Graduate thesis

NTNU
Norwegian University of Science and Technology
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Department of Clinical and Molecular Medicine

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Intestinal epithelial ISG15 interactions with the immune system

Graduate thesis in Medicine
Supervisor: Arne Kristian Sandvik
Co-supervisor: Torunn Bruland

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Table of content

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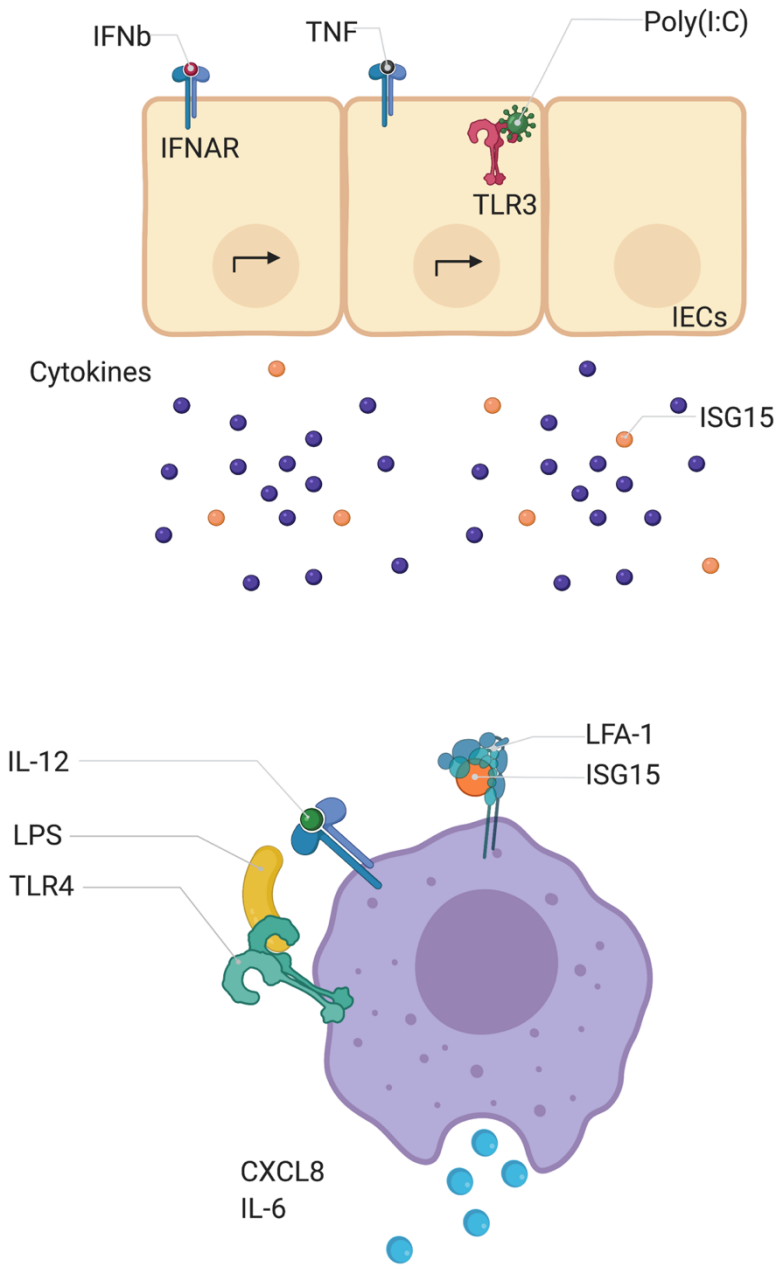
I am forever grateful for the continued support from my supervisors Arne Kristian Sandvik and Torunn Bruland. Since I joined the IBD group, they have always welcomed me with an open door and encouraging support. I also wish to thank Ingunn Bakke for helping me with histology related problems. I want to thank everyone in the IBD group for contributing to a positive learning environment. It is inspiring to learn and discuss with all of you.

Graphical abstract



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Abstract

Introduction

Inflammatory bowel disease (IBD) comprises Crohn's disease and ulcerative colitis. IBD is a state of chronic inflammation in the gut without a cure. Medications aim to suppress the inflammatory response, thus relieving symptoms. However, some patients do not respond. Intestinal epithelial cells (IECs) are conductors for intestinal homeostasis. They are in close contact with both the microbiome and immune cells. IECs mediate microbial signals through pattern recognition receptors (PRRs). The interferon pathway is activated through PRRs and upregulated in IECs during IBD. Interferons induce the expression of interferon-stimulated genes (ISGs). Interferon stimulated gene 15 (ISG15) is one of the ISGs upregulated during IBD. It has cytokine-like properties and enhances the secretion of other cytokines. In this thesis, we study how ISG15 is regulated in an ex-vivo system for human IECs (colonoids) upon pro-inflammatory treatment, how ISG15 affects peripheral blood mononuclear cells (PBMCs) in combination with lipopolysaccharide (LPS) signals and if we can mimic the crosstalk between IECs and immune cells. In addition, we investigate whether ISG15 has potential as a biomarker for IBD.

Materials and methods

The expression of ISG15 in the human-derived colonoids was assessed with immunohistochemistry and immunofluorescence. The presence of ISG15 in conditioned medium from HT-29 cells and colonoids and human plasma were analysed with an Enzyme-Linked Immunosorbent Assay.

Results

ISG15 was detected in plasma from both IBD patients and healthy controls. TNF, poly(I:C), and IFNβ treatment induces ISG15 expression in colonoids. Pro-inflammatory treatment of HT-29 cells and colonoids induces secretion of ISG15. In combination with LPS, ISG15 enhances CXCL8 and IL-6 secretion from PBMCs. Treating PBMCs with conditioned medium from colonoids induced CXCL8 secretion, but the cytokine secretion seemed independent of ISG15.

Conclusion

ISG15 is expressed and secreted by human colonoids upon pro-inflammatory stimulation. Free ISG15 can potentiate LPS induced cytokine expression. ISG15 is present in peripheral blood from IBD patients but lacks potential as a biomarker for IBD duo to overlapping concentrations with healthy controls.

Sammendrag

Introduksjon

Inflammatorisk tarmsykdom (IBD) er en tilstand med kronisk inflammasjon i gastrointestinal traktus som utgjøres av sykdommene Crohns sykdom og ulcerøs kolitt. Det finnes ingen kur for IBD, men behandlingen er rettet mot å dempe inflammasjonen. Et av problemene ved IBD er at ikke alle pasienter responderer på behandling. Intestinale epitelceller (IECs) er sentrale for intestinal homeostase. De er tett knyttet til både mikrobiomet og immunceller. IECs medierer signaler fra mikrobiomet til immuncellene via mønstergjenkjenningsreseptorer (PRRs). Interferoner blir aktivert via PRRs og er oppregulert i IECs under IBD. Interferoner induserer transkripsjon av Interferon Stimulerte Gener (ISGs). Ett av ISG-ene oppregulert ved IBD er interferon stimulert gen 15 (ISG15). Det er vist at ISG15 har cytokin liknende egenskaper og kan forsterke effekten av andre cytokiner. I denne oppgaven undersøker vi hvordan ISG15 reguleres av pro-inflammatoriske cytokiner i tarmepitelceller, hvordan ISG15 affiserer perifere blod mononukleære celler (PBMC) i kombinasjon med lipopolysakkarider (LPS) og om man kan etterligne ekstracellulær kommunikasjon mellom IECs og immunceller. I tillegg, undersøker vi om ISG15 har potensiale som en biomarkør for IBD.

Materiale og metode

Uttrykket av ISG15 ble undersøkt med immunohistokjemi og immunoflouresence. Sekresjon av ISG15 i kondisjonert medium fra HT-29 celler, kolonoider og i humant plasma ble undersøkt med ELISA (Enzymkoblet Immunoabsorberende Analyse).

Resultater

ISG15 kan påvises i humant plasma. Uttrykk av ISG15 blir indusert i kolonoider ved stimulering med TNF, poly(I:C) og IFNβ. Proinflammatorisk stimulering av HT-29 celler og kolonoider medfører sekresjon av ISG15. ISG15 forsterker LPS indusert sekresjon av CXCL8 og IL-6 fra PBMCer. PBMC stimulering med kondisjonert medium fra kolonoidene medfører sekresjon av CXCL8, men mengden er uavhengig av tilstedeværelsen av ISG15.

Konklusjon

ISG15 er uttrykt og blir skilt ut av tarmepitelceller ved pro-inflammatorisk stimuli. Fritt ISG15 potenserer LPS mediert cytokin frigjørelse. ISG15 er til stede i humant plasma. Dog, mangler det potensiale som en biomarkør grunnet store overlapp mellom IBD pasienter og friske kontroller.

Introduction

Inflammatory bowel disease (IBD) is chronic inflammation in the gastrointestinal (GI) tract. Crohn's disease and ulcerative colitis are the subsets of diseases traditionally characterised as IBD. The aetiology of IBD is still unknown but thought to be a dysfunctional immune response to environmental factors or commensal microbes in genetically predisposed individuals(1). There is a disruption of the homeostatic interplay between the immune system and the microbiome, spiralling out of control. As a result, patients commonly experience stomach pain, defecation urgency and blood in the stool(2). IBD is a chronic disease without a cure. Medications aim at suppressing inflammation, thus relieving symptoms. However, patients respond differently to medications. One subgroup of patients might respond to their medication and become symptom-free. The inflammatory process is initially suppressed for others, but the effect diminishes. A third group might not respond to the treatment at all. Consequently, some patients require surgical interventions(3, 4).

Intestinal epithelial cells (IECs) are regulators of intestinal homeostasis(5). Their anatomical locations make them central in the crosstalk between the microbiome and the immune system. The intestinal mucosa comprises a single-layered epithelium residing on a layer of connective tissue named lamina propria. The epithelial cells are polarised, with an apical side facing the lumen, a basolateral side facing the lamina propria and the immune cells residing there (Illustration 1). Previously, IECs were perceived as a static barrier, but growing evidence shows their role as regulators of immune responses(6, 7). IECs express pattern recognition receptors (PRRs), enabling them to detect pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) from their microenvironment(5). The activation of PRRs is vital in both pro-inflammatory and immunotolerogenic responses(8, 9). IECs express several PRRs, e.g. toll-like receptors (TLRs), NOD-like receptors (NLRs) or RIG-1-like receptors(10-12). The TLRs expressed by intestinal epithelial cells are TLR2, TLR3, TLR4, TLR5 and TLR9(10, 13). One of the adaptor proteins essential for TLR signalling is myeloid differentiation primary response protein 88 (MyD88)(14, 15).

Signalling through TLRs has been classified into two pathways. The signal is either MyD88 dependant or independent(14). TLR3 is the only TLR that solely signal through the MyD88 independent pathway(14, 15). The other TLR signal through the MyD88 dependant

pathway(14, 15). TLR4 can activate both pathways(16). Activation of either pathway induces transcription of NF-kB, mitogen-activated protein kinase (MAPK), and interferon regulatory factors (IRFs)(14). Classically, TLRs are thought of as sensors in the inflammatory pathway resulting in a pro-inflammatory response in a target tissue(17). TLRs are sensors utilized by the innate immune system to detect DAMPS and PAMPs thus initiating inflammation. Furthermore, TLRs aid the innate immune system to recruit and shape the adaptive immune response. For instance, TLRs promote antigen uptake, major histocompatibility complex presentation, induce upregulation of co-stimulatory molecules and induce cytokines that stimulate T cell proliferation, survival and differentiation(18). However, TLRs play a multifaceted role in the intestinal epithelium(13). Mice studies have shown that TLR activation by commensal bacteria is crucial for IECs barrier function, proliferation and survival(9). Activation of TLR in IECs has several beneficial anti-inflammatory effects such as expression of epidermal growth factor receptors ligands, cytoprotective heat shock protein and trefoil factor 3. In addition, TLR activation induces enhanced integrity of apical tight-junction complexes(8, 13). IEC interactions with the microbiome regulate the immune response.

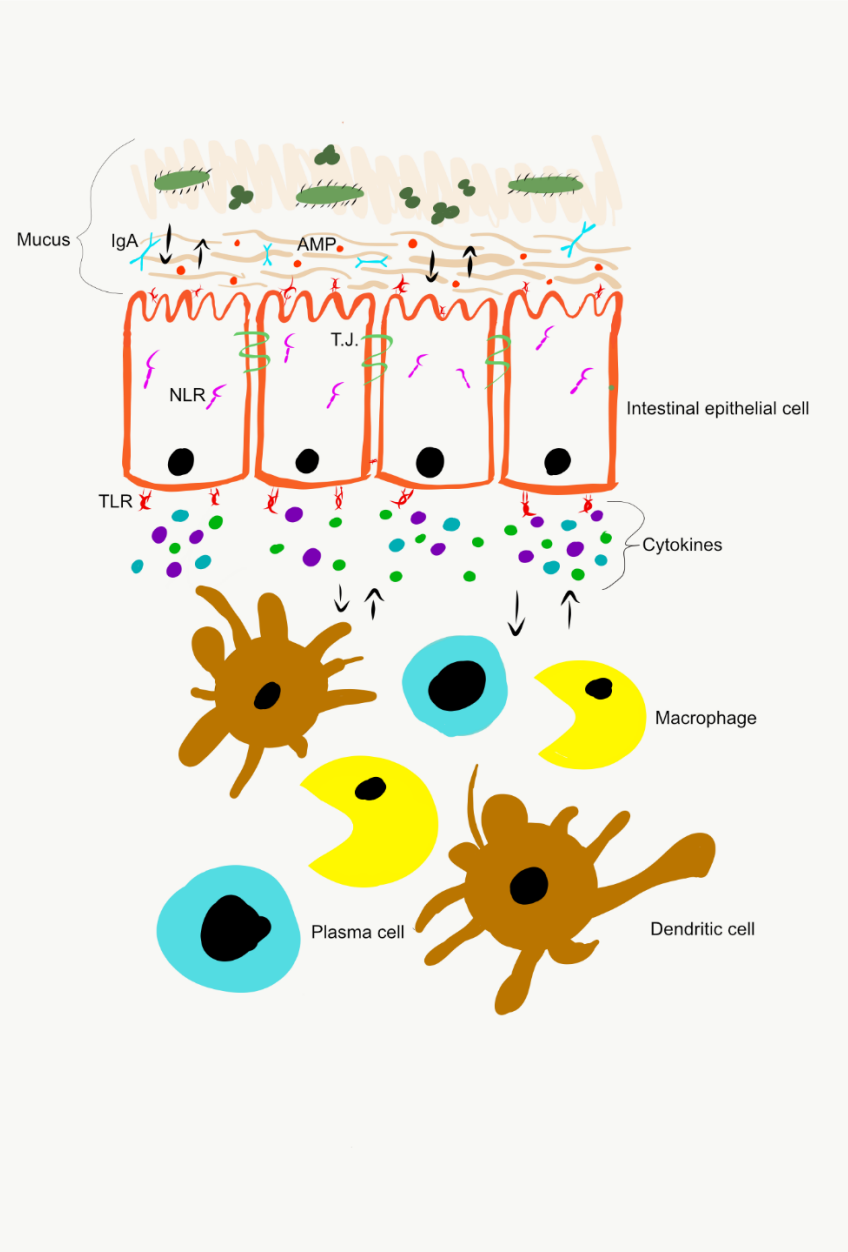


Illustration 1: The intestinal epithelial cells (IECs) constitute a dynamic barrier separating the microbiome from the immune system. The epithelium comprises several different cells; the most abundant are absorptive cells, goblet cells, stem cells, enteroendocrine cells and tuft cells. The intestinal mucosa is an integral part of the innate immune system. Mucus secreted from goblet cells forms a mucus layer apical to the epithelial cells. In the colon, there is a double mucus layer. The outer mucus layer is loosely connected and enables bacteria, viruses, and fungi to reside within it. The inner mucus layer is dense and sterile, separating the IEC from direct contact with the microbiome. Tight junction proteins between the IECs are crucial for the epithelium to form a barrier. Expression of PRRs (e.g. TLR and NLRs) enables the epithelium to sense microbial products and is essential for intestinal epithelium's integrity. By secreting cytokines and by presenting antigens, the epithelium communicates with immune cells in the lamina propria. Figure created by G.A.Walaas. AMP – antimicrobial products, TLR – toll-like receptor, NLR – NOD-like receptors, T.J. – tight junction proteins

