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Title	Investigation of the TNF superfamily of ligands as potential therapeutic targets in inflammatory bowel diseases
Authors	Rajaram, Subhasree
Publication date	2022-05-10
Original Citation	Rajaram, S. 2022. Investigation of the TNF superfamily of ligands as potential therapeutic targets in inflammatory bowel diseases. PhD Thesis, University College Cork.
Type of publication	Doctoral thesis
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Investigation of the TNF superfamily of ligands as potential therapeutic targets in inflammatory bowel diseases

Thesis presented by

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for the degree of

Doctor of Philosophy

University College Cork

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2022

Table of Contents

Table of Contents i
Author's Declaration vi
Data Attribution vii
Acknowledgement viii
List of Poster Presentations and Publications x
List of Figures xii
List of Tables xv
List of Abbreviations xvi
General Abstract xxv
Chapter 1 : Introduction 1
1.1. Inflammatory Bowel Disease 2
1.1.1. Immunobiology and Immunopathogenesis of IBD 2
1.1.1.1. Intestinal Barrier 3
1.1.1.2. Role of T cells in IBD 6
1.1.1.3. The role of cytokines in IBD 7
1.2. IBD therapeutics- success, failures and novelties 13
1.2.1. Classic anti-inflammatory and immunosuppressive drugs 13
1.2.2. Anti-cytokine therapies 14
1.2.2.1. Anti-TNF-α 14
1.2.2.2. Other anti-cytokine therapies 15
1.2.3. Anti-adhesion therapies 15
1.2.4. Small molecule inhibitors 16
1.2.4.1. JAK inhibitors 16
1.2.4.2. Other small molecule inhibitors 17
1.2.5. Challenges in IBD therapeutic success 18
1.3. The TNF superfamily 21
1.4. TNFSF superfamily in Inflammatory Bowel Diseases 27
1.4.1. Role of TNF-α in IBD 27
1.4.2. FasL 30
1.4.3. TRAIL 32
1.4.4. TWEAK 34
1.4.5. LIGHT 36

1.4.6. TL1A 37
1.5. IFN-γ and TNF superfamily ligands 41
1.5.1. Interferon-gamma (IFN-γ) 41
1.5.2. IFN-γ synergism with TNF-α 45
1.5.3. IFN-γ and other TNFSF 49
1.6. Aims of the thesis 52
1.7. References 54
Chapter 2 : IFN-γ and TNF-α synergise to induce STAT1 dependent cell death through pathway cross regulation 95
2.1. Abstract 96
2.2. Introduction 98
2.3. Materials and Methods 102
2.3.1. Cell culture 102
2.3.2. Generation of CRISPR/Cas9-mediated gene knockout line 102
2.3.3. Human studies 103
2.3.4. Primary human colonic organoid culture 103
2.3.5. Recombinant proteins, reagents and antibodies 105
2.3.6. Microarray gene expression profiling 106
2.3.7. Transcriptional regulator prediction 107
2.3.8. Functional enrichment analysis 107
2.3.9. Identification of gene modules using WGCNA 108
2.3.10. Endpoint cell viability and cell death assays 109
2.3.11. Cell cycle analysis 109
2.3.12. RNA isolation and RT-qPCR 110
2.3.13. Western blotting 111
2.3.14. RNAi mediated gene knockdown 112
2.3.15. Homogeneous Time Resolved Fluorescence (HTRF) STAT1 and NF-κB Phosphorylation Assays 113
2.3.16. Statistics 113
2.4. Results 116
2.4.1. IFN-γ and TNF-α synergise to induce cell cycle arrest and cell death 116
2.4.2. IFN-γ and TNF-α treatment differentially regulates a higher proportion genes compared to individual cytokine treatment 116

