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Intestinal Epithelial Cells Express Immunomodulatory ISG15 During Active Ulcerative Colitis and Crohn's Disease

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Abstract

Background and Aims: Intestinal epithelial cells [IECs] secrete cytokines that recruit immune cells to the mucosa and regulate immune responses that drive inflammation in inflammatory bowel disease [IBD]. However, experiments in patient-derived IEC models are still scarce. Here, we aimed to investigate how innate immunity and IEC-specific pattern recognition receptor [PRR] signalling can be involved in an enhanced type I interferon [IFN] gene signature observed in colon epithelium of patients with active IBD, with a special focus on secreted ubiquitin-like protein ISG15.

Methods: Gene and protein expression in whole mucosa biopsies and in microdissected human colonic epithelial lining, in HT29 human intestinal epithelial cells and primary 3D colonoids treated with PRR-ligands and cytokines, were detected by transcriptomics, *in situ* hybridisation, immunohistochemistry, western blots, and enzyme-linked immunosorbent assay [ELISA]. Effects of IEC-secreted cytokines were examined in human peripheral blood mononuclear cells [PBMCs] by multiplex chemokine profiling and ELISA.

Results: The type I IFN gene signature in human mucosal biopsies was mimicked in Toll-like receptor TLR3 and to some extent tumour necrosis factor [TNF]-treated human IECs. In intestinal biopsies, ISG15 expression correlated with expression of the newly identified receptor for extracellular ISG15, LFA-1 integrin. ISG15 was expressed and secreted from HT29 cells and primary 3D colonoids through both JAK1-pSTAT-IRF9-dependent and independent pathways. In

experiments using PBMCs, we show that ISG15 releases IBD-relevant proinflammatory cytokines such as CXCL1, CXCL5, CXCL8, CCL20, IL1, IL6, TNF, and IFNγ.
Conclusions: ISG15 is secreted from primary IECs upon extracellular stimulation, and mucosal ISG15 emerges as an intriguing candidate for immunotherapy in IBD.

Key Words: Human intestinal organoids; innate immunity; ISG15

1. Introduction

Recent evidence suggests that the intestinal epithelium contributes to the development and perpetuation of inflammation in the inflammatory bowel diseases [IBD], ulcerative colitis [UC], and Crohn's disease [CD].[1,2] In addition to having barrier functions, intestinal epithelial cells [IECs] act as both sensors for pathogen- or damage-associated molecular patterns [PAMPs or DAMPS] and as regulators of immune cells.[3,4] Binding of exogenous PAMPs or endogenous DAMPS to pattern recognition receptors [PRRs], such as Toll-like receptors [TLRs], initiate a number of intracellular signalling cascades via, for example, the transcription factor complexes nuclear factor kappa-light-chain-enhancer of activated B cells [NF-κB], mitogen-activated protein kinases [MAPKs], or interferon regulatory factors [IRFs]. Activation of NF-κB and MAPKs in IECs induces secretion of interleukins and chemokines, whereas IRFs are more involved in stimulation of interferon [IFN] production and induction of IFN-stimulated genes [ISGs].[5,6] IFNs are classified into three types: type I [13 subtypes of IFNα, single members of IFNβ κ, ω, ε, δ, and τ], type II [IFNγ], and type III [IFNλ1, IFNλ2, and IFNλ3].[7]

Upon external stimuli, IECs have potential to secrete chemokines that can both recruit immune cells and directly induce secretion of inflammatory cytokines that augment and prolong inflammatory responses. For example, C-X-C motif chemokine ligand 8 [CXCL8], secreted from IECs and immune cells, is considered to be a major chemotactic factor which can attract, for example, CXCR1[+] CXCR2[+]IL-23-producing neutrophils that infiltrate and accumulate in inflamed colon tissue.[8] Previous work from our group has shown that extracellular stimulation of TLR3 can mediate release of immunomodulatory C-X-C motif chemokine ligand 10 [CXCL10][9] and chemokine [C-C motif] ligand 20 [CCL20][10] from colonic epithelial cells. A recent study showed that the human colonic epithelial cell line NCM460 transfected with the TLR3 ligand poly[I:C] released exosomes containing canonical type I IFN-induced antiviral ISGs such as ubiquitin-like protein ISG15[11]. To our knowledge, no study regarding release of ISG15 from primary human IECs upon external stimuli has been reported.

ISG15 exists in three forms: conjugated to proteins [ISGylation], unconjugated intracellular, and secreted free forms.[12] There appears to be functional diversity between species,[13,14] and in humans several immunomodulatory functions of secreted free ISG15 have been postulated, including chemotaxis of neutrophils,[15] recruitment of IL1β-producing CD8α+ dendritic cells,[16] and release of IFNγ[14,17,18] and IL10[19,20] from lymphocytes. Recently, Swaim *et al.*[19] showed that extracellular ISG15 regulates IFNγ and IL10 secretion from the natural killer [NK] cell line NK-92 through the leukocyte function-associated antigen-1 [LFA-1] integrin receptor. The LFA-1 has two subunits, CD11a[αL integrin] and CD18[β2 integrin], encoded by the *ITGAL* and *ITGB2* genes, respectively. Swaim *et al.*[19] suggest that direct binding of ISG15 to CD11a signals and activates SRC family kinases [SFKs], leading to secretion of IFNγ and IL10. In their model, IL12 drives cytokine expression and ISG15 enhances cytokine secretion.

IEC-derived cytokines that augment and prolong inflammatory responses during active IBD are potential targets for immunotherapy. Gene lists in omics studies from our group[21] and others[22–24] show enhanced expression of *ISG15* mRNA in active IBD and in experimental murine colitis. Even though ISG15 may boost mucosal immune activity, ISG15 has not been investigated in the context of primary colonic epithelial cells and IBD. Here, we show increased expression of canonical type I IFN-induced ISGs in IECs during active inflammation. We study how IEC-specific PRR-signalling can contribute to the type I IFN signature found, and demonstrate that extracellular ISG15 has cytokine-like activities that can modulate immune functions in IBD.

2. Methods

2.1. Ethics

The study was approved by the Regional Committee for Medical and Health Research Ethics [reference numbers 5.2007.910 and 2013/212/REKmidt] and was registered in the Clinical Trials Protocol Registration System [identifier NCT00516776]. All subjects included in the study gave informed written consent.

2.2. Clinical material

Patients admitted to St Olav's University Hospital, Trondheim, Norway, for colonoscopy were included after informed consent. They were diagnosed with UC or CD or underwent colonoscopy due to gastrointestinal symptoms with no significant pathology being found. Patients using immunomodulants such as azathioprine or tumour necrosis factor]TNF]-blockers were excluded. Colonic pinch biopsies [Sample set I] and peripheral blood mononuclear cells [PBMCs] [Sample set II], were collected as described previously.[21,25]

2.2.1. Sample set I

The colonic pinch biopsies were from a previously described cross-sectional study biobank[21] including clinical information, blood fractions, and tissue samples. Haematoxylin and eosin-stained sections of all biopsies were examined by an experienced pathologist and classified into normal, inactive, or active inflammation.

2.2.2. Sample set II

PBMCs from non-IBD controls [n = 7], inactive CD [CDi, n = 8], and inactive UC [UCi, n = 9], and from active CD [CDa, n = 8] and active UC [UCa, n = 7], were randomly selected from Sample set I, as described.[25] PBMCs isolated from buffy coat from six healthy blood donors at the Department of Immunology and Transfusion Medicine, St Olav's University Hospital, were included for additional functional assays, as described below.

2.3. PRR-ligands, cytokines, neutralising antibodies, and inhibitors used in the stimulation experiments

The PRR-ligands used were: the lipopeptide Pam3CysSK4 [P3C] [TLR2/1] [300 ng/mL] [#L2000, EMC microcollections], Lipomannan

[LM] [TLR2/6] [30 ng/mL] [#tlrl-hkmt-1], polyinosinic:polycytidylic acid (poly[I:C) [TLR3] [5–70 µg/mL] [#tlrl-pic], lipopolysaccharide [LPS] [TLR4] [100 ng/mL] [#tlrl-peklps], Flagellin [TLR5] [100 ng/mL] [#tlrl-stfla], the antiviral compound R848 [TLR7/8] [100 ng/mL] [#tlrl-r848], the peptidoglycan component muramyl dipeptide [MDP] [NOD2] [1 µg/mL] [#tlrl-mdp], all from InvivoGen, and unmethylated CpG dinucleotides [TLR9] [10 µM] [# 1712649, TibMolBiol].

Recombinant cytokines used were: IL-10 [100 ng/mL] [#200–10], IL-1β [100 ng/mL] [#200-01B], TNF [100–200 ng/mL] [#300-01A], and IFNβ [0.1–10 ng/ml] [#300-02BC] [all from PeproTech], IL12 [20 ng/mL] [#219-IL, R&D], and ISG15 [500 ng/ml] [#12729HNAE, Thermo Fisher Scientific]. JAK-inhibitors used were: filgotinib [10 µM] [GLPG0634] [#S7605, Selleckchem.com] and ruxolitinib [10 µM] [#tlrl-rux, InvivoGen]. Neutralising antibodies used were: rabbit anti-human IFN-β [2.5 µg/ml] [#500-P32B, PeproTech] and isotype control rabbit IgG [2.5 µg/ml] [#X0903, Dako Cytomation]. For details, see Supplementary Table 1, available as Supplementary data at *ECCO-JCC* online.

2.4. Experiments in HT29 human intestinal epithelial cells

HT29 cells [#HTB-38, ATCC, Manassas, VA, USA] were cultured at 37°C, 5% CO_2 in RPMI supplemented with 10% fetal bovine serum, 2 mM glutamine and 0.05% gentamicin. Stimulation experiments were performed on confluent cells in 12-well plates. The day before stimulation, the cells were incubated overnight in serum-depleted medium. Medium was then removed and fresh medium with 2–10% fetal bovine serum and PRR-ligands and cytokines were added for the time indicated in the different experiments.