Another PRR directed response is the upregulation of IRFs(14). There are nine known IRFs named IRF1-IRF9. IRFs are either activated by cytosolic PRRs such as RIG-1 or transmembrane PRRs such as TLR (Illustration 2). Activating IRFs produces interferons and interferon-stimulated genes (ISGs). ISGs are different molecules present at baseline or

induced by interferon stimulation(19). They are involved in anti-viral, anti-bacterial and anti-fungal activity and take on several forms such as PRRs and IRFs.(19) Interferons are divided into three varieties(20), type 1 interferons (IFNα, IFNβ), type 2 interferons (IFNγ), and type 3 interferons (IFNλ1, IFNλ2, and IFNλ3). Type 1 interferons are classically associated with anti-viral functions(20). Two separate IRFs induce IFNα and IFNβ. IFNβ expression is dependent on IRF-3, whereas IFNα requires IRF-7 for expression(20). Intracellular bacterial infections activate type 2 interferons (IFNγ). Type 3 interferons are also associated with anti-viral activity.

Activation of type 1 interferons (IFN1) has primarily been studied under viral infections(14). Cytosolic PRRs recognises viruses, a pathway that has been named the classical pathway for inducing type 1 interferons. The classical pathway is a positive feedback loop, where IRF3 and IRF7 are essential(21). IRF3 is continuously present in a latent form in the cytosol. IRF7 is only present in small amounts in the cytosol but is induced upon IFN1 stimulation(21). When a virus infects a cell, IRF3 and IRF7 form a heterodimer or a homodimer that translocate to the nucleus, inducing IFNα and IFNβ transcription(14). The initial effect of type 1 interferons is autocrine. They induce transcription of IRF7. The type 1 interferon-induced IRF7 is necessary for the full-scale activation of type 1 interferon genes(21).

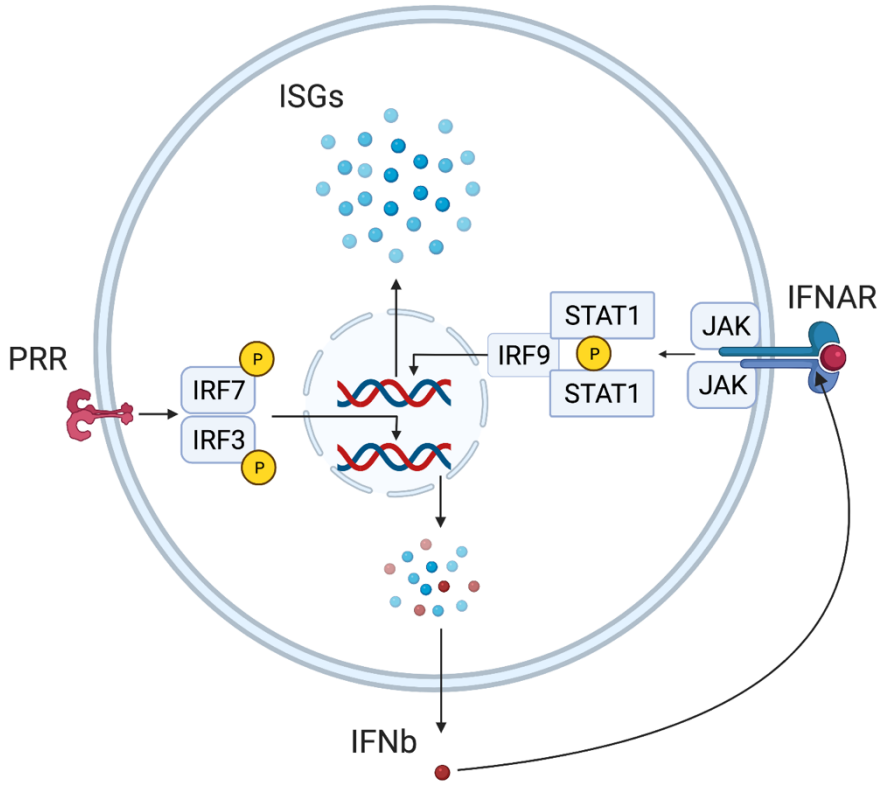


Illustration 2: Transcription of interferon-stimulated genes is a three-step process(14). Initially, the epithelial cells recognise microbial products with PRRs. The recognition activates IRF3, which is continuously present in the cytosol. IRF3 translocates to the nucleus, where it induces transcription of ISGs, among them IFNβ. In this first step, IRF7 also plays a role but is less critical than IRF3. The activation of ISGF3 is the second step in an interferon response. IFNβ secreted from the epithelial cells has a paracrine or autocrine effect. It binds to the interferon receptor IFNAR which is a JAK-STAT dependent receptor. Ligand binding to IFNAR activates the transcription complex ISGF3, which comprises IRF9 and STAT proteins. The transcription complex translocates to the nucleus inducing the transcription of ISGs, among them IRF7. Lastly, IRF7 will drive further transcription of IFNα/β in the third step of the interferon process. Figure created with BioRender.com by G.A.Walaas. PRR – Pattern recognition receptor, IRF – Interferon regulatory factor, IFNb – interferon β, IFNAR – interferon-alpha/beta receptor, JAK – Janus kinase, STAT – Signal transducer and activator of transcription P - phosphorylated

TLR activation of IRF is associated with both myD88 dependent- and myD88 independent pathways. TLR4 recognises lipopolysaccharides from bacteria and induces IFNβ transcription(16). Reports have shown how mice lacking IRF3 do not induce type 1 interferons upon LPS stimulation and that mice lacking IRF7 show regular expression of type 1 interferons. Translation of type 1 interferons from TLR4 activation is mainly dependent on IRF3 instead of IRF7. TLR3 recognises doubled stranded RNA (dsRNA). Studies have shown that mice lacking IRF3 express lower amounts of type 1 interferons upon stimulation of poly(I:C). However, mice lacking both IRF3 and IRF7 do not express type 1 interferons upon

poly(I:C) stimulation. Therefore, TLR3 activation of type 1 interferons requires both IRF3 and IRF7.

Type 1 interferons secreted by the epithelium either have a paracrine or autocrine signalling pathway(14). When a type 1 interferon binds its receptor, it activates a Janus kinase (JAK)–signal transducer of the transcription (STAT) pathway(20). The receptor of type 1 interferon is called IFNAR, which consist of two subunits IFNAR1 and IFNAR2. The activation of IFNAR triggers the induction of a transcription factor complex called ISGF3. The complex ISGF3, together with IRF9, is responsible for inducing ISGs transcription.
To summarise, the interferon pathway is a positive feedback loop initialised by PRRs. The initial response induces transcription of type 1 interferons, thereby stimulating the cell to increase its transcription of further ISGs and interferons. Consequently, the cell can quickly initialise e.g., an anti-viral state.

Several ISGs are upregulated during IBD(22). One of the ISGs with cytokine-like properties is ISG15. It is known to be induced by IFNβ stimulation but also by lipopeptide binding to TLR1/2 and dsRNA viruse binding to TLR3 (23, 24). ISG15 exists in three forms, conjugated to proteins (ISGylation), unconjugated intracellular and extracellular in the free form(25). ISGylation is important in mice for anti-viral immune responses(26). The mechanism was thought to be important in human anti-viral activity until Bogunovic et al., in 2012, reported that ISG15 deficient humans were not more susceptible to viral infections(27). On the contrary, the ISG15 deficient humans showed susceptibility to mycobacterial infections(28). Extracellular ISG15 is proposed to activate and act as a chemoattractant for neutrophils (25, 29). ISG15 induces dendritic cell maturation and the expression of E-cadherin, CD15 and CD86(25, 30). It is not yet clear whether it is the effect of ISG15 alone or synergistic to other signalling molecules(25). Swaim et al. showed how ISG15 synergises with IL-12 in inducing IL-10 and IFNγ secretion from NK-cells. They proposed that IL-12 induces transcription of cytokines, whereas ISG15 induces secretion(31). ISG15 could play a role in the inflammatory cascade during IBD(32).

Hypothesis and aims

Hypothesis

Intestinal epithelial cells (IECs) play a vital role in regulating homeostasis in the gut. We hypothesise that the intestinal epithelial cells are involved in initiating the inflammation observed in IBD. Furthermore, we hypothesise that secretion of ISG15 from intestinal epithelial cells plays a role when the inflammatory cascade spirals out of control.

Aims of the study

This study aims to elucidate how human-derived ex-vivo IECs regulate ISG15 and how it affects immune cells in combination with other signalling molecules. We also aim to explore a method for partially mimicking the crosstalk between the intestinal epithelial and immune cells. Lastly, we investigate whether ISG15 could be utilized as a biomarker for IBD. To aid us in these objectives, we have specified five research questions:

1. How is ISG15 regulated in human IECs?
2. How does ISG15 induce the secretion of cytokines in immune cells?
3. Does ISG15 potentiate the cytokine secretion from immune cells stimulated with LPS?
4. Can ISG15 from IECs stimulate cytokine secretion from immune cells?
5. Is there a difference in ISG15 plasma concentration between IBD patients and healthy controls?

Material and methods

Overview of contribution to the analysed material

Table 1

Experiment number	1	2	3
Design	Investigate if HT-29 cells treated with IBD relevant pro-inflammatory molecules secrete ISG15	Investigate whether colonoids treated with IBD relevant proinflammatory molecules express ISG15	Investigate if proliferating cells express ISG15
Material	C.M. from treated HT-29 cells	Sections of human colonoids treated with pro-inflammatory molecules	Sections of human colonoids treated IFNβ
Conditions	Unstimulated, Poly (I:C) (0.5, 5, 50 μg/ml), TNF (0.1,1.0 μg/ml), IL-1β (0.1 μg/ml)	Unstimulated, Poly (I:C) (50, 75 ng/ml), TNF (0.1ng/ml), IL-1β (0.1ng/ml), TNF + Poly(I:C) (0.1ng/ml + 20 ng/ml)	Unstimulated, IFNβ (10ng/ml),
Number of biological	1-4	1	1
Material collected by	IBD-group	IBD-group	IBD-group
Analysis	Sandwich ELISA	Immunohistochemistry with	Immunofluorescence
Readout	ISG15		
The analysis is done by	Gunnar A. Walaas	Gunnar A. Walaas	Gunnar A. Walaas

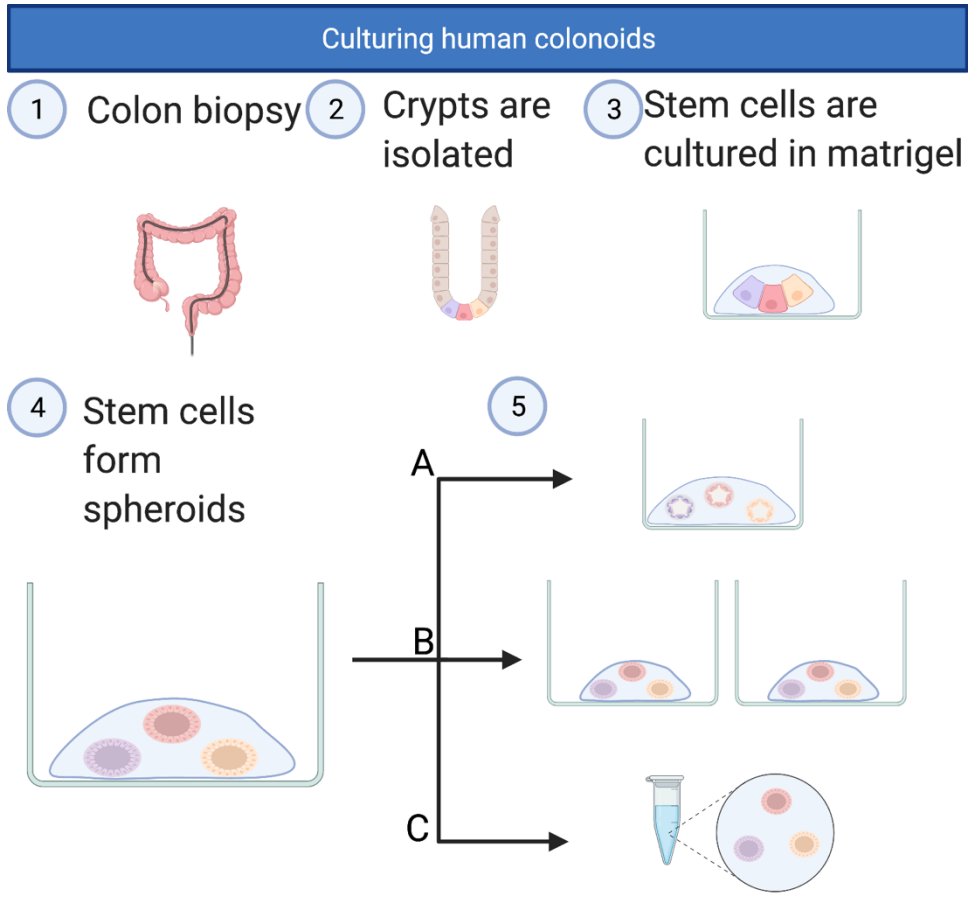
4	5	6	7	8
Investigate if human colonoids treated with IBD relevant pro-inflammatory molecules secrete ISG15	Investigate if Poly(I:C) directly or indirectly induces ISG15 secretion in HT-29 cells	Investigate if ISG15 potentiates cytokine secretion from LPS stimulated PBMCs	Treating PBMCs with C.M. from treated human colonoids.	Investigate whether extracellular ISG15 can be detected in human plasma and if it can be used as a biomarker for IBD
C.M. from treated human colonoids	C.M. from treated HT-29 cells	C.M. from treated human PBMCs	C.M. from treated human PBMCs	Human plasma
Unstimulated, TNF (0.1 µg/ml), TNF +IL17 (0.1 µg/ml), IL17, TNF +Poly(I:C) (0.1 + 20 µg/ml), Poly(I:C) (20 µg/ml), IL22, IL-1β, IL22 + IL1β (0.1 µg/ml)	Unstimulated, IFNβ (10ng/ml), Poly(I:C) (5µg/ml), TNF (0.1µg/ml), Anti-IFNβ antibody + IFNβ, Anti-IFNβ antibody + TNF, Anti-IFNβ antibody + Poly(I:C), Isotype antibody + IFNβ, Isotype antibody + TNF, Isotype antibody + Poly(I:C)	LPS: 0.0ng/ml, 0.01ng/ml, 0.1ng/ml, 1.0ng/ml, 10ng/ml, 100ng/ml + ISG15: 0ng/ml, 50ng/ml, 500 ng/ml	C.M. from untreated colonoids. C.M. from Poly(I:C) (20 µg/ml) treated colonoids. C.M. from untreated colonoids + IL-12 (0.02 µg/ml). C.M. from Poly(I:C) treated colonoids + IL-12. RPMI control. RPMI control + IL-12	Severe active IBD (aIBD), healthy controls (HC)
2-3	1-2	2	1	11 aIBD, 10 HC
IBD-group	IBD-group	Gunnar A. Walaas	Gunnar A. Walaas	IBD-group
Sandwich ELISA	Sandwich ELISA	Sandwich ELISA	Sandwich ELISA	Sandwich ELISA
ISG15	ISG15	CXCL8, IL-6	CXCL8	ISG15
Gunnar A. Walaas	Gunnar A. Walaas	Gunnar A. Walaas	Gunnar A. Walaas	Gunnar A. Walaas

