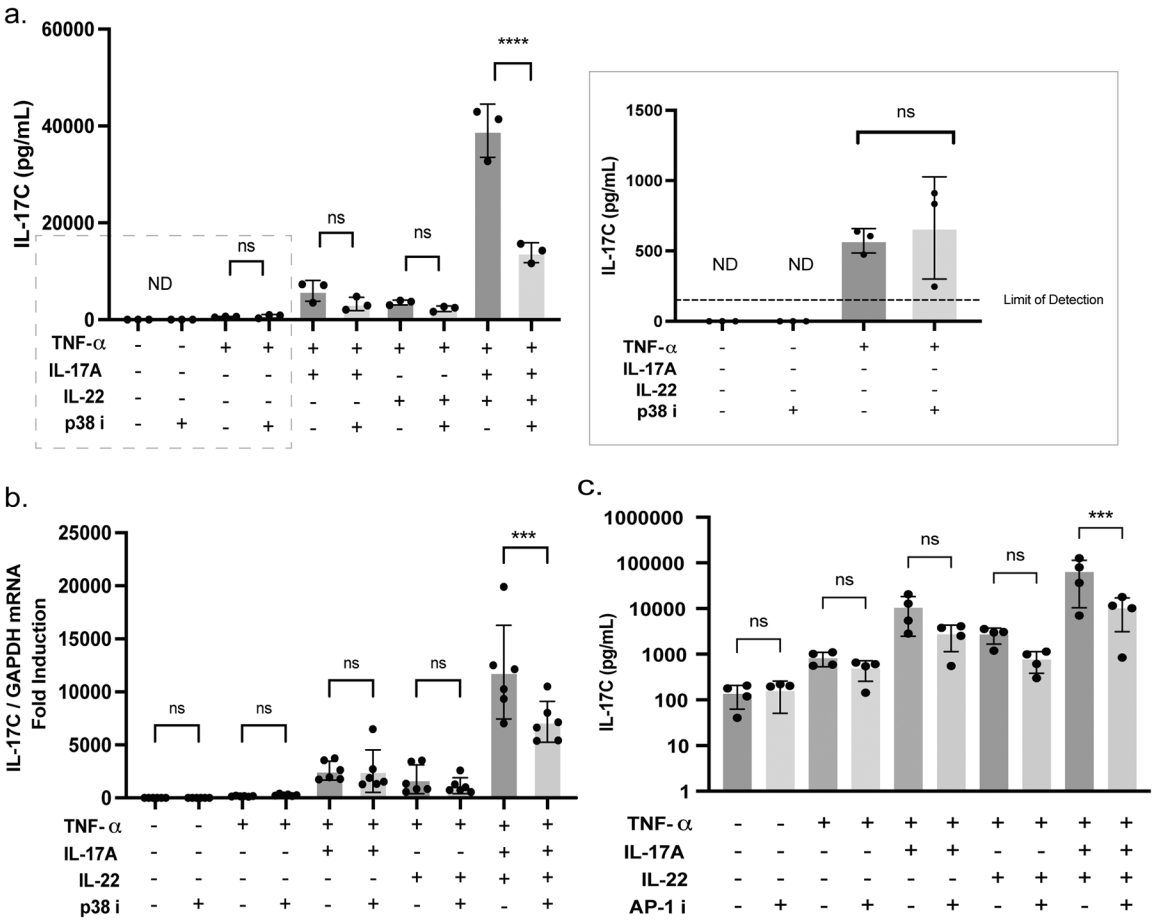

Figure 6: Synergistic induction of IL-17C expression is dependent on p38 and AP-1 activity.