2.5. Experiments in human 3D colonoids

Human 3D colonoid cultures were established from colonic pinch biopsies taken from three non-IBD healthy controls, using an optimised protocol from Mahe *et al*.[26] based on Jung *et al*.[27] Briefly, isolated crypt pellets were resuspended in basement membrane matrix [Corning® Matrigel GFR, #734–1101, Corning, NY, USA], and 50 µl of crypt suspension was added to each well in pre-warmed 24-well plates. The crypts were incubated with complete medium, composed of 50% Wnt-3A conditioned medium [#ATCC® CRL-2647™] and 50% Advanced DMEM/F12 [#12634028, Thermo Fisher Scientific, Bremen, Germany], containing recombinant human r-spondin [1 µg/ml] [#120–38], recombinant human noggin [0.1 µg/ml] [#120-10C] from PeproTech [Rocky Hill, NJ, USA], and other factors critical for stem cell growth, as described.[26] For establishment of colonoids from crypts, the complete medium was supplemented by the GSK3 inhibitor [Wnt-activator] CHIR99021 [0.86 µg/ml] [#72052], and thiazovivin [0.778 µg/ml] [#72252], an inhibitor of rho-associated coiled-coil containing protein kinase [ROCK], both from STEMCELL Technologies.

Undifferentiated cultured colonoids were passaged and expanded every 7 to 10 days after plating, or frozen for biobanking, as described.[26] Before experiments, approximately 8000 cells were cultured in basement membrane matrix [Corning® Matrigel GFR] and grown in complete medium, with the selective ROCK-inhibitor Y-27632 [3.203µg/ml] [#1254, Bio-Techne] added for the first 3 days. Medium was changed every 2–3 days, and the undifferentiated colonoid cultures were differentiated at Day 7 by reducing the Wnt-3A-conditioned medium concentration from 50% to 5%, removing nicotinamide [1221.2 µg/ml] [#N3376-100G, MerckMillipore] and the MAPK inhibitor SB202190 [3.314 µg/ml] [#S7067, Sigma-Aldrich, Missouri, USA], and adding the pan-notch inhibitor DAPT [4.324 µg/ml] [#2634, Bio-Techne] to the medium. Experiments were performed after 4–5 days of differentiation. A-83-01 [0.211 mg/ml] [#SML0788, Sigma-Aldrich, Missouri, USA], an inhibitor of TGFβ type I activin receptor-like kinase [ALK5] was removed during stimulation experiments.

2.6. Experiments in PBMCs

Human PBMCs [Sample set II] were prepared and thawed as previously described.[25] For stimulation experiments, recombinant human ISG15 [500 ng/mL] and/or recombinant human IL12 [20 ng/mL] were added for 48 h before conditioned medium was harvested and stored at -80°C.

2.6.1. Flow cytometry

PBMC subpopulations in 35 samples [Sample set II] had previously been identified by flow cytometry analysis,[25] and the data were used for correlation analyses in the present study. Detailed description of antibody staining and compensation controls are given in Skovdahl *et al*.[25] Flow cytometry was performed on a BD FACS Canto II flow cytometer with FACS Diva software from BD Bioscience [10 000 events per sample] and samples were analysed using FlowJo v10 software from Flow Jo, LLC.

2.7. Enzyme-linked immunosorbent assay and multiplex chemokine profiling

CircuLex Human ISG15 ELISA Kit [#CY-8085, Nordic Biosite]; human IFNγ [#88-7316-86, Invitrogen, Thermo Fisher Scientific, Bremen, Germany]; TNF [#DY210], CXCL10 [#DY266], CCL20 [#DY360] and CXCL8 [#DY208]; all [R&D]; were used according to the manufacturers' protocol. Cytokines in conditioned medium from stimulated PBMCs were detected and analysed by Bio-Plex Pro™ Human Chemokine Panel, 40-Plex [#171ak99mr2, Bio-Rad laboratories] according to the manufacturer's protocol. The Limulus Amebocyte Lysate [LAL]-assay [#50-647U, LONZA Walkersville, MD USA, QCL-100TM] was performed according to the manufacturer's instructions to confirm that the levels of endotoxin were well below 1 EU [<0.1 ng] per µg protein, as stated by the suppliers of both recombinant IL12 and ISG15. Nevertheless, since even 0.02 ng/ml LPS has been shown to significantly stimulate small amounts of cytokine production,[28] we defined cytokine release to be modulated by ISG15 only if the enhanced cytokine concentration in conditioned medium was above 1000 pg/ml compared with untreated control in the multiplex assay.

2.8. RNA extraction and gene expression analysis

The following kits were used for RNA extraction, according to the manufacturers' protocol: Ambion *mir*Vana mRNA isolation kit [Applied Biosystems] [human biopsies], RNeasy micro plus [#74034, Qiagen] [microdissected epithelial monolayer], RNeasy Mini kit [#74106, Qiagen] [HT29 cells and colonoids], and RNeasy mini kit and QiaShredder columns [#ID:79654, Qiagen] [PBMCs].

Microarray gene expression analysis of the human colonic biopsies [Sample set I] has previously been described.[21] A subset [n = 29] with inflamed, active IBD [n = 12:5 CDa + 7 UCa], uninflamed IBD [n = 11:5 CDi + 6 UCi], and non-IBD controls [n = 6] from Sample set I was used for RNA sequencing of laser capture microdissected [LCM] epithelial monolayer. The microdissection was performed on an MMI CellCut LCM microscope [MMI CellCut on Olympus IX71]. Samples were mounted in Tissue-Tek O.C.T compound embedding medium [Sakura Finetek, Europe]. After a period of

30 min in the cryotome [Leica CM3050 S, Leica], 10-µm cryosections were cut and mounted on a pre-chilled LCM membrane slide. The slide was briefly exposed to room temperature to let sections adhere and was placed in chilled 100% EtOH [30 s]. Before LCM, samples were stained and de-humidified with H_2O + ProtectRNA RNAse inhibitor [#R7397, Sigma-Aldrich, Missouri, USA] [2 × 5 s], Histogene staining solution [# KIT0401, Life Technologies] [30 s], 100% EtOH [3 × 30 s], and xylene [5 min], and left for desiccation in a vacuum chamber containing silica gel [30 min]. Following the pre-processing steps, an area corresponding to approximately 10 000 epithelial cells [1 mm^2] underwent LCM, was immediately lysed by adding 300 µL lysis buffer, and was stored at -80°C. The RNA sequencing [RNASeq] was done on libraries prepared using Illumina TruSeq RNA access library kit [Illumina, Inc., San Diego, CA, USA] according to the manufacturer's protocol.

Microarray gene expression analysis of HT29 cells was performed using Illumina human HT-12 expression BeadChips [Illumina, Inc., San Diego, CA, USA] on an Illumina BeadStation, following standard protocols.

For RNASeq of human 3D colonoids, RNASeq libraries were generated using SENSE total RNASeq library prep kit [with RiboCop rRNA depletion] according to manufacturer's instructions [Lexogen GmbH]. Single-read sequencing was performed for 75 cycles on a NextSeq 500 instrument [Illumina]. Base calling was done on the NS500 instrument by RTA 2.4.6. FASTQ files were generated using bcl2fastq2 Conversion Software v2.17 [Illumina, Inc., San Diego, CA, USA].

For real-time quantitative polymerase chain reaction [RT-qPCR] of *ITGAL* and *ITGB2* expression in PBMCs [Sample set II], RNA extraction and reverse transcription were done as previously desrcibed.[25] cDNA was analysed for LFA-1 subunits *ITGAL* and *ITGB2* transcripts by quantitative real-time PCR using Taqman probes [*ITGAL*: probe ID Hs_00158218_m1; *ITGB2*: probe ID Hs_00164957_m1] with the reference genes *beta actin* [ACTB: probe ID Hs01060665_g1], *TATA box binding protein* [TBP: probe ID Hs00427620_m1] and *eukaryotic 18S* rRNA [18S: probe ID Hs99999901_s1] [Applied Biosystems]. PerfeCTa assay with PerfeCTa qPCR FastMix [Quantabio], and StepOnePlus™ Real-Time PCR System and StepOne™ software v2.1 [Applied Biosystems] were used for all PCR procedures.

2.9. Bioinformatics and Gene set enrichment analysis

In general, expression analysis was done in the R software environment for statistical computing.[29] Analysis of the microarray gene expression data from Sample set I has been previously described.[21] For gene expression analysis in colonic pinch biopsies, LCM epithelial monolayer, and colonoids, differential expression between groups was identified using LIMMA linear models with least squares regression and empirical Bayes moderated t statistics.[30] Temporal gene expressions in treated and untreated HT29 cells were identified using LIMMA linear models with least squares regression and empirical Bayes moderated nested F statistics[30]; *p*-values were adjusted for multiple comparisons using the Benjamini-Hochberg false-discovery rate correction [FDR]. Enrichment analyses were done by MetaCore™ version 6.34 build 69200.

2.10. Western blotting

Cells were washed twice in ice-cold PBS before they were lysed for 2 h on ice in lysis buffer [50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 1 mM DTT, 1 x Complete® EDTA-free protease inhibitor, and 1 x phosphatase inhibitor cocktail I and III, respectively [Sigma-Aldrich, Missouri, USA]. Protein lysate was obtained by centrifugation at 14 000 × *g* for 20 min at 4°C, and protein concentration was measured using the Bradford protein assay [Bio-Rad]. Conditioned medium from HT29 cells was concentrated from 500 µL to 50 µL using Amicon® ultra-0.5 centrifugal filter devices, 10 000 NMWL [Millipore], according to the manufacturer's recommendations.

Samples were denatured in 1 × NuPage LDS sample buffer supplemented with 40 mM DTT for 10 min at 70°C before they were separated on 4–12% NuPage Bis-Tris gels [Invitrogen] and electroblotted onto Immobilon PVDF membranes [Millipore]. The membranes were blocked using 5% BSA in PBS/0.01% Tween 20 [PBS-T] and incubated with primary antibodies as listed in Supplementary Table 2, available as Supplementary data at *ECCO-JCC* online. For visualisation, the blots were incubated with IRDye 800CW goat anti-mouse IgG [1:20 000, LI-COR, LCR-926–32210] or with horseradish peroxidase [HRP] conjugated swine anti-rabbit IgG and HRP-conjugated rabbit anti-mouse IgG [both 1:5000, Dako Cytomation] developed with Super Signal West Femto substrate [Pierce]. Images were obtained with LI-COR Odyssey Fc and analysed using Image Studio Software [LI-COR].