C.M. = Conditioned medium

Human colonoids

Human colonoids were established from pinch biopsies from the colon (Illustration 3) with an optimised protocol from Mahe et al., 2015(33) based on Jung et al., 2011(34). Colonic crypts are isolated from the biopsy and grown in a basement membrane matrix (#734-1101, Matrigel, Growth Factor Reduced (GFR) Basement Membrane Matrix, phenol red-free, Corning®, New York City, NY). Initially, the growth medium contains factors promoting stem cell proliferation. The growth medium contains 50% Minigut A (Wnt-3A (CRL-2647, RRID: CVCL_0635, ATCC, Manassas, VA), BSA, GlutaMAX 100X, HEPES, Penicillin-Streptomycin, N2 and B27) and 50% Minigut B (Advanced DMEM (#12634028, Thermo Fischer Scientific, Bremen, Germany), BSA, GlutaMAX 100X, HEPES, Penicillin-Streptomycin, N2 and B27) with Nicotinamide (1:100), N-acetyl-L-cysteine (1:1000), Noggin (1:1000) (#120–10c, PeproTech, Rocky Hill, NJ), R-spondin (HA-R-Spondin1-Fc 293T Cells; # AMS. RSPO1-CELLS, RRID: CVCL_RU08, AMS Biotechnology, Abington, United Kingdom), A-83-01 (1:1000), SB202190 (1:3000), human EGF (1:10000), 15-Gastrin 1 (1:10000) and factor y-27632 (1:100). Proliferating stem cells from the colon form spherical structures called spheroids. After day 8-10 the medium is altered into a differentiation medium. The differentiation medium contains 5% Minigut A, 75% Minigut B with Noggin (1:1000), R-spondin, A-83-01 (1:1000), human EGF (1:10000), 15-Gastrin 1 (1:10000) and DAPT (1:10000). The stem cells differentiate into the functional cells of the colonic mucosa. Thus, a colonoid is formed containing stem cells, goblet cells, enteroendocrine cells and colonocytes.

A



B

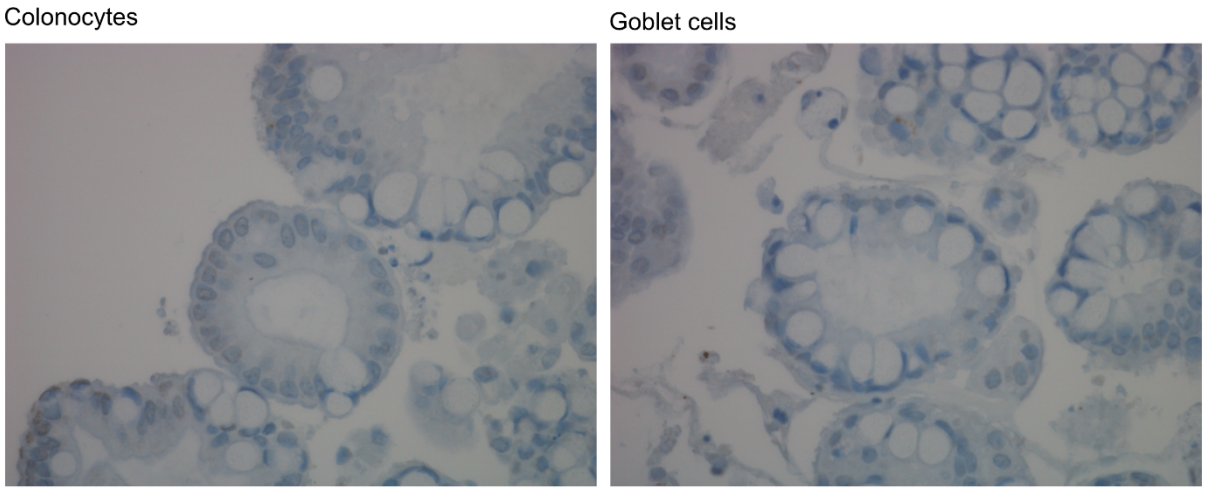


Illustration 3A: 1: Colonic pinch biopsies are collected from patients at St. Olav's University Hospital. 2: Stem cells reside in the bottom of colonic crypts. Therefore, crypts are isolated. 3: Initially, stem cells are dissociated into single cells and cultured in a proliferation-inducing medium. 4: Single stem cells form spheroids. 5: After the proliferation phase, the spheroids can either be further processed for differentiation (A), split into single stem cells for expansion (B) or frozen for storage (C). Created by G.A. Walaas with Biorender.com
Illustration 3B: H&E staining of human colonoids. The colonocytes are polarised with and the apical part facing the lumen and the nucleus at a basal position. Goblet cells have large mucus vacuoles and a characteristic small and curved nucleus. Staining and visualisation by G.A. Walaas

Peripheral blood monocytes

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats from healthy donors acquired from St. Olav's University Hospital, Department for Immunology and Transfusion Medicine (Illustration 4) (Supplementary Figure 1). The buffy coat and PBS (room temperature) were mixed in 1:1 proportion. The buffy coat-PBS solution was slowly added to a 50 ml tube containing Lymphoprep mononuclear cell separation medium (Axis-Shield, Oslo, Norway) to have two separate layers, one with buffy coat and one with Lymphoprep. The 50 ml tube was centrifuged (20 minutes, 800g) to separate plasma, mononuclear cells (PBMCs), and red blood cells. The PBMCs were extracted into a 50 ml tube, centrifuged at 950g for 10 minutes. After that, the supernatant was discarded, and 1-2 ml of RPMI was added to the PBMCs. The 1-2 ml cell solution was transferred to another 50 ml tube, and 30 ml of RPMI was added. The tube was centrifuged for 8 minutes at 150g. The supernatant was discarded, and 20 ml of RPMI was added.

In total, PBMCs from three donors were acquired. Donor 1 yielded 290 million PBMCs with 95% viability. Donor 2 yielded 380 million PBMCs with 94% viability. Donor 3 yielded 340 million PBMCs with 95% viability. From each donor, 14 million cells were extracted and plated in flat bottomed 96 well plates. The PBMCs were cultured in RPMI with L-glutamine (1%) and Penicillin-Streptomycin (1%) for 24 hours at 37,0°C in 5% CO_2 in a humid environment before stimulation.

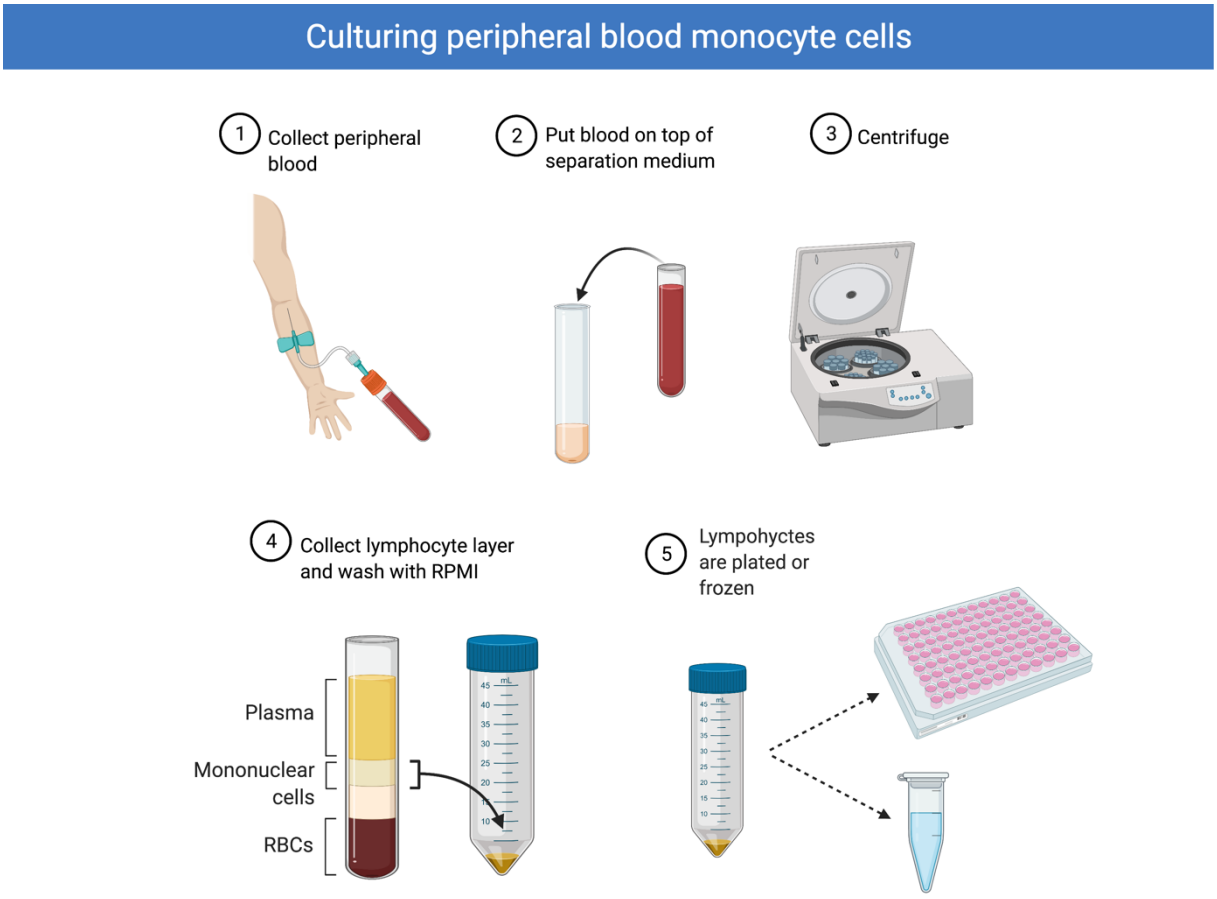


Illustration 4: 1) Peripheral blood was delivered by St. Olav's University Hospital, Department for Immunology and Transfusion Medicine. 2) Blood was transferred over to a 50 ml tube containing Lymphoprep. It was carefully discharged in order not to mix Lymphoprep and blood. 3) The tubes were centrifuged at 800g for 20 minutes. 4) After centrifugation, the red blood cells (erythrocytes) had moved through the Lymphoprep into a bottom RBC layer. Mononuclear cells were aggregated on top of the Lymphoprep. Plasma was also separated into its layer. With a pipette, the lymphocytes were collected and transferred into another 50 ml tube. The tube was centrifuged for 10 minutes at 950g and resuspended in HANKS. This was repeated three times. 5) The lymphocytes were either directly plated in an experiment or frozen in liquid nitrogen for later use. Figure created with BioRender.com by G.A.Walaas

Immunohistochemistry and -fluorescence

Fixed colonoids were embedded in paraffin and cut into 4-µm thick sections for histology; Haematoxylin and Eosin (H&E) staining, IHC and immune fluorescence (Illustration 5). The sections underwent standard pre-treatment with deparaffinisation, quenching of endogenous peroxidase and antigen retrieval antigens by 15 minutes boiling in citrate buffer (pH 6.0) in a microwave oven. Quenching of endogenous peroxidase is required to avoid background staining if the tissue contains endogenous peroxidase. Antigen retrieval breaks methylene bridges formed by the paraffin, thus exposing the antigen. Table 2 contains a list of primary antibodies. The immunoreactions were visualised using the secondary antibody rabbit/mouse EnVision-HRP/DAB+ kit (#K5007, Dako Agilent, Santa Clara, CA) and counterstained with haematoxylin.