Cells were pretreated with p38 inhibitor SB202190 (1 μM) (**a,b**), AP-1 inhibitor SR 11302 (50μM) (**c**), or DMSO for 1 h, followed by addition of cytokines, as noted. IL-17C protein at 24 h (**a**) and mRNA at 5 h (**b,c**) were quantified as previously described in the legend to Fig 1. The insert in Fig 6b is an expanded view of the first 4 treatment conditions. Statistical analysis was done using a one-way ANOVA with Dunnett's multiple comparisons. **, $p<0.01$; ****, $p<0.0001$. Each data point represents three separate experiments of biological replicates.

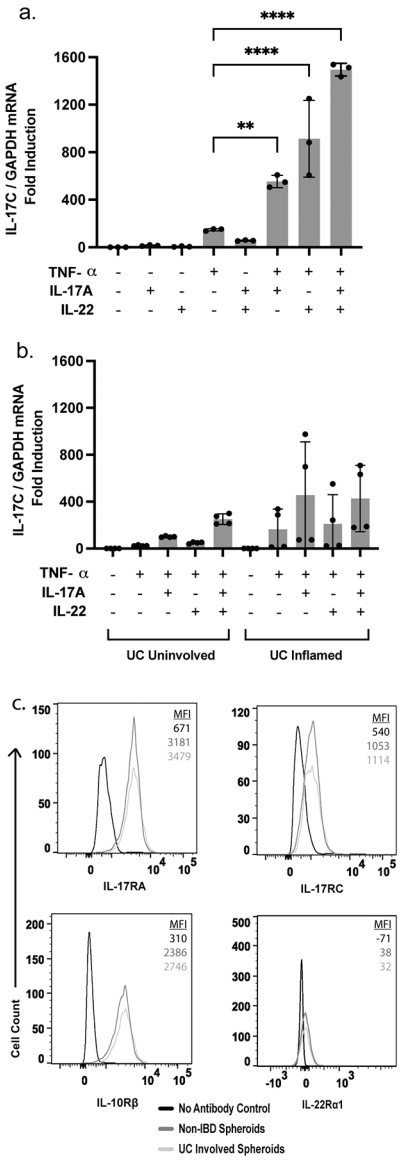

Figure 7: Primary non-IBD and UC uninvolved human colonic spheroids, but not UC involved cells, stimulated with Th17-derived cytokines synergistically induce IL-17C expression.

Primary human colonic spheroids from non-IBD affected individuals (**a**) and UC donors (**b**) were stimulated with TNF-α (50 ng/mL), IL-17A (100 ng/mL), and IL-22 (10 ng/mL). RNA was collected at 5 h, and IL-17C mRNA quantified as previously described in the legend to Fig 1. Statistical analysis was completed using a one-way ANOVA with Dunnett's multiple comparisons. **, p=0.0035; ****, p<0.0001. Each data point represents separate experiments of biological replicates for distinct spheroids derived from different donors. Spheroids from non-IBD individuals and UC donors were prepared for flow cytometry analysis detecting IL-17RA, IL-17RC, IL-10Rβ, and IL-22Rα1 (**c**).

Table 1:

Participant Demographics

Patient ID	Patient Diagnosis	Involved/Uninvolved tissue region	Age @ Access	Sex (Male/Female)	Race/Ethnicity
H292	N	n/a*	57	F	White
H262	N	n/a*	56	F	White
Hu303	UC	Uninvolved	N/A**	N/A**	N/A**
Hu252	UC	Uninvolved	18	M	Hispanic
Hu144	UC	Involved	37	F	White
Hu178	UC	Involved	50	M	White

(N= non-IBD/healthy; UC= ulcerative colitis; n/a*= Not applicable; N/A**= Not available; M= male; F=female)

Table 2:

Primer Sequences

Gene	Primer Sequence (5'->3')
GAPDH	AAATTGAGCCCGCAGCCT
	TAAAAGCAGCCCTGGTGACC
IL-17C	AGGTGTTGGAGGCAGACA
	CATCGATACAGCCTCTGCAC
CXCL1	CACACTCAAGAATGGGCGGAAAG
	CCTCTGCAGCTGTGTCTCTCTT