2.11. Immunohistochemistry and *in situ* hybridisation of human biopsies and colonoids

The human colonic biopsies were fixed in 10% buffered formalin for 3–6 days before embedding in paraffin. For fixation of human colonoids, the Matrigel was depolymerised using Cell Recovery Solution, and the colonoids were washed with PBS and centrifuged [500 × *g*] three times. The colonoid pellets were either resuspended in 50 µl Richard-Allan Scientific™ HistoGel™ Specimen Processing Gel [# HG-4000–012, Thermo Fisher Scientific] or small amounts [1:5 µl] of plasma and thrombin that was allowed to coagulate before the pellets were fixed in formalin for 48 h and embedded in paraffin. The formalin-fixed, paraffin-embedded [FFPE] biopsies or colonoids were cut into 4-µm sections before standard pretreatments and staining with haematoxylin and eosin.

FFPE sections were processed through standard de-paraffinisation and quenching of endogenous peroxidase before immunohistochemistry [IHC]. Antigen retrieval was achieved by 15 min boiling in citrate buffer [pH 6.0] [a-ISG15, E-cadherin] or Tris-EDTA buffer [pH 9.0] [a-pSTAT, a-CD11a, a-FABP1, a-MUC2, a-CgA] using a commercial microwave. All sections were incubated overnight at 4°C with primary antibodies diluted in PBS with 0.25% Triton-X and 0.25% BSA. Primary antibodies and dilutions are listed in Supplementary Table 2. Immunoreactions were visualised using the rabbit/mouse EnVision-HRP/DAB+ kit [#K5007, Dako] and counterstaining with haematoxylin. Negative controls were omission of primary antibody and similar concentration of identical non-immunised IgG.

In situ hybridisation [ISH] was performed with the RNAscope 2.5 HD Reagent Kit [Brown] for FFPE tissue [#322300] and human *ISG15* probe [#467741] [Advanced Cell Diagnostics, Inc.,] according to the manufacturer's instructions. Images were captured using Nikon E400 microscope, DS-Fil U2 camera, and NIS-Elements BR imaging software [Nikon]. Further processing was done using Fiji.[31]

2.12. Statistical analyses

Except for the global gene expression analyses described in Methods section 2.9, statistical analyses were performed in GraphPad Prism 8.0 [GraphPad Software, Inc., San Diego, CA, USA]. For numerical values and log2 transformed data, differences between groups were

evaluated by analysis of variance [ANOVA] with multiple comparison tests, as indicated in the figure legends. Briefly, Dunnett's test was used when comparing multiple treatments versus a control, Tukey test when comparing every treatment with every other treatment, and Šídák test when selected groups were compared. Pearson's correlation coefficient or Spearman rank correlation was used to examine correlations; p <0.05 was considered significant for all analysis.

2.13. Data availability

Transcriptome analysis of Sample set I is available at Array Express E-MTAB-184. Selected gene expression data are shown in Supplementary Files 1–4, available as Supplementary data at *ECCO-JCC* online. The authors declare that all data supporting the results presented in this study are available within the article and supplementary material or are available from the corresponding author upon reasonable request.

3. Results

3.1. Expression of type I IFN signature genes is increased in IECs from patients with active IBD

Exploration of an in-house microarray gene expression dataset on well-characterised and controlled colon pinch biopsies from non-IBD controls [n = 20] and inactive and active UC [n = 81] and CD [n = 26][21] revealed that several canonical ISGs were significantly upregulated in mucosa during active disease [Figure 1 and Supplementary File 1]. We could not detect upregulation of *IFNα* or *IFNβ* mRNAs in mucosa from IBD patients compared with non-IBD controls [Supplementary File 1]. However, significantly enhanced expression of signal transducer and activator of transcription 1 and 2 [*STAT1*, *STAT2*] and IFN-regulatory factors 1 and 9 [*IRF1*, *IRF9]* in both active UC [n = 37] and CD [n = 7] indicated induction of type I IFN signalling pathways during active disease. Signalling through interferon *α/β* receptor [IFNAR] activates Janus kinase 1 [JAK1] and tyrosine kinase 2 [TYK2] which phosphorylate STAT1 and STAT2. STAT1/STAT2 heterodimer can interact with IRF9 to form IFN-stimulated gene factor 3 [ISGF3] which binds to IFN-stimulated response DNA element [ISRE] and induces transcription of ISGs.[32] The upregulated ISGs shown in Figure 1 have been reported to be strongly enriched for the ISGF3-binding ISRE motif.[33,34]

To examine whether the ISGs were produced by IECs, we analysed an in-house RNASeq-dataset showing gene expression in laser-captured, microdissected, epithelial monolayer from inflamed, active IBD [n = 12*]* versus uninflamed IBD [n = 11] and non-IBD controls [n = 6]. Several type I IFN genes [*IFNAR2*, *STAT1*, *IRF1*, *ISG15*, *ISG20*, *IFI16*, *IFIT3*, *IFITM1*, *IFITM2, IFITM3*, *OAS2*, *OAS3*, and *USP18*] were upregulated also in the separated IECs during active IBD [Figure 2A and Supplementary File 2].

Since ISG15 in particular has potential to boost IBD-relevant immune activities,[12,19] we proceeded to examine localisation of pSTAT1, ISG15 proteins, and the ISG15 binding receptor unit CD11a [*ITGAL*][19] in colon mucosa biopsies from non-IBD controls and active IBD by IHC [Figure 2B–D]. Although we observed increased levels of pSTAT1 and ISG15 in the colonic epithelium monolayer from active IBD compared with non-IBD controls, the newly identified receptor subunit for extracellular ISG15, CD11a,[19] was mainly restricted to lamina propria cells. This is in line with the gene expression data from microdissected colonic epithelial lining [Figure 2A]. The IEC localisation and increased expression of ISG15 in active UC and CD were confirmed by ISH examining *ISG15* mRNA [Figure 2E].

Since ISG15 can enhance IL12-induced IFNγ release[14,17,18] and IFNγ is upregulated in mucosa during active IBD,[9] we analysed correlation between expression of *ISG15*, *IFN*γ, and the LFA-1 receptor units *ITGAL* and *ITGB2* in mucosa from non-IBD controls [n = 20] and inactive [n = 65] and active IBD [n = 49]. We found a significant correlation between *ISG15* and *IFN*γ mRNA expression in inactive [r = 0.34, p = 0.0064] and active [r = 0.5, p <0.0001] IBD that was not observed in controls [r = 0.17, p = 0.463] [Figure 2F]. Expression of *ISG15* mRNA correlated strongly with both *ITGAL* [Figure 2G] and *ITGB2* [Figure 2H] mRNA when analysing all individuals [n = 134]. Overall, this indicated a possible correlation between ISG15 expression in IECs, expression of LFA-1 in immune cells, and enhanced expression of IFNγ in mucosa during active IBD.

3.2. Poly[I:C] and TNF activate type I IFN signalling pathway and successive regulation of ISG15 in HT29 human intestinal epithelial cells

To investigate whether innate immunity and IEC-specific PRR-signalling could contribute to type I IFN signalling pathway and regulation of ISG15 in IECs during IBD, we first performed a transcriptome time-series screen in colonic epithelial HT29 cells treated with TLR1-9 ligands, NOD2-ligand, IL10, IL1β, or TNF. HT29 cells showed strongest response to TNF, IL1β, poly[I:C] [TLR3], flagellin [TLR5], and LPS [TLR4], as demonstrated by induction of mRNAs coding for the IBD-related chemokines *CXCL8* and *CXCL10* [Supplementary Figure 1A, available as Supplementary data at *ECCO-JCC* online]. Gene enrichment analysis revealed that TNF, IL1β, and the TLR3 ligand poly[I:C] elicited canonical type I IFN signature genes that encode antiviral and inflammatory mediators[32,35] [Supplementary Figure 1B and Supplementary File 3]. Both regulatory and effector ISGs[33] were significantly induced by the three ligands compared with untreated controls [Figure 3A].

We then continued to examine regulation of ISG15 protein expression by TNF, poly[I:C], and IL1β in HT29 cells. Western blot analysis confirmed phosphorylation of STAT1 and expression of IRF9 and intracellular free ISG15 protein in poly[I:C], IL1β, and TNF-treated HT29 cells, as well as after treatment with the known IFNAR ligand IFNβ used as positive control [Figure 3B]. We detected significantly enhanced levels of intracellular free ISG15 in the ligand-treated cells at 24 h [Figure 3C]. ISG15 conjugates [ISGylated proteins] were not observed [Supplementary Figure 2, available as Supplementary data at *ECCO-JCC* online]. Poly[I:C] induced a significant release of free ISG15 [Figure 3D].

To test whether poly[I:C] and TNF induced an autocrine/paracrine JAK1-STAT signalling loop, we pre-treated the cells with the JAK1 inhibitor filgotinib or the JAK1/JAK2 inhibitor ruxolitinib [Figure 3E] and measured expression of pSTAT1, total STAT, IRF9, and ISG15 at 24 h. The levels of all proteins, including intracellular and secreted free ISG15, were strongly impaired after pharmacologically blocking the JAK-STAT pathway, indicating that the signalling went through JAK1. We also detected small amounts of poly[I:C]-induced IFNβ in up-concentrated medium [1:5], which was again reduced by JAK inhibition [Figure 3E]. When we pre-treated the cells with neutralising antibody against IFNβ 1 h before ligand stimulation, the levels of pSTAT1, IRF9, and free ISG15 in TNF- and IFNβ-treated cells were almost similar to untreated controls [Figure 3F–G], indicating that the signalling went through IFNAR/JAK, as illustrated in Supplementary Figure 1D. In poly[I:C] treated cells, densitometric analysis of intracellular expression of free ISG15 protein, and detection of free ISG15 in conditioned medium