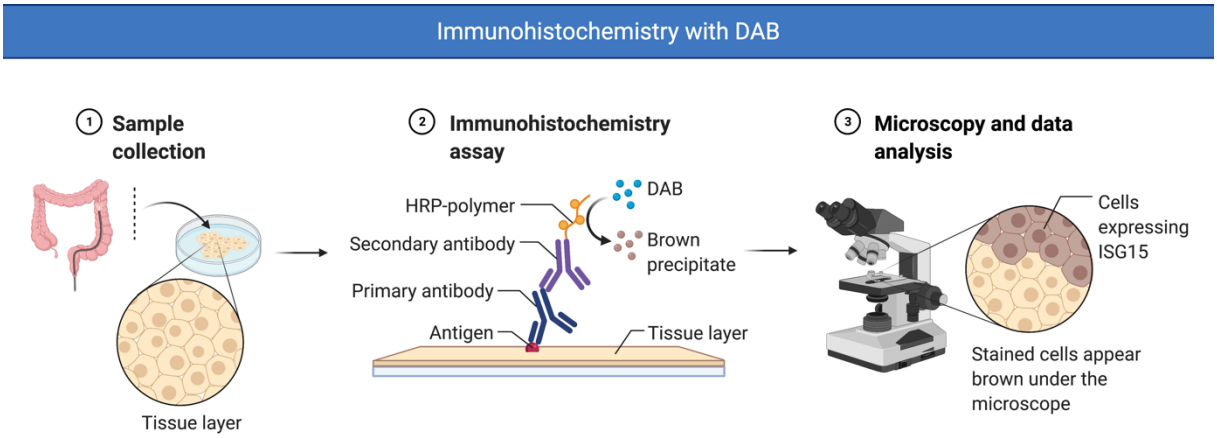


Illustration 5: 1) Biopsies are cut into sections for histological analysis. 2) The sections are treated with a primary antibody specific to the antigen of interest. Secondary antibodies bind to the primary antibodies and are attached with an enzyme (HRP). The enzyme oxidases DAB, creating brown precipitates that can be visualised. 3)The stained sections are investigated and imaged with a light microscope. Figure created with BioRender.com by G.A.Walaas

The immunofluorescence technique is an imaging technique that enables single molecules to be highlighted within a singular cell (Illustration 6)(35). Some molecules have a natural fluorescence, more commonly referred to as autofluorescence. Molecules that do not have an autofluorescent ability can be targeted by antibodies connected to a fluorochrome. Fluorochrome excitation requires ultraviolet (UV) light of a specific wavelength being directed at the fluorochrome. From a multispectral light source, light is emitted towards an excitation filter. The filter only allows UV light of a specific wavelength to pass. The UV light travels towards a dichromatic mirror and is directed towards the tissue with the fluorochromes. The excitation light excites the fluorochromes. Subsequently, the fluorochrome emits UV light in a lower energy state called emission light. The emission light is gathered by the dichromatic mirror and passes an emission filter. The filter only allows UV light of a known and wanted wavelength to pass. The result is a digital visualisation of the fluorochromes in a specific colour (red, green, blue) on a black background. It is possible to detect several fluorochromes within a tissue layer. This is achieved by having multiple emission filters. One picture is acquired with a specific emission filter. Then the emission filter changes and a new emission filter that enables UV light of a different wavelength to pass is used for the second picture. The two images can be combined, thereby visualising several fluorochromes within a specimen.

Immunofluorescence was performed using a double staining kit from Max Vision Bioscience Inc. Slides for immunofluorescence were blocked after antigen retrieval, then incubated with two primary antibodies ISG15 and Ki-67 (Table 2), overnight. The immunoreactions were

visualised using fluorescent marked secondary antibodies (MaxVision bioscience, Washington) and counterstained with DAPI (Max Vision bioscience, Washington) (Table 2). The immunofluorescent pictures were acquired with a confocal microscope from the Cellular & Molecular Imaging Core Facility centre at NTNU.

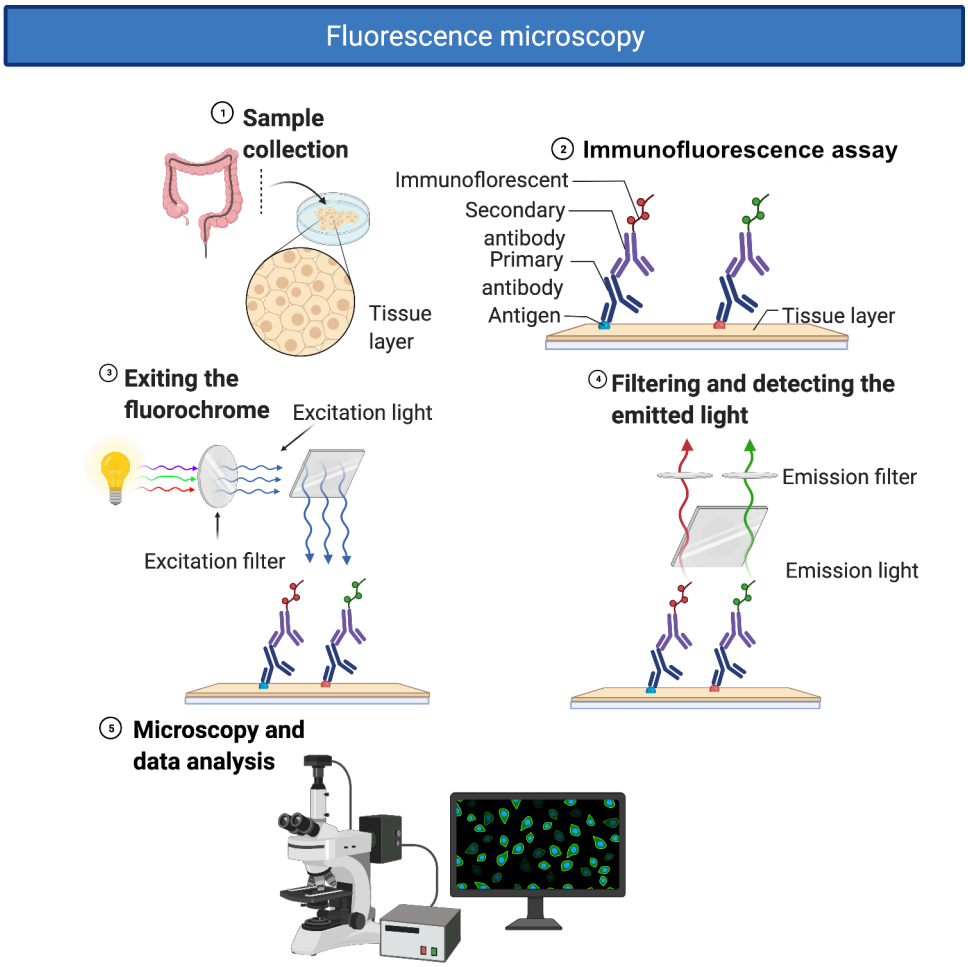


Illustration 6: 1) Biopsies are cut into sections. 2) The sections are treated with a primary antibody specific to the antigen of interest. Treating the sections with different primary antibodies enables the visualisation of multiple proteins. Secondary antibodies, labelled with a fluorochrome, bind to the primary antibody 3) Excitation light travels through an excitation filter. The filter attenuates light waves longer than a specific wavelength and transmits shorter wavelengths. The excitation light then gets reflected by the dichroic mirror and is directed towards the sample. The excitation light excites the fluorochromes. 4) To return to a normal state, the fluorochromes release photons (emission light). The emission light is transmitted through the dichroic mirror. The emission filter attenuates unwanted wavelengths and transmits the emission light. Lastly, the emission light hits a detector that forms an image 5) The image is processed with a computer program before the final image is ready. Figure created with BioRender.com by G.A.Walaas

Table 2: Immunohistochemistry reagents

Name	RRID	Dilution	Reference number	Manufacturers
ISG15 Rabbit pAb (human reactivity)	AB_2549338	1:200 and 1:300	PA5-31865	Thermo Fischer Scientific, Bremen, Germany

Prolong. Diamond antifade with DAPI		Prediluted	P369666	Thermo Fischer Scientific, Bremen, Germany
Dako. Monoclonal mouse. Anti-Human Ki67 antigen	AB_2142367	1:50	M7240	Agilent Technologies Inc., California, CA.
Immunofluorescent kit: MaxDouble. M&R Immunofluorescence double staining kit (w/MaxFluor 488 & MaxFluor 650)		Prediluted	DSMR-H3	MaxVision Biosciences Inc., Washington

Enzyme-linked immunosorbent assay (ELISA)

ELISA was used as a quantitative method for measuring different proteins. Materials used for analysing human plasma, HT-29 and colonoid conditioned medium, were collected by other members of the IBD group at NTNU. Materials were collected and analysed as stated in Table 1. There are four main types of ELISA: direct, indirect, sandwich and competitive(36). With direct ELISA, the target antigen is immobilised and detected with an antibody. The antibody enables a substrate to signal the reaction proportional to the amount of antigen captured by the antibody. Indirect ELISA resembles direct ELISA but requires an extra amplification step. The primary antibody is amplified by a secondary antibody which in turn reacts with the substrate. Sandwich ELISA is the most specific but also the most time-consuming ELISA protocol (Illustration 7). It requires capture antibodies to be added before the antigen. The capture antibodies are coated in a well. The antigen is added afterwards and binds to the pre-coated antibody. Detection antibodies are added after the antigen to visualise the antigen. Competitive ELISA is used to detect molecules too small for sandwich ELISA. The wells are coated with a primary antibody. The antigen is added at the same time as a competitive secondary antibody. A competitive secondary antibody is required to produce the signal. Therefore, the amount of antigen present is inversely related to the colour of the well. We chose a sandwich ELISA (Table 3) for our experiment because it is sensitive and specific, compatible with the conditioned medium, and available for the antigens of interest.

All samples were analysed in duplicates. 96 well plates were pre-coated with capture antibody and incubated overnight. The plate was washed with a wash buffer (0,05% tween 20 in PBS) between every step until adding the cleaving enzyme. The plate was blocked with reagent

diluent (1% BSA in PBS, R&D systems, #DY006, Minneapolis, MN) and incubated for at least 1 hour. The sample solution and standard were added and incubated for 2 hours at room temperature. Detection antibodies were added and incubated for two hours. "Streptavidin-HRP" were added and incubated for 20 minutes. Second, to last, a substrate solution (1:1 mixture of colour reagent A (H_2O_2) and colour reagent B, R&D systems, #DY999) was added and incubated for 20 minutes. The enzyme cleaves the substrate solution producing colour. Lastly, a stop solution (2N H_2SO_4 R&D System) was added to stop the cleaving process.

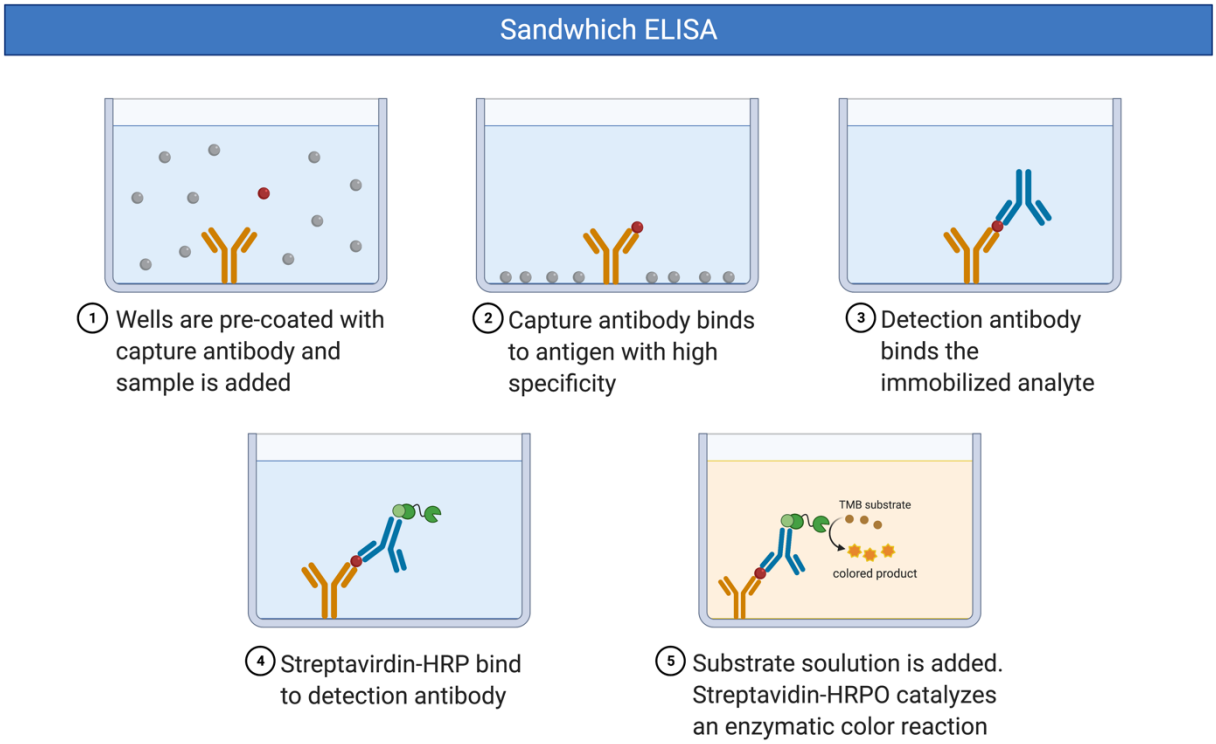


Illustration 7: Sandwich ELISA: 1) The wells are pre-coated with a capture antibody, and any excess capture antibodies are washed off. The sample is added afterwards. 2) Proteins in the sample, specific for the capture antibody, will bind to the capture antibody. Any excess proteins in the sample are washed off. 3) Detection antibody binds to the target antigen 4: Streptavidin-HRP is an enzyme that binds to the detection antibody 4) Substrate solution (1:1 H2O2 and Tetramethylbenzidine) reacts with streptavidin-HRP and creates a coloured product. Figure created with BioRender.com by G.A.Walaas

Table 3: ELISA reagents

ELISA target	ELISA type	RRID	Reference number	Company
ISG15	Pre-coated sandwich ELISA		CY-8085	Circulex
CXCL8	Sandwich ELISA	AB_2892143	DY-208	R&D
IL-6	Sandwich ELISA	AB_2814717	DY-206	R&D
IFN-y	Sandwich ELISA		88-7316-88	Invitrogen

Reagents	Components	Company
Wash buffer	PBS + 0.05% Tween 20	In house

Reagent diluent	PBS + 1% BSA	In house
Substrate solution	1:1 H_2O_2 + Tetramethylbenzidine	In house
Stop solution	2N H_2SO_4	In house

Statistics

Statistical analyses were performed with Prism 8 (GraphPad Software, San Diego, CA). Significant values were defined as p-value <0.05 (two-sided). The datasets were analysed for normality with the Andersons-Darlings test, D'Agostino & Pearsons test, Shapiro-Wilk test, and Kolmogorov-Smirnov test. Anderson-Darling's test compares the cumulative distribution of a sample dataset to the cumulative distribution of a Gaussian distribution(37). D'Agostino & Pearsons test correlates the skewness and kurtosis of the sample dataset compared to a Gaussian distribution(37). Skewness is the lack of symmetry compared to a Gaussian distribution, while kurtosis is the peakedness of a distribution(38). Shapiro-Wilk correlates observations in a dataset to corresponding normal scores. The Kolmogorov-Smirnov test is a type of empirical distribution function that correlates a dataset's cumulative distribution to a reference dataset.

The datasets were not normally distributed. It is important to analyse the distribution of datasets for normality because it directs further analyses. Normally distributed datasets can be analysed with parametric analyses. Parametric analyses could also be used if the sample number within a dataset is great enough. The non-parametric test does not require information about the distribution of the samples. Therefore, the non-parametric test can be used for datasets without normal distribution. Datasets with two variables and without normal distribution were analysed with the Mann-Whitney test. In addition to failing the normality test, the datasets did not have a large enough sample size to be analysed with students T-test. Datasets with multiple variables were analysed with the Kruskal-Wallis test. After that, they were analysed with the post hoc test Dunn's multiple comparisons test. The Kruskal-Wallis test determines whether medians of three or more groups are different but does not specify which groups showed a difference. Dunn's test dissects which groups of the Kruskal-Wallis were significantly different.