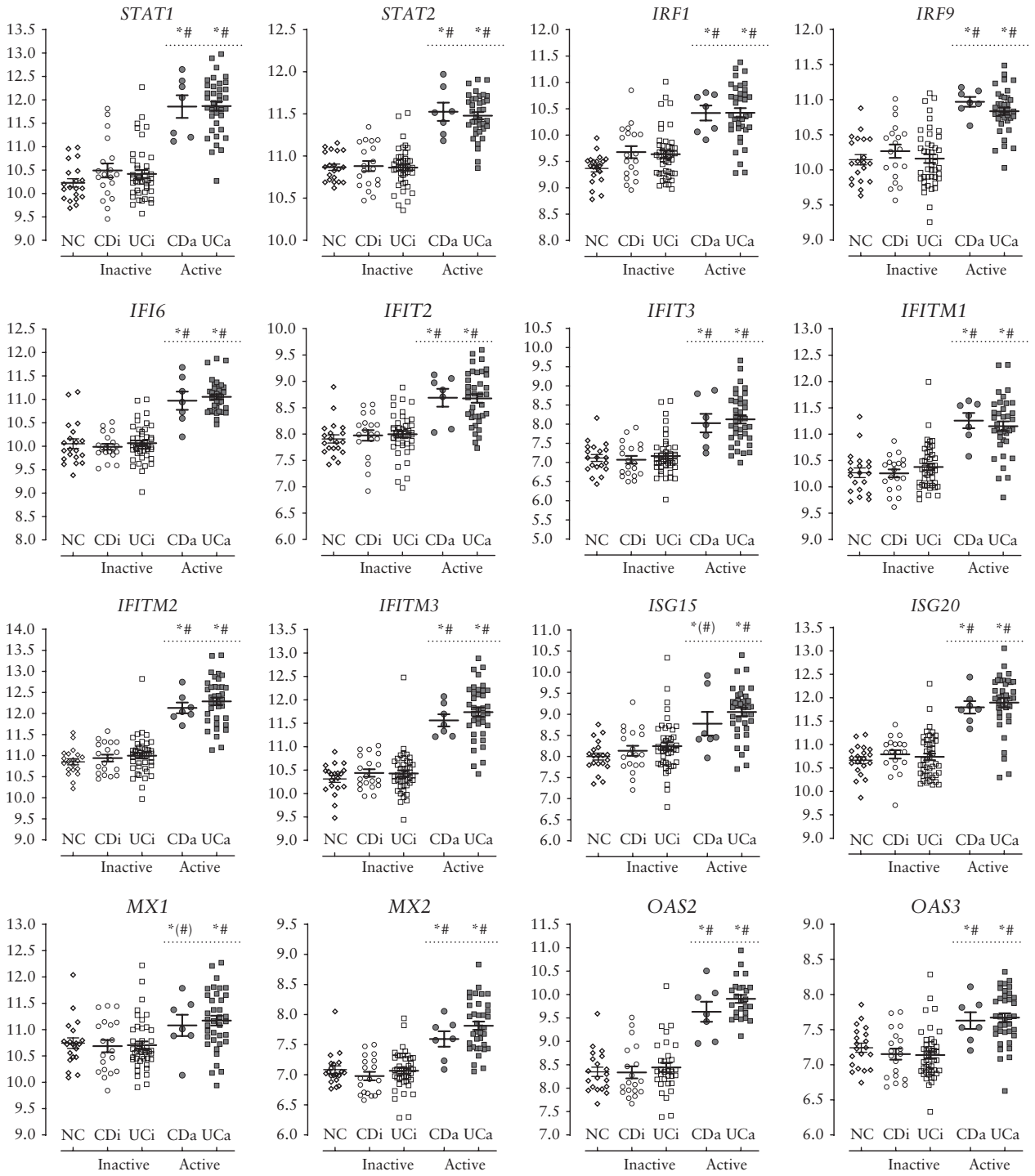

Figure 1. Type I IFN-stimulated genes [ISGs] in whole colonic mucosal pinch biopsies from non-IBD controls [NC], and inactive [i] or active [a] ulcerative colitis [UC] and Crohn's disease [CD]. Microarray-derived mRNA expression levels are shown as log2 signal intensities [Y-axis]. The panels show mean and individual values for each gene in the different groups. Active UC [*n* = 37] versus NC [*n* = 20] and active CD [*n* = 7] versus NC [*n* = 20]: adjusted *p*-values *<0.05. Active UC [*n* = 37] versus inactive UC [*n* = 44] and active CD [*n* = 7] versus inactive CD [*n* = 19]: adjusted *p*-values #<0.05 or [#]*p* <0.2. Differential gene expression analysis was performed as described in the Methods section 2.9; *p*-values were adjusted for multiple comparisons using the Benjamini-Hochberg false-discovery rate correction [FDR]. The selected gene expression data are shown in Supplementary File 1, available as Supplementary data at *ECCO-JCC* online. IBD, inflammatory bowel disease; UC, ulcerative colitis; CD, Crohn's disease.

by ELISA, showed that the response was only partly impaired by neutralising antibody against IFNβ versus IgG isotype control [Figure 3G–H]. This is in line with other *in vitro* studies showing that some poly[I:C]-induced antiviral genes, including *CXCL10* and *ISG15*, can be activated directly by TLR3-signalling, as well as by autocrine IFNβ-signalling.[36,37] Overall, our results showed that extracellular TNF and poly[I:C] activated type I IFN-signalling through JAK1-pSTAT-IRF9 with successive induction of both intracellular and secreted free ISG15 in HT29 cells. In addition, free ISG15 can be induced by IFN-independent signalling.

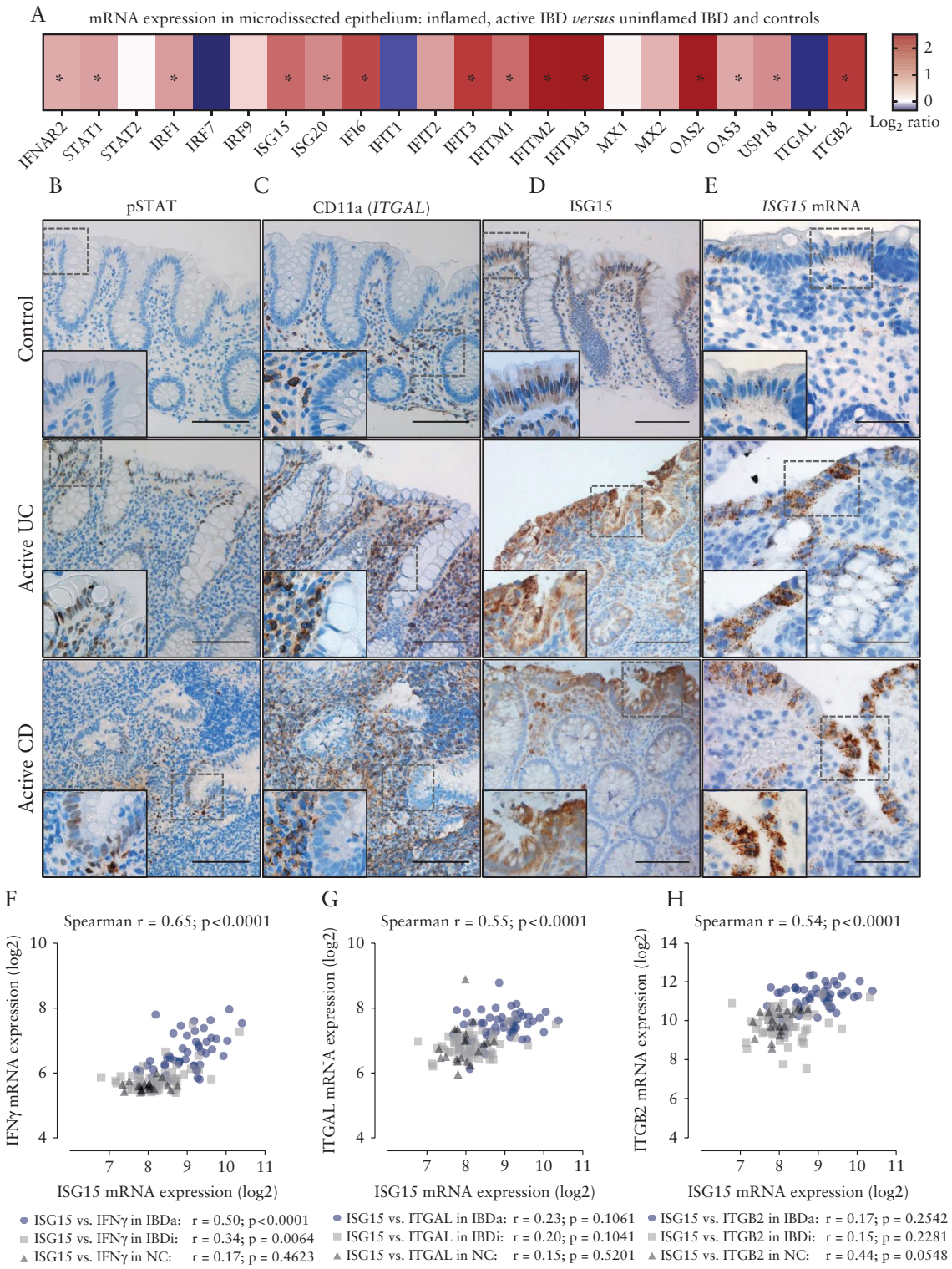

Figure 2. Expression of ISGs in colonic epithelium during active IBD. [A] RNASeq data of ISGs in laser-captured, microdissected epithelial monolayer [*n* = 29]. Heat map shows log2 ratios of gene expression in microdissected epithelial cells from inflamed, active IBD [*n* = 12] compared with expression in epithelial cells from uninflamed IBD and normal controls [*n* = 17]. Differential gene expression analysis was performed as described in the Methods section 2.9; *adjusted *p*-values <0.05. The gene lists are shown in Supplementary File 2, available as Supplementary data at *ECCO-JCC* online. [B–E] Expression of pSTAT, CD11a, and ISG15 [brown] in colonic mucosa from active UC and CD compared with non-IBD control [representative pictures from *n* = 3–4 in each group]. Insets show higher magnification of areas as indicated. Scale bars 100 μm in [B–D], 50 μm in [E]. IHC showing that in active IBD there are [B] increased expression of pSTAT1 in both epithelial cells and immune cells infiltrating the lamina propria, [C] infiltration of CD11a [gene name *ITGAL*] expressing immune cells in in the full depth of lamina propria, and [D] increased expression of ISG15 in particularly the surface epithelium. [E] ISH of ISG15 mRNA confirming increased expression in colonic epithelial cells during active IBD. [F–H] Scatter plot and Spearman rank correlation analysis of *ISG15* mRNA expression versus *IFNγ* [F], *ITGAL* [G], and *ITGB2* [H] mRNA expression in colonic pinch biopsies from individual non-IBD controls [NC] [*n* = 20], inactive IBDi [*n* = 65] and active IBDa [*n* = 49] [total *n* = 134]. IBD, inflammatory bowel disease; UC, ulcerative colitis; CD, Crohn's disease; IHC, immunohistochemistry; ISH, *in situ* hybridisation.

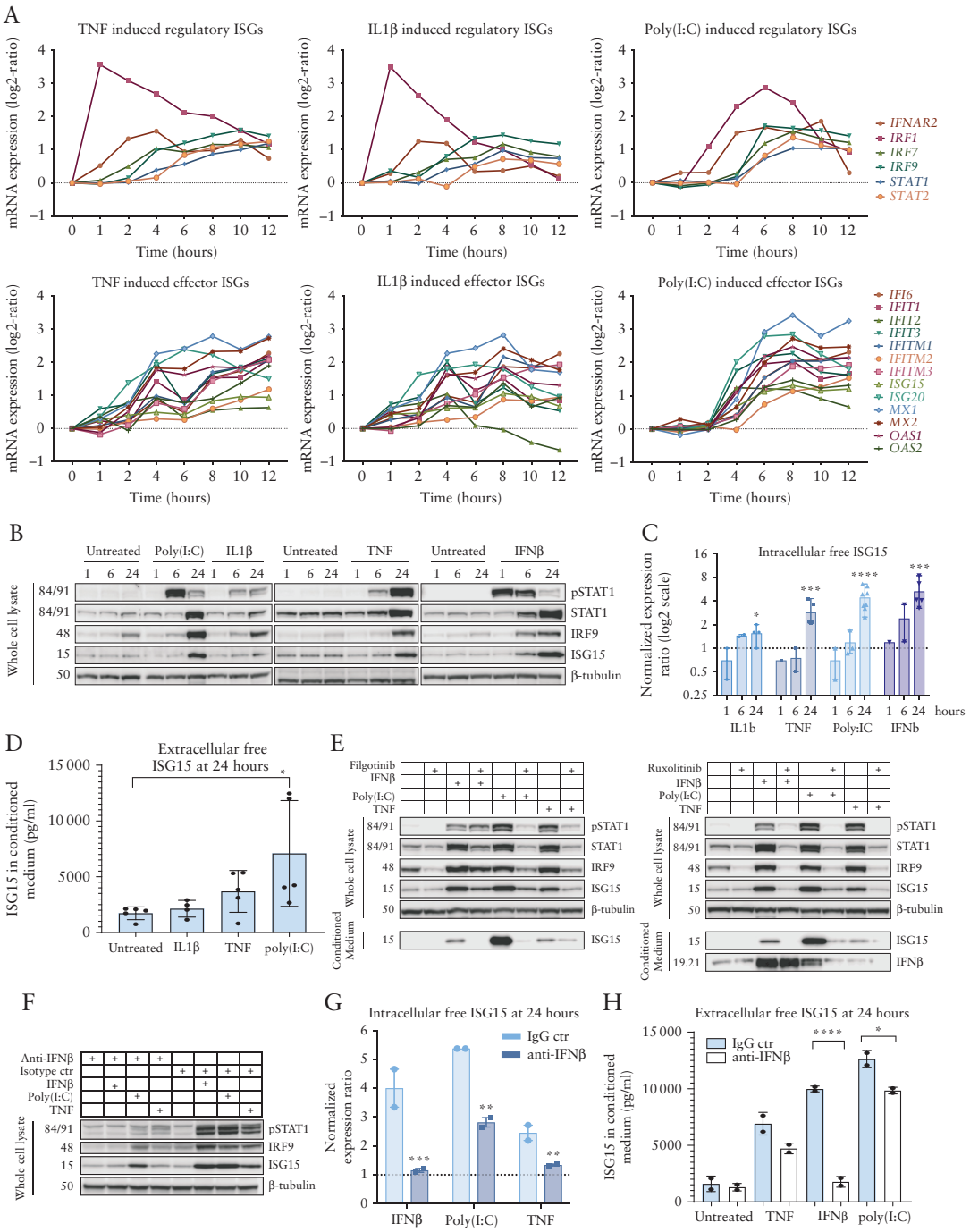

Figure 3. Extracellular signals induce expression of ISGs in HT29 human intestinal epithelial cells. [A] Temporal expression of induced regulatory [upper panel] and effector [lower panel] ISGs after TNF [100 ng/ml], IL1β [100 ng/ml], or poly[I:C] [5 μg/ml] treatment. Data shown: pool of triplicate wells. Statistical analysis was performed on temporal profiles as described in the Methods section 2.9. Independent validation of TNF [$n = 3$] and poly[I:C] [$n = 5$] gene expression at 6 h are shown in Supplementary Figure 1 and Supplementary File 3, available as Supplementary data at *ECCO-JCC* online. [B] pSTAT1, total STAT1, IRF9, and ISG15 in whole-cell lysates of cells treated with poly[I:C] [5 μg/ml], IL1β [100 ng/ml], or TNF [100 ng/ml] for 1, 6, and 24 h were analysed by western blots. [C] Densitometric analysis of intracellular free ISG15 at 1 h, 6 h [$n = 2$–3], and 24 h [$n = 5$–8]. [D] Extracellular free ISG15 in conditioned medium detected by ELISA [$n = 4$–5]. [E] Effect of 10 μM filgotinib [JAK1 inhibitor] or 10 μM ruxolitinib [JAK1/JAK2 inhibitor] on intracellular levels of pSTAT1, total STAT, IRF9, and ISG15, 24 h after treatment with IFNβ [10 ng/ml], poly[I:C] [5 μg/ml], or TNF [100 ng/ml]. Extracellular ISG15 and IFNβ were detected in up-concentrated conditioned medium [1:5]. [F] Analysis of autocrine IFNβ signalling. Cells were pre-treated for 1 h with IFNβ-specific neutralising antibody [anti-hIFNβ, 2.5 μg/ml] or isotype control [rabbit IgG, 2.5 μg/ml] before treatment with IFNβ, poly[I:C], or TNF. Levels of pSTAT, IRF9, and ISG15 in whole-cell lysate were measured at 24 h. [G] Densitometric analysis of the levels of intracellular free ISG15 at 24 h. [H] Extracellular free ISG15 [mean pg/ml + SD] in conditioned medium detected by ELISA; *p*-values were calculated with one-way ANOVA followed by Dunnett's multiple comparisons test. Each bar in [C] and [G] represents the mean expression ratio of ISG15 in ligand-treated cells relative to the reference value of 1 [dotted line] subsequent to normalisation against β-tubulin [loading control]; *p*-values for each treatment group were calculated on log2 transformed values with one-way ANOVA followed by Dunnett's multiple comparisons test [in C] and by two-way ANOVA followed by Šídák [IgG versus ablFNβ] or Tukey [between ligands] multiple comparison test [in G]. Uncropped blot images are shown in Supplementary Figure 2, available as Supplementary data at *ECCO-JCC* online; *p <0.05, **p <0.01, ***p <0.001, ****p <0.0001. ELISA, enzyme-linked immunosorbent assay; SD, standard deviation; ANOVA, analysis of variance.

3.3. Human 3D colonoids release free ISG15 upon extracellular stimulation

To examine if poly[I:C] and TNF also could induce type I IFN signature genes in a more physiologically relevant model, we performed additional experiments in primary human 3D colonoids established from colonoscopic pinch biopsies.[26] The colonoids were expanded and differentiated into 3D multicellular structures containing absorptive, goblet, and enteroendocrine cells [Figure 4A]. After having established that the human colonoids were responsive to poly[I:C] and TNF treatment [Supplementary Figure 3A, available as Supplementary data at *ECCO-JCC* online], duplicate RNA samples from colonoids treated with poly[I:C] or TNF for 1, 6, and 24 h were analysed by whole transcriptomic shotgun sequencing [RNASeq]. MetaCore™ pathway enrichment analysis revealed that mainly the TLR3 ligand poly[I:C] elicited prototypical type I IFN signature genes in colonoids [Supplementary Figure 3B]. Whereas TNF induced fewer ISG mRNAs in human colonoids than in the cell line HT29 [Supplementary File 4], several ISGs, including *ISG15*, were significantly upregulated in colonoids treated with poly[I:C] for 6 and 24 h [Figure 4B].

We then examined whether ISG15 protein expression was enhanced in the colonoids after extracellular stimulation. IHC staining showed varying intensities of both nuclear [ISGylation] and cytoplasmic ISG15 expression in poly[I:C]-, poly[I:C] + TNF-, and IFNβ-treated colonoids at 24 h [Figure 4C]. ISG15 seems to be expressed in both goblet cells and absorptive cells.

Western blot analysis showed that poly[I:C] induced pSTAT1, IRF9, and free ISG15 proteins [Figure 4D–E], but with different time-dependent expression compared with the positive control: In IFNβ-treated cells, pSTAT was detected at as early as 10 min [Figure 4D], wheres a similar level of pSTAT was not detected until 3–6 h after poly[I:C] treatment [Figure 4E]. Organoids pre-treated with neutralising antibodies against IFNβ before IFNβ treatment had reduced levels of pSTAT1, total STAT1, IRF9, and intracellular and secreted free ISG15 at 24 h [Figure 4F]. Levels of the same proteins were not impaired in colonoids pre-treated with antibody against IFNβ before poly[I:C] treatment [Figure 4F]; and we were not able to detect poly[I:C]-induced regulation of *IFN*β mRNA in colonoids. This indicated that poly[I:C] can induce ISG15 independently of an autocrine/paracrine type I IFN signalling loop in both primary human IECs and HT29 cells. In line with the observed nuclear staining of ISG15 by IHC [Figure 4C], IFNβ and poly[I:C] induced ISGylation as well as intracellular and secreted free forms of ISG15 in human colonoids [Figure 4F–H and Supplementary Figure 4, available as Supplementary data at *ECCO-JCC* online]. Interestingly, only intracellular and secreted free ISG15 were observed in HT29 cells [Supplementary Figure 2]. Densitometric analysis of western blots of whole-cell lysates showed that although TNF induced only minor expression of intracellular free ISG15 protein at 24 h, we observed a variable, but significant expression of free ISG15 in poly[I:C]-treated colonoids, and a robust and relatively high expression of free ISG15 in IFNβ-treated colonoids [Figure 4G]. ELISA analysis showed that free ISG15 was released to the medium after treatment with TNF and poly[I:C] as well as IFNβ [Figure 4H].