Ethical approval

All human material is collected after informed consent. These projects go under an existing REK approval (5.2007.910 and 2013/212/REKmidt). Animals are not used for this thesis.

Results

HT-29 cells secrete ISG15 upon pro-inflammatory treatment

Extracellular ISG15 has immune regulating qualities by potentiating the effect of other cytokines(31). The role of ISG15 in the crosstalk between the IECs and the immune system depends on it being secreted by the IECs. HT-29 is an epithelial cancer cell line derived from human colonic adenocarcinoma with shared qualities with moderately well differentiated, human IECs. Therefore, we investigated whether ISG15 was secreted by HT-29 cells upon treatment with IBD relevant pro-inflammatory ligands (Table 1, experiment 1) We analysed HT-29 conditioned medium from four different stimulation assays and combined them to visualise inter-assay variability and the differences in ISG15 secretion between each condition (Figure 1).

Several of the pro-inflammatory cytokines induced an increased ISG15 release in HT-29 cells. Most notably, the TLR3 ligand poly (I:C) induced the strongest secretion of ISG15. TNF also induced higher levels of ISG15 secretion in comparison to the untreated group. IL-1β induced approximately the same release of ISG15 as the control group. There was also a lack of dose-response for poly(I:C) and TNF treatment. The control group, without pro-inflammatory treatment, secreted a detectable amount of ISG15. Thus, the cancer cell line HT-29 secretes ISG15, and the cells increase their secretion of ISG15 in response to pro-inflammatory treatment.

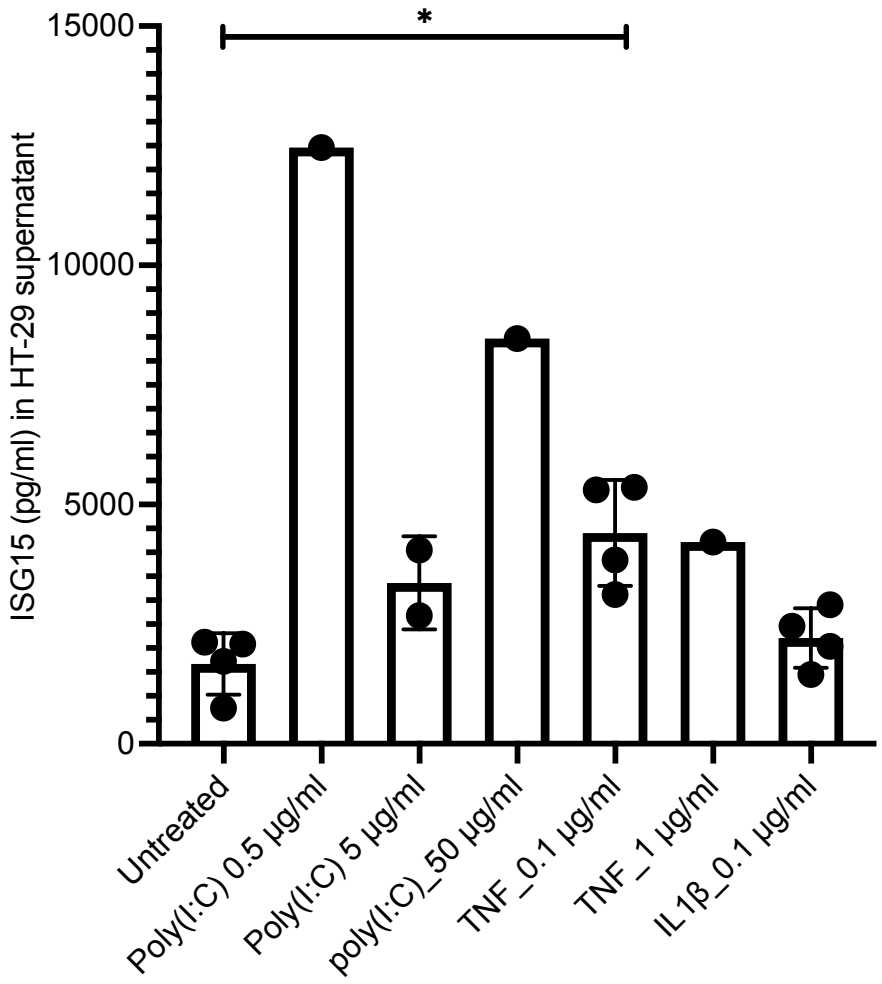


Figure 2: Treated HT-29 cells increase their ISG15 secretion. HT-29 cells were treated with Poly(I:C), TNF and IL-1β in different concentrations. The values were visualised in a scatter plot with a bar. Each dot represents a biological replicate. The X-axis shows the different conditions, and the Y-axis the amount of secreted ISG15. Some of the conditions had too few biological replicates to enable statistical comparisons. Kruskal-Wallis test with Dunn's multiple comparisons post-test was performed on those conditions with three or more biological replicates (TNF 0.1µg/ml and IL-1b 0.1µg/ml). There was a significant difference between unstimulated HT-29 cells and HT-29 cells stimulated with 0.1µg/ml TNF. P-value = 0.016

Intracellular ISG15 expressed by human colonoids

The expression of ISG15 in HT-29 implicates that ISG15 is regulated in intestinal epithelial cells. However, HT-29 is a cancer cell line that does not reliably reflect how IECs are regulated in vivo. Therefore, we investigated whether our findings in the HT-29 cells could be replicated in human colonoids.

The analysis of conditioned medium from HT-29 cells showed that pro-inflammatory treatment upregulates the secretion of ISG15. We, therefore, stained colonoids stimulated with different pro-inflammatory ligands (Table 1, experiment 2), (Figure 3). The results showed how unstimulated colonoids have a scarce expression of ISG15. Furthermore, different types of pro-inflammatory stimulation such as poly(I:C), TNF, and IFNβ induced

increased protein expression of ISG15. Visually, we did not observe an altered ISG15 expression upon stimulation with different concentrations of Poly(I:C) or the combination of Poly(I:C) and TNF.

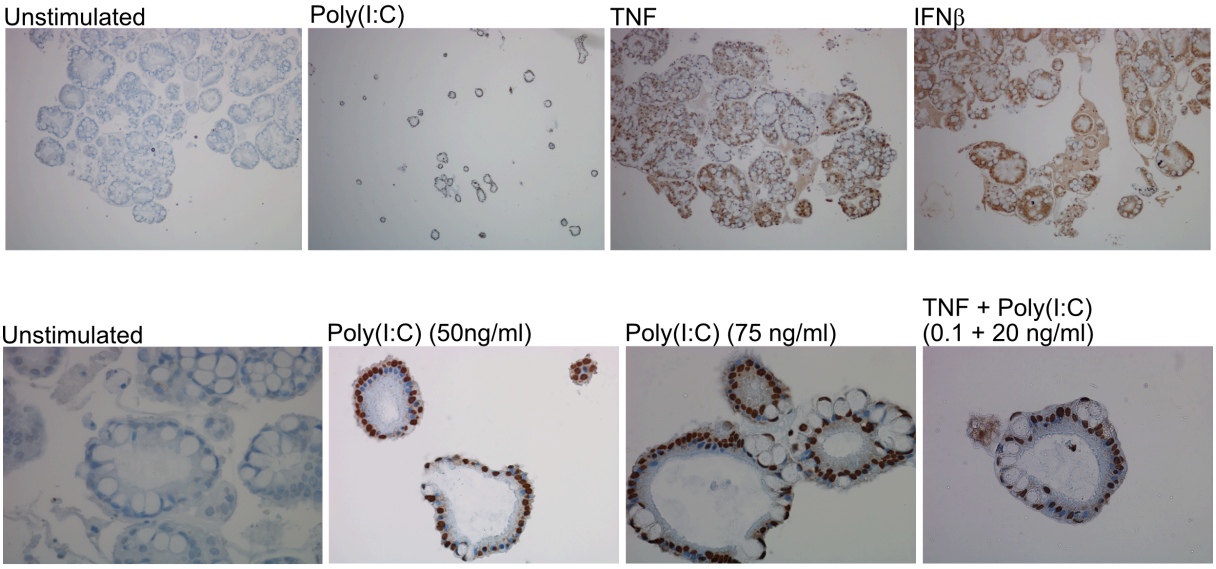


Figure 3: Intracellular ISG15 is expressed in human colonoids after pro-inflammatory stimulation. IHC was performed on sections of human colonoids. The colonoids were either unstimulated, stimulated with poly(I:C), TNF or IFNβ or stimulated with an increasing dose of poly(I:C). The figure shows representative images of colonoids with different conditions. Unstimulated colonoids did not express visible amounts of intracellular ISG15. IFNβ was a positive control since it is a known inducer of ISG15 transcription. Poly(I:C) and TNF also induce the expression of intracellular ISG15. Visually, there was no clear dose-response dynamic when stimulating the colonoids with different concentrations of poly(I:C). The combination of TNF and poly(I:C) also induced the expression of intracellular ISG15.

We further wanted to assess if there is a difference between cells with a potential for proliferation and differentiated cells in their expression of ISG15. Therefore, we stained colonoids with ISG15 in addition to the cell marker Ki-67 (Table 1, experiment 3) (Figure 4). Ki-67 is a marker for proliferation potential. The expression of Ki-67 indicates that the cell is not in a G_0 phase. Most of the cells expressing Ki-67 did not express ISG15 simultaneously, as evident by the clear green colour of some of the epithelial cells. However, some cells were coloured yellow, indicating a co-expression of ISG15 and Ki-67. The co-expression of Ki67 and ISG15 was more abundant in colonoids stimulated with IFNβ compared to unstimulated colonoids. The role of ISG15 in cells with a proliferation potential was not further addressed. Visually, IFNβ stimulated organoids expressed higher amounts of ISG15. However, the expression intensity was not quantified.

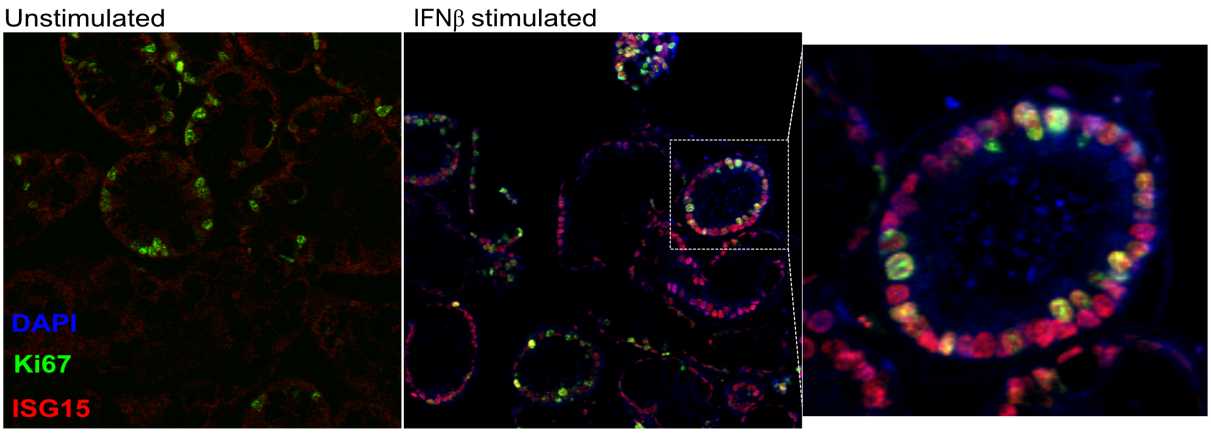


Figure 4: Immunofluorescence performed on unstimulated and IFNβ stimulated human colonoids. The fluorochromes were visualised with a confocal microscope. The blue fluorochrome represents DAPI, the green Ki-67 and the red ISG15. Ki-67 represents cells with a potential for proliferation. DAPI has a high affinity for DNA. Ki-67 is scattered within each colonoid. DAPI is present in each cell as well as in the lumen of the colonoids.

Human colonoids secrete free ISG15 upon pro-inflammatory stimuli

The IHC and immunofluorescence analysis showed that ISG15 is expressed within the IECs after pro-inflammatory stimulation. The question was, therefore, whether human colonoids also secreted ISG15 after pro-inflammatory stimulation. A preliminary experiment showed that pro-inflammatory stimulation upregulates the secretion of ISG15 (Figure 5a) in human colonoids. Subsequently, multiple pro-inflammatory ligands were analysed (Table 1, experiment 4) (Figure 5b). Different pro-inflammatory stimulations induced different ISG15 responses in the colonoids. The combination of poly(I:C) and TNF stimulation induced a significant secretion of ISG15 compared to unstimulated (p-value = 0.017).

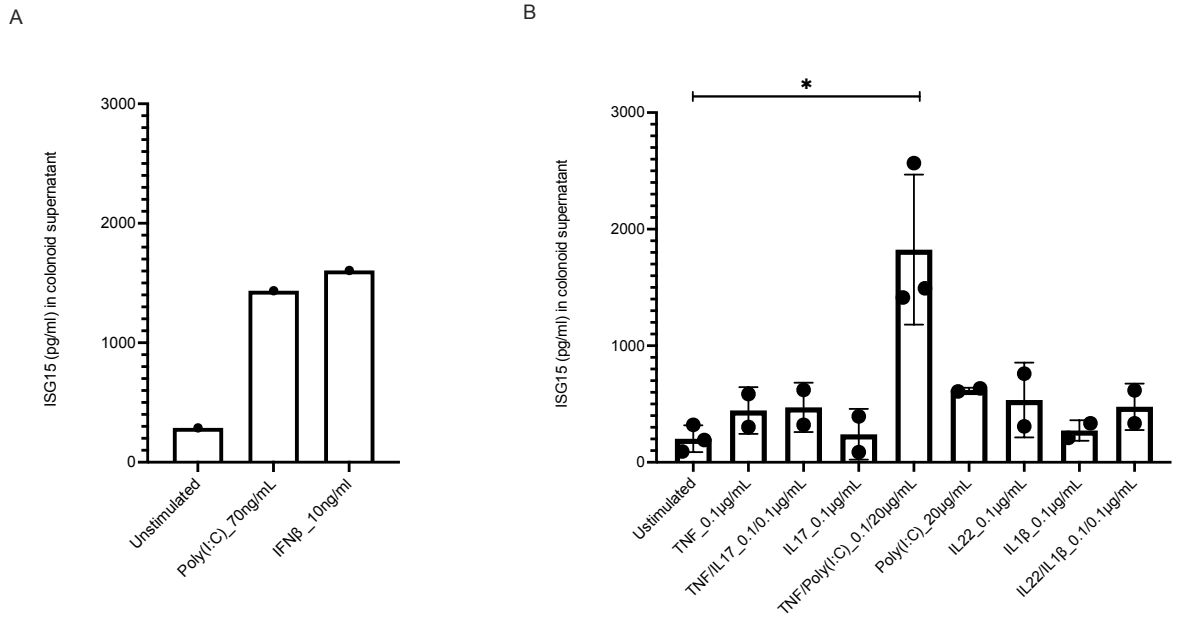


Figure 5: Pro-inflammatory stimuli increase ISG15 secretion from human colonoids. The x-axis displays the different conditions. The y-axis visualises the concentration of ISG15 present in the conditioned medium. Each dot represents a biological replicate.
A: A pilot study with human colonoids stimulated with poly(I:C) and IFNβ. The amount of secreted ISG15 increased after stimulation.
B: A follow up study with a larger stimulation panel. The colonoids were stimulated with TNF, TNF in combination with IL-17, IL-17, TNF in combination with poly(I:C), poly(I:C), IL-22, IL-1b and IL-22 in combination with IL-1b. Mann-Whitney test for non-parametric variables was performed on conditions with three or more biological replicates. Colonoids stimulated with the combination of TNF and poly(I:C) had a significant increase in supernatant ISG15. P-value = 0.017

Poly(I:C) induced secretion of ISG15 is IFNAR independent in HT29 cells

The combination of TNF and poly(I:C) was the most potent inducer of ISG15 secretion. Conditioned medium is a cocktail of cytokines where it can be challenging to assess whether the effect of one ligand is direct or through the induction of another mediating cytokine. The mechanism of IFNβ induction of ISGs is by paracrine or autocrine binding to IFNAR. Poly(I:C) stimulation resulted in a stable secretion of ISG15 with or without the presence of an anti-IFNβ antibody (Tabel 1, experiment 5) (Figure 6). TNF induced secretion of ISG15 was decreased upon treatment with an IFNβ antibody. With the isotype control, TNF induced secretion of ISG15 similar to TNF alone. IFNβ induced secretion of ISG15, but in the presence of an IFNβ antibody, the response was equal to untreated HT-29 cells. Thus, Poly(I:C) induces secretion of ISG15 is IFNAR independent in HT-29 cells.