Overall, RNASeq-data, IHC, and western blot analysis showed that the type I IFN gene signature and ISG15 protein expression observed in human mucosal biopsies [Figures 1 and 2] were mimicked in human epithelial 3D colonoids [Figure 4] which are devoid of any immune cells. We also demonstrated that ISG15 can be secreted from primary IECs after extracellular stimulation.

3.4. Correlation between expression of the LFA-1 receptor unit *ITGAL* and ISG15-regulated release of IFNγ from PBMCs

Since IL12 and IFNγ, as well as the LFA-1 receptor unit CD11a [*ITGAL*], are well-known IBD susceptibility genes,[38,39] it was of interest to analyse if extracellular ISG15 could modulate IL12-induced release of IFNγ in immune cells from IBD patients. We first confirmed[14,19] that extracellular ISG15 and IL12 reproducibly and synergistically stimulate release of IFNγ in PBMCs derived from three healthy donors [Figure 5A]. We then proceeded to measure IFNγ release from PBMCs isolated from non-IBD controls [n = 6], inactive IBD patients [n = 16] and active IBD patients [n = 13] after treatment with ISG15, IL12, or ISG15 + IL12 combined. Whereas PBMCs from both non-IBD controls and IBD patients showed almost undetectable basal and ISG15-induced levels of IFNγ release into media, IL12 induced variable release of IFNγ from PBMCs derived from different individuals, ranging 67–10.500 pg/ml [Supplementary Figure 5, available as Supplementary data at *ECCO-JCC* online]. Although we observed great inter-individual differences in sensitivity to IL12 and ISG15 treatment within all groups, 83% and 55% of PBMC cultures from non-IBD controls and IBD patients, respectively, showed enhanced IFNγ release after ISG15 + IL12 treatment compared with IL12 alone [Figure 5B].

It has been reported that IFNγ is released mainly from natural killer [NK] cells and T cells upon presence of ISG15 and IL-12, although unsorted PBMCs respond better to ISG15 + IL12 than fractioned primary NK or T cells.[14] We therefore examined whether the variations in synergistic release of IFNγ was due to the relative frequency of PBMC subpopulations present in the individual samples, using flow-cytometric analysis.[25] However, we did not find any correlation between IFNγ release in response to IL12 + ISG15 ***versus*** IL12 alone and frequency of CD16+ NK-cells or other PBMC subpopulations [CD4+, CD8+, CD19+, and CD14+] in PBMCs [Figure 5C].

We then examined for a possible correlation between ISG15-modulated secretion of IFNγ and expression of LFA-1 subunits in the cells. Expression levels of *ITGAL* and *ITGB2* [Figure 5D] mRNAs in PBMCs derived from the same sample population used in the stimulation experiments shown in Figure 5B were quantified by RT-qPCR. The expression of both subunits was mostly equal in the different groups. When we linked expression levels of *ITGAL* and *ITGB2* mRNAs with release of IFNγ from matching PBMC samples after treatment with IL12+ISG15 versus IL12 alone, we found a significant correlation between *ITGAL* expressions in PBMCs and IFNγ concentrations in culture media [r = 0.39, p = 0.02] [Figure 5E].

3.5. ISG15 boosts release of several IBD-related cytokines from PBMCs

To investigate whether ISG15 could regulate secretion of additional IBD-related cytokines, PBMCs from three healthy donors were treated with recombinant human ISG15, IL12, or IL12 + ISG15 combined for 48 h, and secreted cytokines were detected by the Bio-Plex Pro Human Chemokine Panel featuring 40 magnetic bead-based immunoassays. Consistent with findings by others,[14,19,20] IL12 + ISG15 combined elicited a strongly synergistic effect on the secretion of IFNγ, and ISG15 alone induced IL6 [Figure 6A] and IL10 [Supplementary Figure 6, available as Supplementary data at *ECCO-JCC* online]. Additionally, we found that IL12 alone induced strong release of CXCL9 and CXL10, and that ISG15 appeared to be a very potent modulator for several other IBD-related cytokines

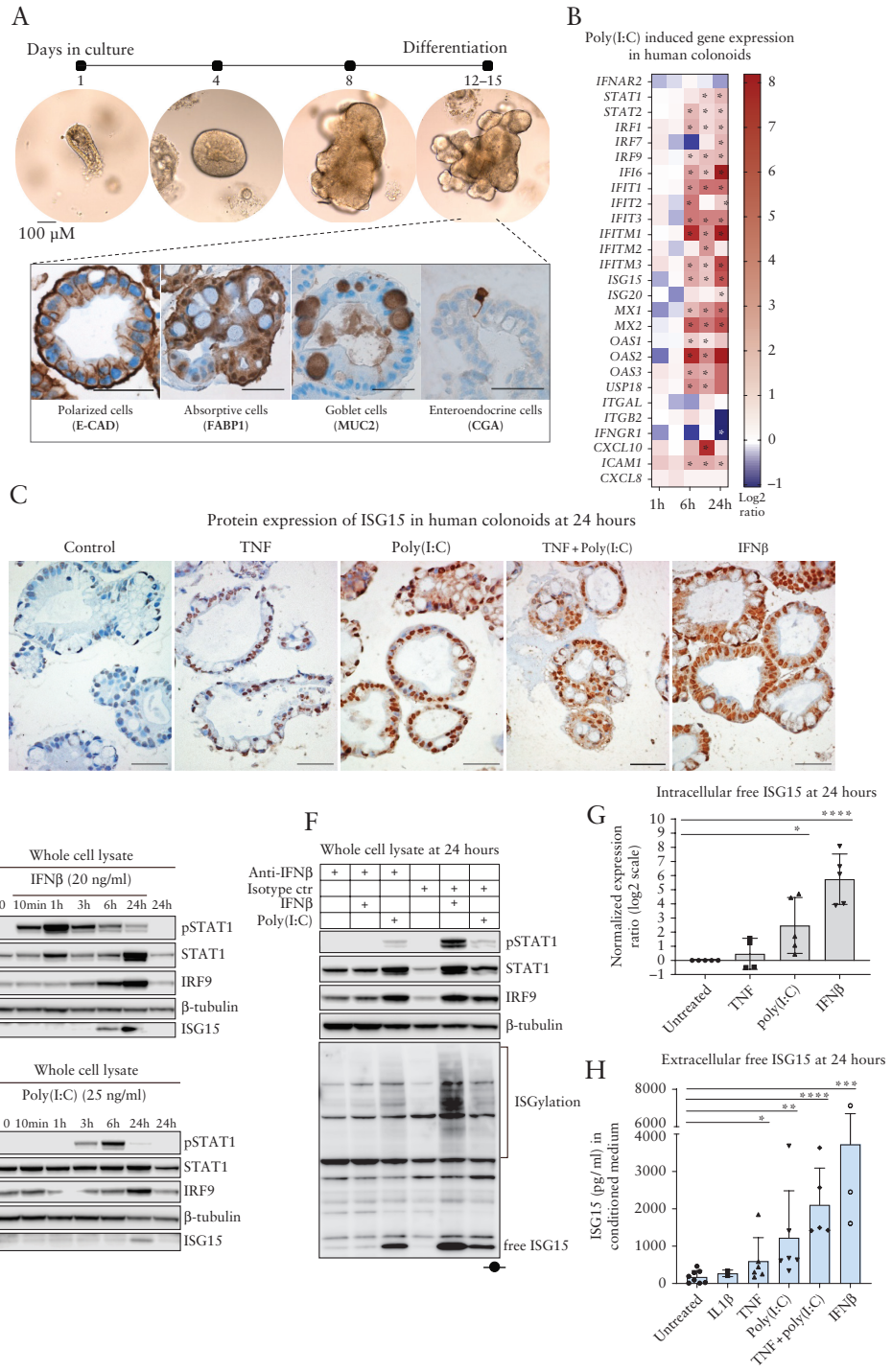

Figure 4. Induction of type I IFN pathway in human colonoids. [A] Colonoids were established from non-IBD control colon crypts, passaged and differentiated from Day 10 into multi-cell 3D structures containing central lumen and polarised cells of the major colonic cell types, as indicated. [B] Heatmap showing log2 ratio of ISGs in colonoids treated with poly[I:C] for 1, 6, and 24 h versus untreated controls at each time point. Separately independent experiments shown were run in duplicates with pools of at least 8 wells. Transcriptome data for poly[I:C]- and TNF-treated colonoids, including MetaCore™ pathway map, are shown in Supplementary File 4, available as Supplementary data at *ECCO-JCC* online. [C] IHC showing expression of ISG15 protein [brown] in differentiated epithelial cells in colonoids [Day 15] treated with the indicated ligands for 24 h [representative pictures from n = at least 5 for each condition]. Scale bars 50 µm. [D–E] Western blot analysis of pSTAT1, total STAT1, IRF9, and ISG15 in whole-cell lysates of colonoids treated with IFNβ [20 ng/ml] or poly[I:C] [25 µg/ml] for 10 min, 1 h, 3 h, 6 h, and 24 h. Untreated controls at T0 and T24 were loaded in the first and last wells, respectively. [F] Analysis of autocrine IFNβ signalling using IFNβ-specific neutralising antibody [anti-hIFNβ, 2.5 µg/ml] or isotype control [rabbit IgG, 2.5 µg/ml] 1 h before treatment with IFNβ [20 ng/ml] or poly[I:C] [25 µg/ml]. Levels of pSTAT, total STAT1, IRF9, ISGylation, and free ISG15 in whole-cell lysate were measured at 24 h. [G] Densitometric analysis of intracellular free ISG15 at 24 h in TNF- [0.2–0.4 µg/ml, n = 4], poly[I:C]- [10–50 µg/ml, n = 6], and IFNβ- [10–20 ng/ml, n = 5] treated colonoids; p-values were calculated as in Figure 3C and G. [H] Detection of free ISG15 in conditioned medium from untreated and treated colonoids detected by ELISA [mean + SD; individual experiments indicated by dots]; p-values were calculated with one-way ANOVA followed by Dunnett's multiple comparisons test on log2 transformed data. Uncropped blot images are shown in Supplementary Figure 4, available as Supplementary data at *ECCO-JCC* online; *p <0.05, **p <0.01, ***p <0.001, **** p <0.0001 treated versus untreated controls or as indicated. IBD, inflammatory bowel disease; IHC, immunohistochemistry; ELISA, enzyme-linked immunosorbent assay; SD, standard deviation; ANOVA, analysis of variance.