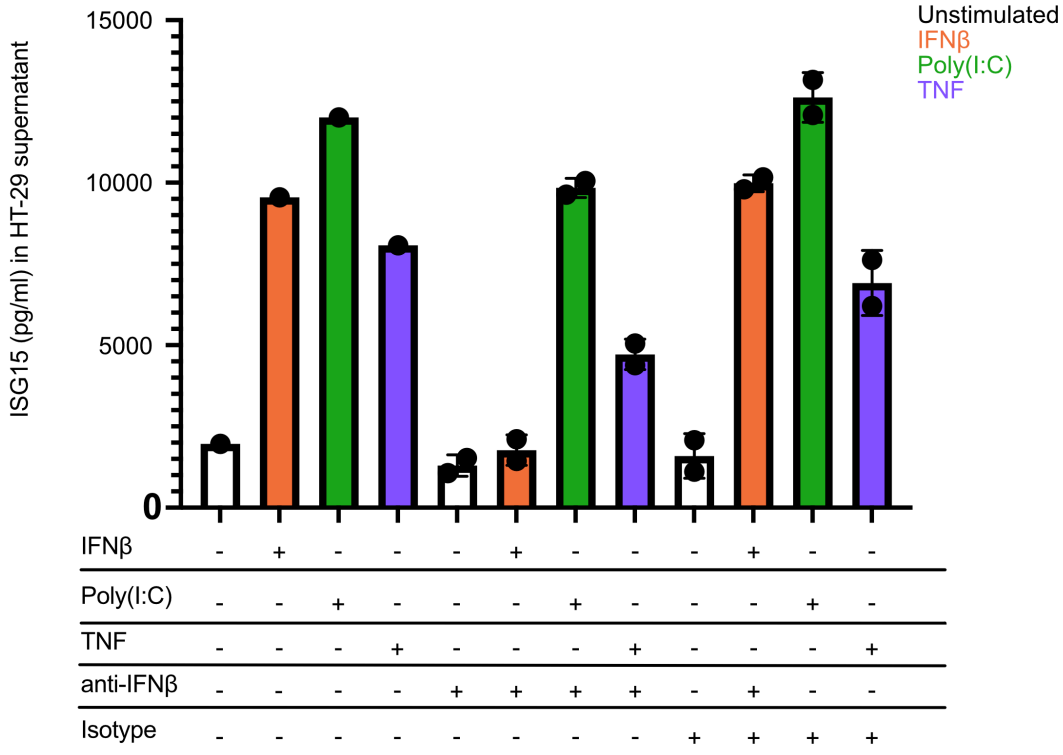


Figure 6: Poly(I:C) induced secretion of ISG15 is IFNAR independent in HT-29 cells. ISG15 secreted from stimulated HT-29 cells in combination with an IFNβ antibody. HT-29 cells were stimulated with IFNβ, poly(I:C) and TNF. In addition, an IFNβ antibody was added to the conditions and an isotype antibody control. Without the IFNβ antibody, poly(I:C) and TNF treated HT-29 cells have increased ISG15 secretion compared to unstimulated HT-29 cells. In combination with an IFNβ antibody, the amount of ISG15 secreted from IFNβ treated cells decreased. TNF stimulated HT-29 cells were partially affected by the IFNβ antibody. Poly(I:C) induced ISG15 secretion was unaffected by the IFNβ antibody.

Extracellular ISG15 potentiates cytokine release from PBMC

The secretion of extracellular ISG15 from IECs indicates a role in immune regulation in the intestinal mucosa. Therefore, we investigated what effect stimulation with ISG15 had on PBMCs combined with a bacterial antigen, LPS (Table 1, experiment 6) (Illustration 8). The PBMCs were treated with LPS and ISG15 and incubated for 48 hours.

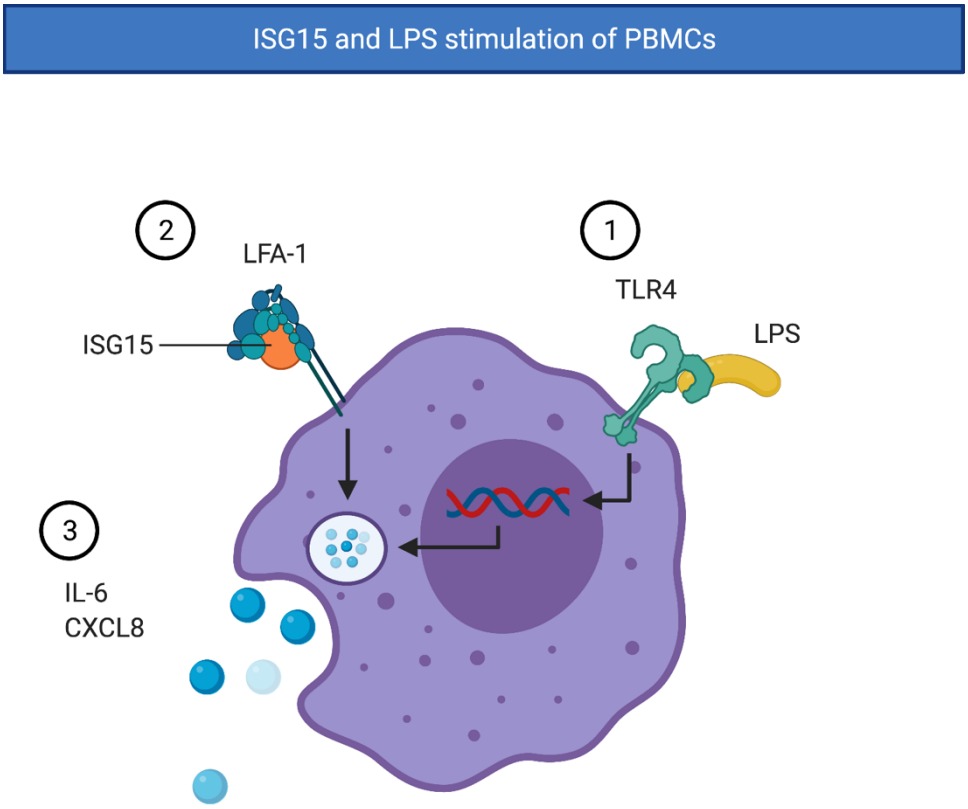


Illustration 8: Experimental design of ISG15 and LPS co-stimulation. 1: First, PBMCs were stimulated with LPS, a protein found on gram-negative bacteria. Innate immune cells express TLR4, which is the receptor for LPS. TLR4 activation induces transcription of several cytokines. 2: Secondly, the PBMCs were stimulated with ISG15, which binds to its receptor LFA1 to induce vesicle exocytosis. 3: Lastly, the amount of secreted IL-6 and CXCL-8 in the PBMC supernatant were quantitatively analysed with ELISA. Figure created with BioRender.com by G. A.Walaas

The PBMC results showed donor-dependent variability. The combination of high concentrations of LPS and ISG15 seemed to have a synergistic effect on the secretion of

CXCL8 and IL-6 (Figures 7 and 8). Furthermore, the more ISG15 present, the greater the secretion of CXCL8 and IL-6. At lower LPS concentration, ISG15 did not show the same potentiating effect. ISG15 alone did not induce secretion of either CXCL8 or IL-6.
There is also a positive correlation between the LPS dose and the secretion of CXCL8 and IL-6. However, cytokine secretion is saturated at LPS concentration between 10-100ng/ml, evident in the similar cytokine secretion between 10ng/ml and 100ng/ml LPS.

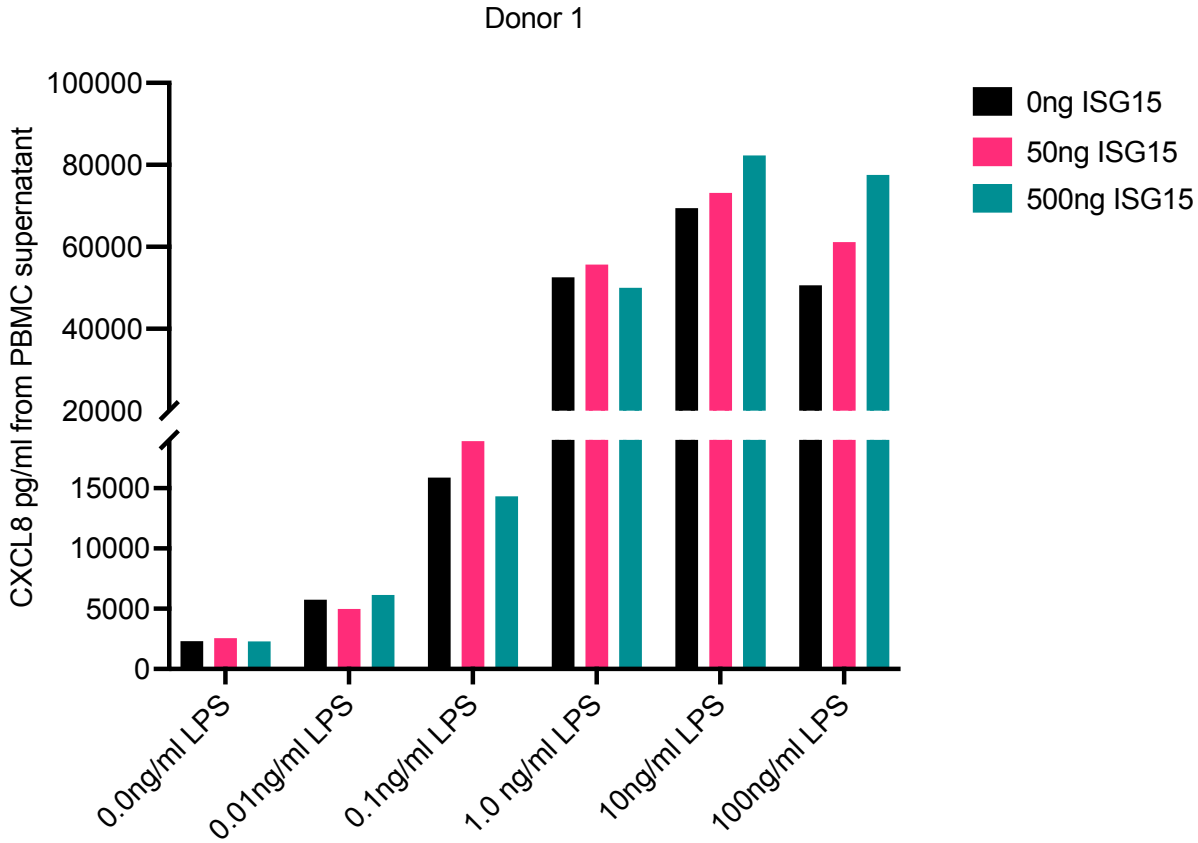


Figure 7: ISG15 potentiates CXCL8 secretion from PBMC with high doses of LPS treatment. CXCL8 secretion from PBMCs stimulated with an increasing dose of LPS in combination with an increasing dose of ISG15. PBMCs were stimulated with LPS and ISG15 and incubated for 48 hours before the supernatant was harvested and analysed with ELISA. The x-axis represents increasing doses of LPS treatment. The y-axis represents the concentration of CXCL8 in the PBMC conditioned medium after treatment. The colours represent different concentrations of ISG15 treatment. CXCL8 secretion increased in a dose-response manner with increasing LPS stimulation. CXCL8 secretion is similar between 10ng/ml and 100ng/ml LPS. ISG15 secretion potentiates CXCL8 secretion in combination with 10- and 100n/ml LPS.

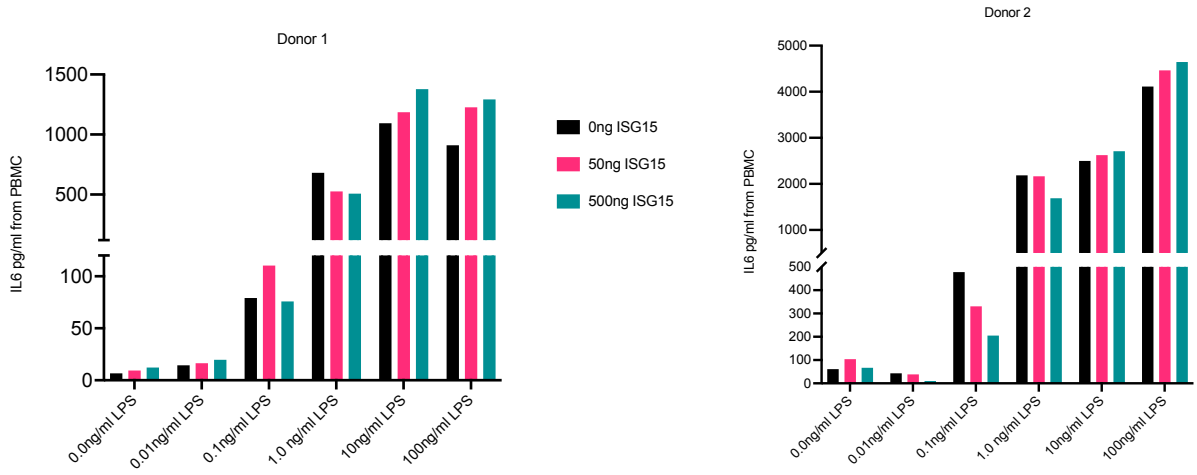


Figure 8: IL-6 secretion from PBMCs stimulated with LPS and ISG15. PBMCs were stimulated with LPS and ISG15 and incubated for 48 hours before the supernatant was harvested and analysed with ELISA. The x-axis represents increasing doses of LPS treatment. The y-axis represents the concentration of IL6 in the PBMC conditioned medium after treatment. The colours represent different concentrations of ISG15. There is a dose-response between increasing LPS concentration and IL-6 secretion. For donor 1, IL-6 secretion is similar when stimulated with 10- and 100ng/ml LPS. For donor 2, IL-6 secretion increases between 10- and 100ng/ml. The potentiating effect of ISG15 becomes evident with higher doses of LPS.

Crosstalk between colonoids and PBMC

To partially mimic mucosal crosstalk between IECs and intestinal immune cells, we stimulated PBMCs with a conditioned medium from colonoids stimulated with pro-inflammatory ligands (Table 1, experiment 7) (Illustration 9). We hypothesised that colonoids stimulated with poly(I:C) would secrete more ISG15 which in combination with IL12 would yield a higher pro-inflammatory response from the PBMCs. IL12 was chosen because other groups have shown how ISG15 has a potentiating effect on immune cells combined with IL12(31).

The colonoids were either treated with poly(I:C) to induce ISG15 secretion, or unstimulated. The PBMCs were incubated with conditioned media from the colonoids for 5 hours before IL-12 was added. Then, the PBMCs were incubated for 24 hours. See Table 1 for an overview of the different conditions.

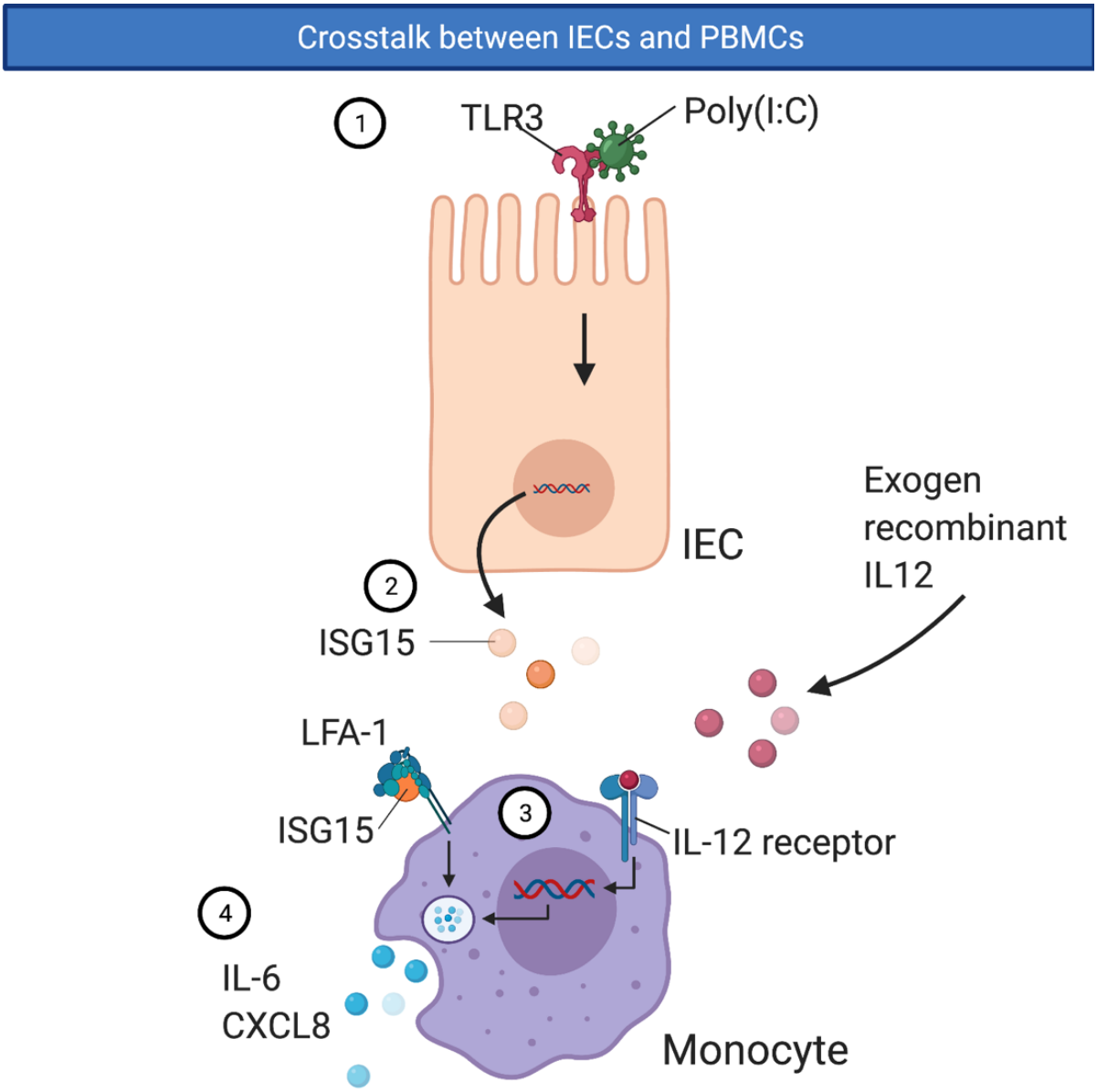
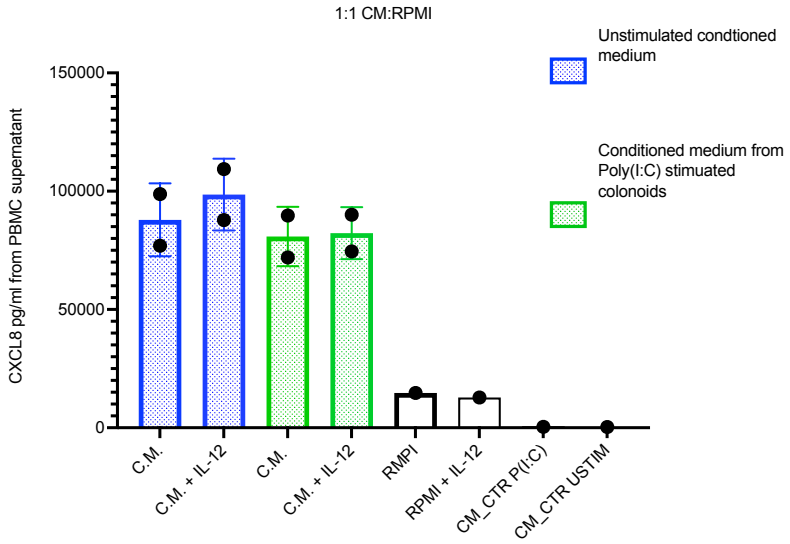
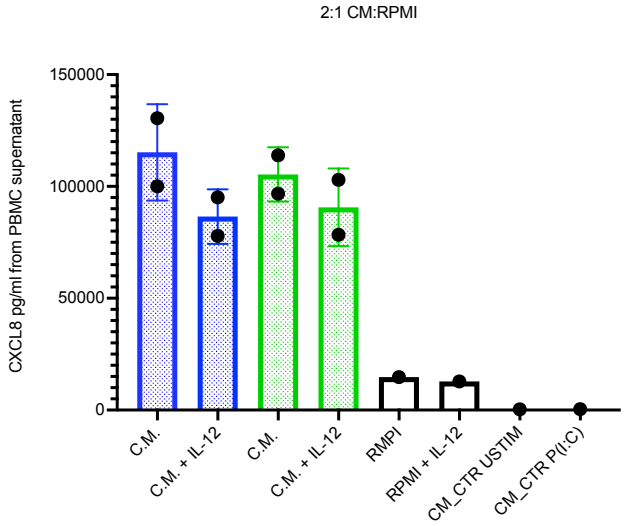


Illustration 9: Experimental design for stimulating PBMC with conditioned medium from colonoids. 1 and 2: As previously shown, poly(I:C) stimulation of IECs induces ISG15 secretion from IECs. 3: The PBMCs were stimulated with conditioned medium from the colonoids with ISG15 present, combined with exogenous recombinant IL-12. Swaim et al. (2017) showed that exogenous ISG15 potentiates cytokine secretion in combination with IL-12. 4: The PBMC supernatant was analysed with ELISA for IL-6 and CXCL8. Figure created with BioRender.com by G.A.Walaas

Both PBMCs groups stimulated with colonoid conditioned medium had increased, equal, secretion of CXCL8. Additional IL-12 stimulation did not affect the amount of CXCL8 secreted (Fig. 9). The RPMI stimulated PBMCs had a lower CXCL8 response. The control media did not contain any CXCL8.


1:1 CM:RPMI
Unstimulated condtioned medium
Conditioned medium from Poly(I:C) stimuated colonoids
CXCL8 pg/ml from PBMC supernatant
150000
100000
50000
0
C.M.
C.M. + IL-12
C.M.
C.M. + IL-12
RMPI
RPMI + IL-12
CM_CTR P(I:C)
CM_CTR USTIM


2:1 CM:RPMI
CXCL8 pg/ml from PBMC supernatant
150000
100000
50000
0
C.M.
C.M. + IL-12
C.M.
C.M. + IL-12
RMPI
RPMI + IL-12
CM_CTR USTIM
CM_CTR P(I:C)

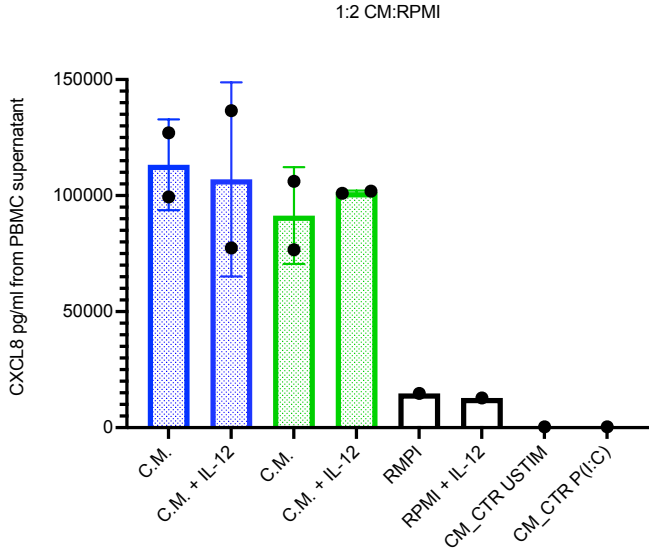

1:2 CM:RPMI
CXCL8 pg/ml from PBMC supernatant
150000
100000
50000
0
C.M.
C.M. + IL-12
C.M.
C.M. + IL-12
RMPI
RPMI + IL-12
CM_CTR USTIM
CM_CTR P(I:C)

Figure 9: Stimulating PBMCs with conditioned media from colonoids increases CXCL8 secretion.
Firstly, human colonoids were either unstimulated or stimulated with poly(I:C). The conditioned medium from the colonoids was harvested and used to stimulate PBMCs in combination with IL-12. As a control, the PBMCs were stimulated with RPMI in combination with IL-12. The conditioned medium from the colonoids was also analysed separately, without contact with PBMCs. The conditions are visualised in a bar graph. The blue bars represent CXCL8 secretion from PBMCs stimulated with conditioned medium from unstimulated colonoid. The green bars represent CXCL8 secretion from PBMCs stimulated with poly(I:C) stimulated colonoid conditioned medium. The uncoloured bars represent control conditions. The three separate graphs represent PBMCs stimulated with different concentrations of conditioned medium from the colonoids. 2:1 CM:RPMI represents the highest concentration of the conditioned medium, and 1:2 CM:RPMI is the lowest conditioned medium.
The CXCL8 response is increased in PBMCs stimulated with conditioned media compared to RPMI treatment. There was no difference in CXCL8 secretion from PBMCs stimulated with unstimulated conditioned medium and poly(I:C) stimulated conditioned medium. The concentration of CXCL8 was low in the conditioned medium from the colonoids. Increasing concentrations of conditioned media did not affect the absolute value of CXCL8 secretion.

Extracellular ISG15 is detectable in human plasma

ISG15 is present in the intestinal mucosa and upregulated during IBD(32). We confirm previous reports that ISG15 is secreted extracellularly. (31). Amoung the cell types that secrete ISG15 are IECs. Therefore, we wanted to investigate if ISG15 could be detected in blood and if there was a difference in the amount present in healthy controls vs IBD patients. We performed a quantitative ELISA with plasma from 11 patients with severe active IBD (aIBD) and ten healthy controls (Figure 1). The same patient cohort was used in a previous publication from our lab (Supplementary Table 1)(39). The ELISA analysis showed that ISG15 is present in circulating blood but with overlapping concentrations between healthy controls and IBD patients. The differences in ISG15 concentrations were not significant (IBD vs HC p-value = 0.0583).

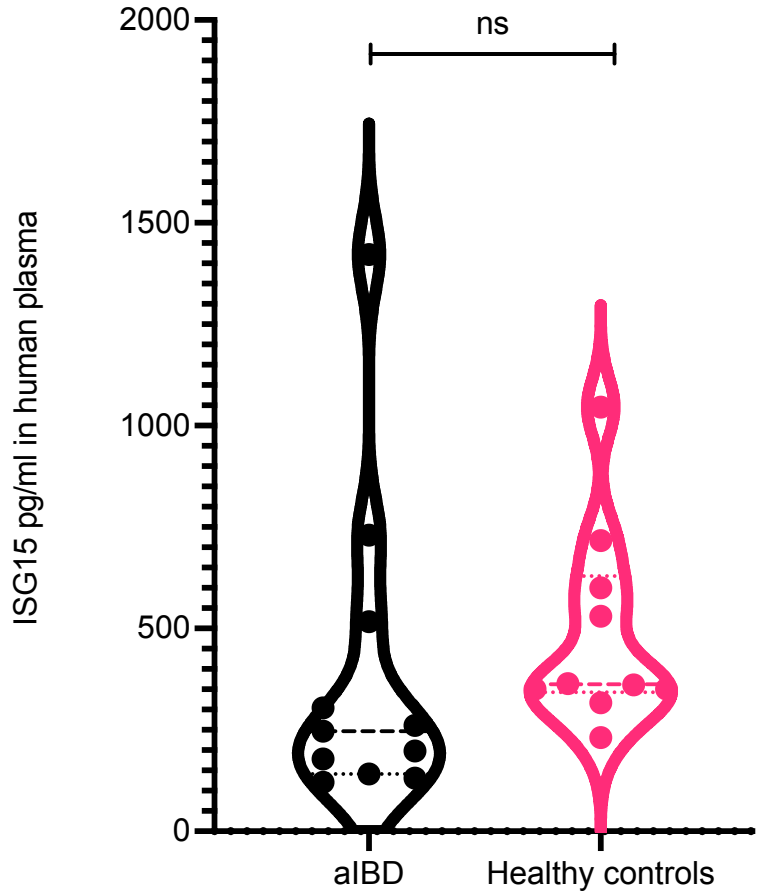


Figure 1: Extracellular ISG15 is present in human plasma. Plasma from patients with active IBD (aIBD) and healthy controls was analysed with ELISA to quantify the amount extracellular ISG15 present. The violin plot visualizes the distribution frequency of each sample. In addition, the median is shown (striped line) and the upper and lower quartile (dotted lines). The groups did not show a normal distribution and thus analysed with Mann-Whitney test. The concentration of ISG15 in the plasma was not significantly different between aIBD and healthy controls. Although the extracellular ISG15 values were overlapping, ISG15 is present and detectable in human plasma. P-value = 0.058

Discussion

Initially, we specified five research questions that are partially answered in this thesis.