[Figure 6A, Supplementary Figure 6]. Using a relatively strict selection criterion as described in the Methods section, the 40-plex analysis indicated that ISG15 modulated release of CXCL1, CXCL2, CXCL5, CCL1, CCL3, CCL7, CCL8, CCL20, CCL25, IL1β, IL6, and TNF. In addition, high levels of CXCL8 [out of range] were observed [Supplementary Figure 6]. Robust ISG15- and/or IL12-induced secretion of TNF, CCL20, CXCL8, and CXCL10 was confirmed by ELISA analysis of conditioned media from 4–5 independent experiments performed on PBMCs from three other healthy donors [Figure 6B]. Overall, these results suggest that ISG15 can potentiate the release of IBD-related cytokines.

4. Discussion

In this study we show an increased expression of type I IFN-related ISGs in colon epithelium from patients with active IBD. The group of ISGs found to be upregulated during active disease is included in a subset of ISGs strongly enriched for the ISRE promoter element[33,34]

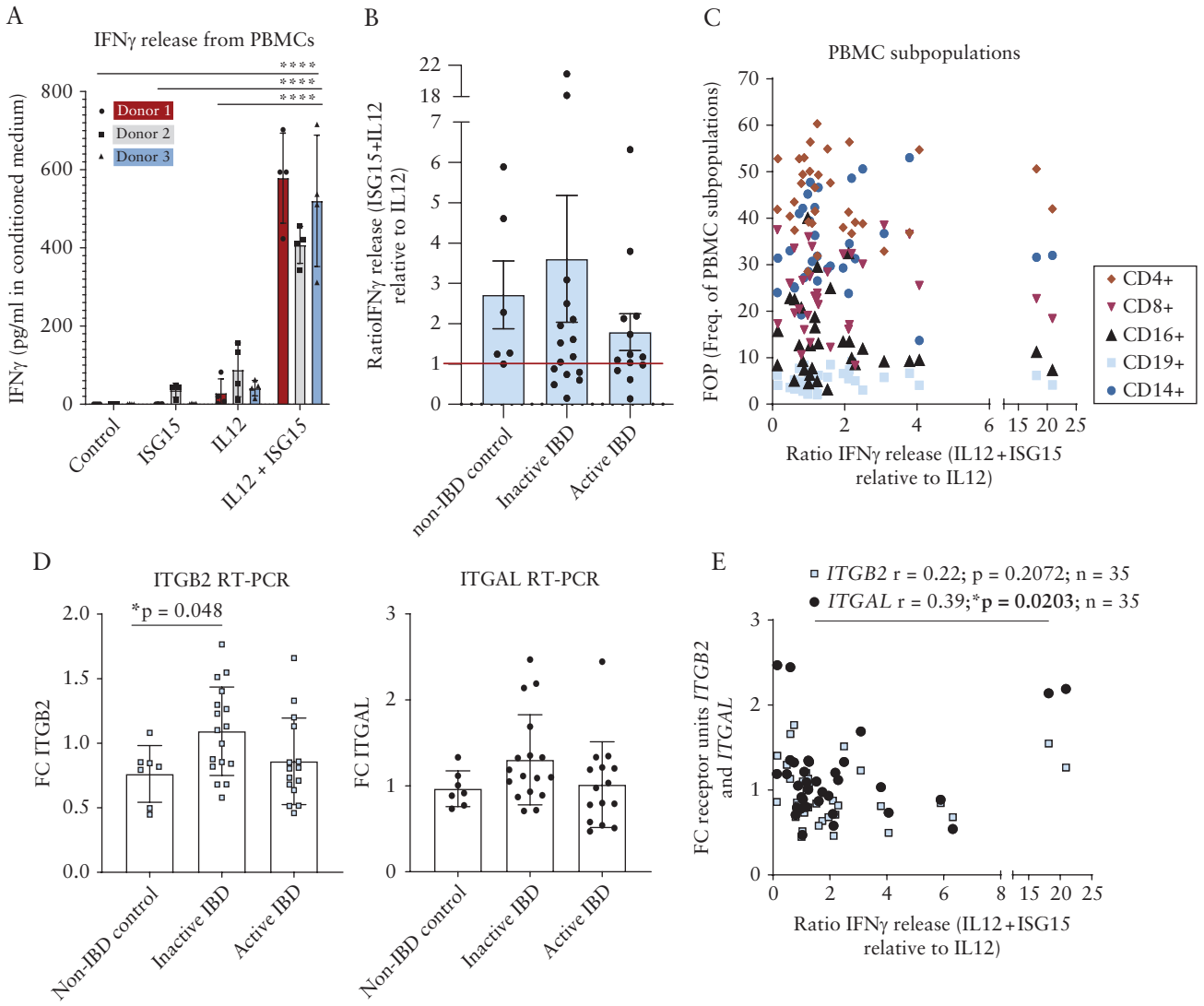

Figure 5. ISG15 and IL12 regulate IFNγ release from PBMCs. [A] IFNγ release from PBMCs was detected by ELISA after treatment with ISG15 [500 ng/ml], IL12 [20 ng/ml], or ISG15 + IL12 [500 + 20 ng/ml] for 48 h. The plot shows mean + SD IFNγ release from three healthy donors with independent experiments marked as dots [$n = 4$]. [B] ISG15-regulated enhanced release of IFNγ from PBMCs isolated from non-IBD controls [$n = 6$], inactive IBD [$n = 16$], and active IBD [$n = 13$]. The plot shows ratio IFNγ release [mean + SEM] between ISG15 + IL12 and IL12 alone in the three groups, with individuals indicated by dots. [C] No significant correlation between PBMC subpopulations and ratio IFNγ release from PBMCs after treatment with IL12 + ISG15 versus IL12 alone. The plot show PBMC subpopulations in 35 samples [Sample set II]. FOP: frequency of parental, CD4+ [T helper cells, monocytes, macrophages, dendritic cells], CD8+ [cytotoxic T cells], CD16+ [natural killer cells, monocytes, and macrophages], CD19+ [B cells], CD14+ [co-receptor, PRR]. [D] Expression of ISG15 receptor units,[19] *ITGAL* and *ITGB2* mRNAs in PBMCs from non-IBD controls [$n = 7$], inactive IBD [$n = 17$], and active IBD [$n = 15$] quantified by RT-qPCR. Fold changes were calculated using the ΔΔCT method, where individual expression levels were determined relative to the mean of reference genes and further relative to the mean in the healthy non-IBD control group. The figures show mean expression +SD and individual fold changes determined relative to the reference gene [*beta actin*] for each group. [E] Correlation between fold change levels of ISG15 receptor units [*ITGB2* and *ITGAL*] mRNAs and ratio IFNγ release from PBMCs after treatment with IL12 + ISG15 versus IL12 alone. Pearson correlation coefficients and *p*-values are shown; *p*-values for each group in A and D were calculated with one-way ANOVA followed by Dunnett's multiple comparison test; *$p < 0.05$, **** $p < 0.0001$. IBD, inflammatory bowel disease; PMBC, peripheral blood mononuclear cells; ELISA, enzyme-linked immunosorbent assay; SD, standard deviation; SEM, standard error of the mean; ANOVA, analysis of variance; PRR, pattern recognition receptor; RT-qPCR, real-time quantitative polymerase chain reaction.

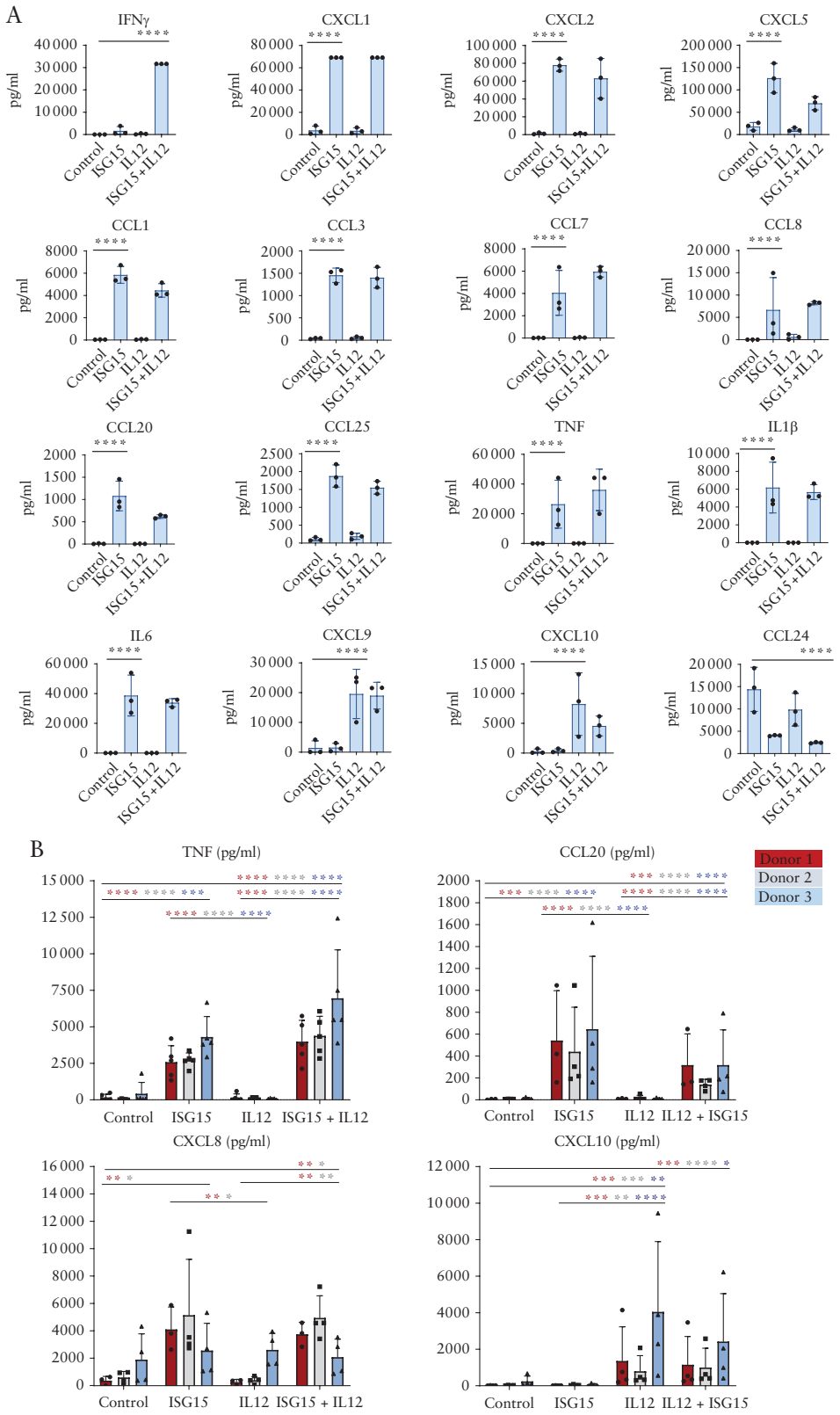

Figure 6. ISG15 regulates release of IBD-related cytokines from human immune cells. [A] Cytokine profile in conditioned medium from PBMCs treated with ISG15 [500 ng/ml], IL12 [20 ng/ml], or ISG15 + IL12 [500 + 20 ng/ml] for 48 h and analysed by Human Chemokine Panel featuring 40 magnetic bead-based immunoassays. [A]The plots show cytokines [pg/ml] significantly regulated by IL12, ISG15, or combination of both, in PBMCs derived from three healthy donors. All data are shown in Supplementary Figure 6, available as Supplementary data at *ECCO-JCC* online. [B] Validation of multiplex-data by ELISA. The panels show results from independent experiments with PBMCs derived from three other healthy donors. The bars show mean cytokine level [pg/ml] + SD in conditioned medium, $n = 4–5$, indicated by dots. In [A] p-values were calculated by two-way ANOVA followed by Tukey's multiple comparisons test [40 cytokines with six comparisons per cytokine]. In [B], p-values for each donor were calculated with one-way ANOVA followed by Dunnett's multiple comparisons test on log2 transformed data; *p <0.05, **p <0.01, ***p <0.001, ****p <0.0001. IBD, inflammatory bowel disease; PMBCs, peripheral blood mononuclear cells; ELISA, enzyme-linked immunosorbent assay; SD, standard deviation; ANOVA, analysis of variance.

that Mostafavi *et al.*[33] defined as sensitive to tonic type I IFN signals at baseline. We did not detect enhanced IFNβ mRNA in colonic mucosa during active IBD, but the overall mRNA abundance for IFNβ mRNA is minute and close to the lower end of the dynamic range of the gene expression assay used. The problem of measuring IFNβ regulation directly is well known and has been encountered in previous studies,[40] and IFNβ is found distributed in epithelium and lamina propria in human colonic tissue.[41] Although we, like others, were unable to show that IFNβ gene expression is regulated during inflammation, the transcriptional pattern observed in active IBD is consistent with a robust interferon response.

In humans, the impact of type I IFN signalling is reported to be both pro-and anti-inflammatory.[7,40,42] Slight alterations in the type I IFN signalling network may contribute to imbalanced immune responses in IBD,[7] but the role of type I IFNs and ISGs in IECs during IBD in the human has not been thoroughly investigated. It has been suggested that constitutive type I IFN signalling induced by the microbiota modulates homeostatic balance through tonic signalling in a cell- and context-dependent manner.[34,43] Low constitutive amounts of IFNβ in the absence of acute infections can prime cells in mucosa for strong responses to cytokines, probably through steady-state abundance of components in the ISGF3 complex [STAT1, STAT2, and IRF9].[34] It was therefore of interest to examine if innate immunity could regulate expression of the ISGs observed in active IBD through PRR-signalling. By combining examination of human colon biopsies with transcriptomic analysis, and studies in a human colon cell line and in primary human 3D multicellular colonoids, we show that especially the TLR3-ligand poly[I:C] induces a type I IFN response in IECs, with enhanced expression of pSTAT1, IRF9, and ISG15 proteins. TLR3 senses endogenous dsRNA from damaged tissues as well as RNA from virus,[44] and emerging evidence indicates TLR3-signalling as a major factor in homeostasis and wound repair.[45] Extracellular poly[I:C]-induced expression and secretion of free ISG15 seemed to be partly independent of an IFNβ autocrine/paracrine loop, especially in human colonoids, signifying a possible mechanism for endogenous DAMP-induced inflammation in IBD.

Although we observed enhanced levels of ISG15 in conditioned medium from both HT29 cells and human colonoids after treatment with poly[I:C], TNF, and the positive control IFNβ, there were some differences between the responses in primary IECs and the cell line. TNF induced stronger type I IFN signature genes in HT29 cells than in human colonoids; and IFNβ and poly[I:C] induced both free ISG15 and ISGylation in colonoids, whereas only free ISG15 was observed in HT29 cells. Examination of fibroblasts from controls and individuals with ISG15 deficiency has shown that human intracellular ISG15 can prevent IFNα/β over-amplification and autoinflammation.[46] In further studies, it would therefore be interesting to investigate the impact of ISG15 as an intracellular regulator of aberrant expression of type I IFNs and ISGs in human primary IECs.

In the present study, we focused on regulation and immunomodulatory effects of extracellular free ISG15. We show for the first time that ISG15 can be released from primary human IECs upon external stimulation, and that extracellular ISG15 can regulate release of several IBD-related cytokines from PBMCs. Although the cytokine responses in PBMCs seemed reproducible within the six healthy donors examined, we observed large inter-individual variation in IFNγ release from ISG15- and IL12-treated PBMCs derived from non-IBD controls and IBD patients. We found that ISG15-modulated secretion of IFNγ correlated weakly, but significantly with mRNA expression levels of the LFA-1 integrin receptor unit CD11a [*ITGAL*] in PBMCs. The LFA-1 integrin receptor is expressed on T cells, B cells, macrophages, neutrophils, and NK cells.[47] In the context of IBD, LFA-1 on T cells is best known as receptor for ICAM1. Swaim *et al.* 2017[19] showed that extracellular ISG15 induces IFNγ and IL10 secretion through the LFA-1 integrin receptor in an NK-92 cell line-based assay and in CD11a KO mice, but no data regarding ISG15 receptors on human primary cells are published. Our transcriptome data from human colon biopsies indicated a correlation between *ISG15* and *ITGAL* expression and enhanced expression of IFNγ in colon mucosa during active IBD. Whereas more investigation is needed to address ISG15 receptors on human primary immune cells, we propose a model based on our data and literature[12,19] where dsRNA[TLR3], PAMPs, or DAMPs signalling and induced ISGs contribute to inflammation in active IBD by, for example, enhanced expression of IEC-derived free ISG15. The secreted free form of human ISG15 can boost IBD-relevant immune activities, including chemotaxis of neutrophils[15] and regulation of cytokines such as IFNγ, CXCL1, CXCL5, CXCL8, CCL20, TNF, IL6, and IL1β released from infiltrating immune cells [Supplementary Figure 7, available as Supplementary data at *ECCO-JCC* online]. To date, around 240 single nucleotide polymorphisms [SNPs] associated with increased IBD risk have been identified, comprising genes related to intestinal epithelial function, as well as innate and adaptive immune responses.[38,39,48,49] Central genes in the IFNAR1-STAT1-ITGAL-ISG15-cytokine network are associated with IBD-related genetic polymorphisms [SNPs],[38,39,48] such as IFNAR1 [rs2284553], STAT1 [rs1517352], IRF1 [rs2188962], IL12B [rs6871626], IFNγ [rs7134599], ITGAL [rs11150589], the major ligand for LFA-1 on T-cells: ICAM1 [rs5498], IL12B [rs6871626], CCL20 [rs111781203[48]_ rs1811711[39]], and the chemokines CXCL1, CXCL5, and CXCL8 [all rs2472649].

D'Cunha *et al.*[50] showed already in 1996 that administration of IFNβ to healthy individuals increased serum ISG15 levels significantly, and suggested that free ISG15 may represent an important mediator of the host response to IFN*α/β* by amplifying the immunomodulatory effects of IFNs. ISG15 has later been suggested as biomarker and/or target for treatment in human diseases such as systemic lupus erythematosus,[51] active tuberculosis,[20] oesophageal squamous cell carcinoma,[52] and pancreatic cancer.[53] Treatment with IFNβ in IBD has shown contradictory results,[7] but inhibition of the JAK-STAT pathway represents a promising therapeutic target for moderate to severe UC.[54] However, JAK proteins can bind to various types of cytokine receptor families[55]; and the overall effect of JAK inhibitors can be difficult to predict in humans. We argue that a better understanding of cell- and context-dependent activation of type I IFN signalling and expression of ISGs in human intestinal mucosa can identify effector ISGs to be targeted in IBD treatment, hopefully without the side effects following treatments directed at broader upstream signalling components such as IFNβ and/or JAK1-STAT. Here we showed that ISG15 can modulate release of a variety of IBD-related cytokines and is therefore an intriguing candidate for immunotherapy in IBD.

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Conflict of Interest

The authors declare that there are no conflicts of interest to disclose.

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Author Contributions

BG, AKS, and TB supervised the study. AEO, TDS, AvBG, BD, HKS, IB, ST, WA, GAW, TEM, AKS, and TB designed experiments, generated data, and analysed data. AEO, ST, BG, and AKS collected and characterised patient samples. TDS and TB made the figure panels. TB wrote the original draft. AEO, TDS, AvBG, BD, HKS, IB, ST, and AKS reviewed and edited the manuscript. All authors have read and approved the final manuscript.

Supplementary Data

Supplementary data are available at *ECCO-JCC* online.

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