1. How is ISG15 regulated in human IECs?
2. How does ISG15 induce the secretion of cytokines in immune cells?
3. Does ISG15 potentiate the cytokine secretion from immune cells stimulated with LPS?
4. Can ISG15 from IECs stimulate cytokine secretion from immune cells?
5. Is there a difference in ISG15 plasma concentration between IBD patients and healthy controls?

We show that ISG15 is regulated in IECs upon treatment with TNF, Poly(I:C) and both in combination. Traditionally ISG15 is thought to be induced by type 1 interferons such as IFNβ. However, our findings support the findings of others that ISG15 can be induced by different types of pro-inflammatory stimuli(24, 32, 40, 41). These findings were confirmed both with the cancer cell line HT-29 and human colonoids. An important note is the lack of dose-response dynamic by the HT-29 cells upon pro-inflammatory stimuli. The 0.5 μg/ml Poly(I:C) treatment a induced secretion of more ISG15 compared to 5 μg/ml and 50 μg/ml. Higher doses of Poly(I:C) could have toxic effect on the IECs affecting the amount of ISG15 secreted. Saturation upon pro-inflammatory stimuli is also important to consider in the interpretation of quantitative results.

ISG15 secretion upon poly(I:C) treatment was not dependent on IFNβ signalling in contrast to TNF, which seemed partially dependent on IFNβ. Our observation that ISG15 secretion upon poly(I:C) treatment was independent on IFNβ and its receptor IFNAR, stands somewhat in contrast to the findings of Swaim et al. (24). They showed that IFNAR$^{-/-}$ mice splenocytes did not secrete significant concentrations of IFNγ compared to splenocytes from wild types, which showed significant secretion of IFNγ upon poly(I:C) and ISG15 co-stimulation. A difference between the model system of Swaim et al. and our model system is that Swaim et al. used mice derived splenocytes, whereas, in this thesis, we used human-derived IECs. One explanation for the observed difference is that identical PRRs initiates different responses depending on which cell type they are expressed on, which tissue the cell resides in and where in the tissue the cell resides. A danger signal (PAMP or DAMP) recognised in the blood stream rquires a more severe response than a danger signal recognised in the intestinal lumen. Splenocytes are white blood cells located in the spleen. Therefore, splenocytes might respond differently to the same danger signal. Another explanation is the fact that Swaim et al. investigated mice whereas we investigated HT-29 cells. TLR3 recognises poly(I:C)(42) and could be responsible for the induction of ISG15. However, it is important to note for further investigation that poly(I:C) can activate other signalling pathways such as retinoic acid-inducible gene 1, melanoma differentiation-associated protein 5 and protein kinase R(43).

Investigating ISG15's effect on immune cells is central to unveiling its role in mucosal inflammation. Swaim et al. showed that ISG15, in combination with IL-12, potentiates IL-10 and IFNγ secretion in natural killer cells(31). We further investigated whether ISG15 could have the same function in combination with LPS. PBMCs were stimulated with ISG15 and

LPS. The analysis showed that ISG15 potentiated CXCL8 and IL-6 secretion in combination with LPS. ISG15 did not induce cytokine secretion without LPS. For donor 1, we observed saturation of cytokine secretion between an LPS dose of 10ng/ml and 100ng/ml. The potentiating role of ISG15 suggests that ISG15 could drive the inflammatory cascade in the presence of other inflammatory stimuli. Supporting the potentiating role of ISG15 is a recent publication by Sanyal et. al in Nature Immunology(44). They show that Sars-Cov2 induced extracellular ISG15 amplifies cytokine release in PBMC-derived macrophages. Interestingly they found that human bronchial epithelial cells (NHBE cells) express ISG15 on a gene level, but these epithelial cells do not secrete ISG15.

Biology is complicated. It is difficult to extrapolate results from cancer cells and animal studies to humans. Therefore, methods resembling in vivo like conditions could yield results transferable to humans. We investigated a method for partially mimicking crosstalk between human IECs and human PBMCs by treating immune cells with a conditioned medium from colonoids in combination with IL-12. We hypothesised that pro-inflammatory stimulation of IECs would affect the cytokine response from the PBMCs. The colonoids were stimulated with poly(I:C) to induce an increased ISG15 secretion. Thereafter, the PBMCs were treated with a combination of conditioned medium and IL-12. The assay showed that it is possible to stimulate PBMCs with a conditioned medium from colonoids. Our hypothesis, on the other hand, was not confirmed. The PBMCs response was equally strong in conditioned medium from unstimulated colonoids compared to poly(I:C) stimulated colonoids. This indicates that something in the conditioned medium induces a cytokine response in the PBMCs, but it was independent of ISG15 present. The controls showed that it was not IL-12 itself inducing CXCL8 secretion and that the conditioned medium from the colonoids did not contain substantial cytokine concentrations.

Since ISG15 can be secreted from IECs, we investigated whether free ISG15 was present in the plasma of patients with active IBD compared to healthy controls. A clinical biomarker requires a distinct separation between groups to have sufficient sensitivity and specificity for a disease. Because of the overlap between the two groups, ISG15 seems unsuitable as a biomarker for IBD. However, extracellular ISG15 was present and detectable in human blood. The number of patients in the assay was small. Evaluating a biomarker requires a larger cohort than presented in this thesis. A common challenge when investigating potential biomarkers is donor-dependent variability. In our study for ISG15 as a biomarker for IBD, we

only included 11 IBD patients and ten healthy controls. As seen in Figure 1, the donor differences are substantial. However, larger studies are expensive and time-consuming. Thus, a balance between cost and utility should always be considered.

There are some other limitation and challenges in the present study: The minimum requirement for statistical analysis is three biological replicates. For several of the assays in this study, we had too few biological replicates, which compromised the biological insight gained from these studies. Another challenge appears when biological replicates do not behave in a similar fashion. Genetic differences are always present with human donors. For the PBMC studies, we observed inter-donor variability, which could be explained by genetic differences between the donors. Another cause of inter-donor variability is a variation of PBMC composition. The proportion of the different leukocytes within each donor was not investigated. Thus, a difference in composition could contribute to inter-donor variability. A third challenge when using PBMCs in mucosal immunology studies is that PBMCs might behave differently than immune cells residing in the gut. Due to time limitations, it was difficult to establish a new method for studying gut immune cells. In our assay stimulating PBMC with conditioned medium from colonoids we did not include a Matrigel control. Matrigel contains, among other things, transforming growth factor β, epidermal growth factor, insulin-like growth factor 1 and fibroblast growth factor. In hindsight, we should have included a control for conditioned medium cultured in Matrigel without colonoids.

The potentiating effect of ISG15 was not consistent between the doses of LPS stimulation. It was only for the higher LPS doses that ISG15 showed a potentiating effect. This could be related to the specific function of ISG15. Perhaps because ISG15 induces exocytosis, a potentiating secretory effect is not seen unless a certain amount of a cytokine is present intracellularly. Future investigations of ISG15s function in a similar assay should be repeated multiple times for each donor, with an increased number of donors. Other co-stimulatory ligands should be investigated to disclose whether ISG15 has a general potentiating effect or only in combinations with specific ligands. A common concern when utilizing recombinant protein is contamination. Recombinant proteins are often produced in bacteria and could be contaminated with microbial products. Previously, endotoxin levels in the recombinant ISG15 we used was found to be well below recommended limit(32). Further, since ISG15 alone did not induce CXCL8 or IL-6 secretion, it appears that the recombinant protein used was acceptably purified.

Our findings reflect the complexity of biology and the need to investigate molecular functions in more comprehensive systems. It is often easier to interpret results in an isolated systems but more challenging to interpret in a biological system. A possible cause for this phenomenon is that isolated systems lack the biological crosstalk present in complex systems. Another cause for the discrepancy is a functional difference between recombinant and biological proteins. A newly published article in The Journal of immunology by Nilsson et al. addresses the issue of drawing conclusions based on recombinant proteins(45). Recombinant proteins undergo purification processes that could alter their function compared to biological proteins. Whether this is the case for ISG15 as well remains to be seen. Due to time limitations, the method was only tested with one colonoid donor and one PBMC donor. Assays with multiple donors are essential for interpreting the result with certainty. Future investigations could introduce antibodies in the colonoid conditioned medium to pinpoint what drives the cytokine response from the PBMCs.

Conclusion

We set out to investigate what role ISG15 has in the crosstalk between IECs and immune cells. We show that human IECs express and secrete ISG15 upon poly(I:C), TNF and IFNβ treatment. Once secreted, ISG15 can potentiate cytokine secretion from immune cells in combination with LPS. How ISG15 potentiates LPS is not yet discovered, but our results indicate that ISG15 does not induce CXCL8 and IL-6 secretion without another pro-inflammatory stimulus. We show that it is possible to stimulate PBMCs with a conditioned medium from colonoids. However, we found no difference in cytokine secretion from PBMCs stimulated with conditioned medium containing ISG15 compared to conditioned medium without ISG15. Free ISG15 us present in human peripheral blood but seems unsuitable as a biomarker for IBD. Future work could investigate the mechanistic function of ISG15 induced cytokine secretion. It would also be interesting to further develop the method for stimulating immune cells with a conditioned medium from human colonoids. Then, with more insight into the components in the conditioned medium from the colonoids, the results might be easier to interpret.

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Supplementary

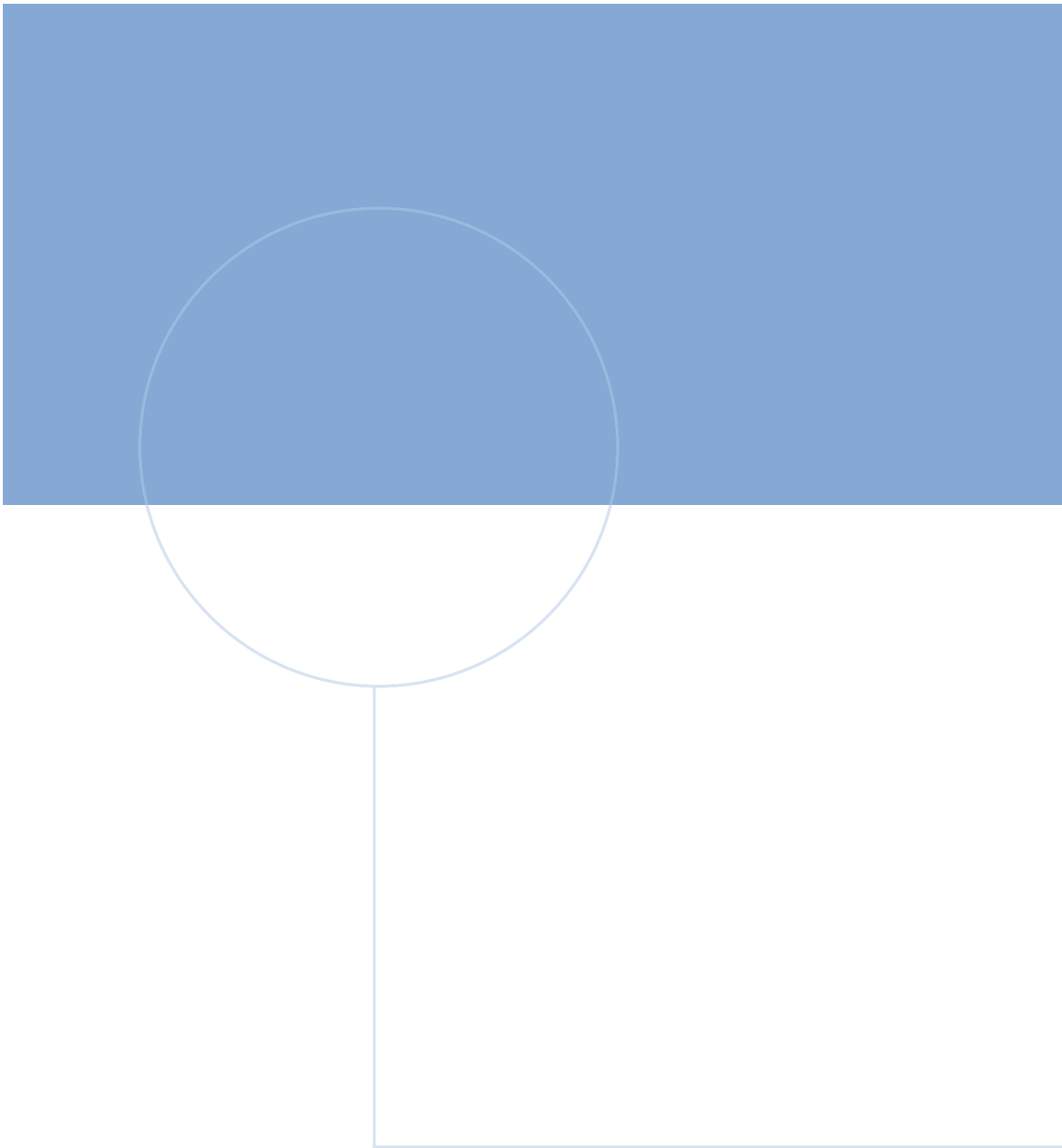
Supplementary table 1. This table is aquired from a previous publications by the IBD group at NTNU. (39)

	Controls	IBD	*P*
No. of subjects	9	9	NS
Age (range)	34 (23–66)	37 (25–65)	NS
Female sex (%)	2 (22.2)	2 (22.2)	NS
Duration of disease (range)	0	0 (0–12)	NA
5-ASA (%)	0	4 (44.4)	NA
Systemic corticosteroids (%)	0	0	NS
hsCRP (range)	1.1 (0.2–4.9)	27.7 (0.2–222.6)	0.01*

Age, duration of disease, and hsCRP (mg/L) are given as median and gender and medication are given as numbers.

*Significantly higher serum hsCRP in IBD versus control subjects.

ASA, aminosalicylic acid; hsCRP, high sensitive C-reactive protein; NA, not available; NS, nonsignificant.



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