2.4.3. IFN-γ and TNF-α regulate genes associated with multiple cell death pathways, DNA replication and repair and immune response 122
2.4.4. IFN-γ and TNF-α induced inflammatory cell death is independent of canonical death pathways 123
2.4.5. WGCNA identifies fourteen highly co-expressed gene modules associated with individual and combination cytokine treatments 126
2.4.6. IFN-γ and TNF-α cross regulate each other's receptors and signalling components 131
2.4.7. IFN-γ and TNF-α induced cell death is dependent on STAT1 134
2.4.8. IFN-γ and TNF-α cross-regulate each other signalling pathways and synergise to induce cell death through the JAK1/2-STAT1 pathway in colonic organoids 138
2.5. Discussion 143
2.6. References 149
Chapter 3 : Screening of TNF superfamily ligands identifies TNF-α like ligands that synergise with IFN-γ 160
3.1. Abstract 161
3.2. Introduction 162
3.3. Materials and Methods 167
3.3.1. Cell Culture 167
3.3.2. Human Studies 167
3.3.3. Primary human colonic organoid culture 168
3.3.4. Recombinant proteins, reagents and antibodies 168
3.3.5. Endpoint cell viability assay 169
3.3.6. Crystal violet staining 169
3.3.7. Screening Protocol Optimisation 169
3.3.8. ELISA for chemokine analysis 172
3.3.9. Meso Scale Discovery (MSD) immunoassay for chemokine analysis 172
3.3.10. Heat map generation 173
3.3.11. Statistics 173
3.4. Results 174
3.4.1. IFN-γ and TNF-α synergise to induce Th1 chemokine production and cell death in colonic epithelial cancer cell lines 174
3.4.2. Optimisation of cell-based assays for screening TNFSF ligands for TNF-α-like biology 176
3.4.3. FasL, LT-α/β, TRAIL, TWEAK, BAFF and LIGHT synergise with IFN-γ to induce inflammatory cytokine/chemokine production and cell death 180

3.4.4. Validation screen confirms TRAIL, TWEAK and LIGHT as TNF-α-like ligands 186

3.4.5. TRAIL, TWEAK and LIGHT are confirmed as potential TNFSF ligand targets in human colonic organoids 190

3.5. Discussion 194

3.6. References 199

Chapter 4 : IFN-γ and TRAIL synergise to induce Th1 chemokines and cell death in human colon cancer cells in a caspase8-JAK1/2-STAT1 dependent manner 215

4.1. Abstract 216

4.2. Introduction 218

4.3. Materials and Methods 223

4.3.1. Cell Culture 223

4.3.2. Generation of HT-29 knockout lines 223

4.3.3. Generation of DLD-1 knockout lines 224

4.3.4. Colorectal cancer organoid line culture 225

4.3.5. Reagents and Antibodies 226

4.3.6. Cell Viability 227

4.3.7. Chemokine Analysis by ELISA 229

4.3.8. Meso Scale Discovery (MSD) immunoassay for chemokine analysis 229

4.3.9. RNAi mediated gene knockdown 229

4.3.10. RNA isolation and RT-qPCR 230

4.3.11. Western Blotting 231

4.3.12. Homegenous Time Resolved Fluorescence (HTRF) assay 231

4.3.13. Statistics 232

4.4. Results 235

4.4.1. Differential sensitivity of a panel of colon cancer cell lines to TRAIL, IFN-γ or the combination of IFN-γ+TRAIL 235

4.4.2. TRAIL and IFN-γ induced inflammatory chemokine signature in colon cancer cell lines is not aligned with their effects on cell viability 243

4.4.3. IFN-γ and TRAIL induced synergistic cell death is independent of canonical death-receptor mediated apoptotic and necroptotic pathways 247

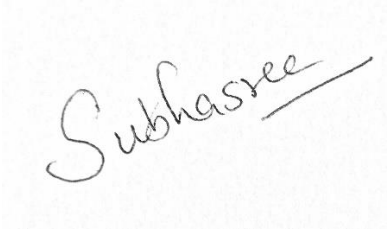
4.4.4. Synergistic cell death induced by the combination of IFN-γ and TRAIL is dependent on the JAK1/2-STAT1 pathway 255

4.4.5. IFN-γ and TRAIL induced cell death and CXCL10 production is dependent on caspases and the JAK1/2-STAT pathway in the TRAIL-sensitive DLD-1 cell line 264

4.4.6. Reduced STAT1 activation in colon cancer cell lines is associated with resistance to IFN-γ and TRAIL induced cell death 270
4.4.7. Colorectal cancer organoids are resistant to IFN-γ and TRAIL induced cell death 273
4.5. Discussion 275
4.6. References 281
Chapter 5 : General Discussion and Future Directions 294
5.1. Discussion 295
5.2. References 302

Author's Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism.



Subhasree Rajaram, MSc.

Data Attribution

I would like to acknowledge the following people for their help in generating data and figures in part for this thesis:

1. Dr Jerzy A. Woznicki for Fig 2.1 and performing the Affymetrix microarray experiment in Chapter 2
2. Dr Vijaykumar Muley for performing bioinformatics analysis and generating Fig 2.2, 2.3, and 2.4 of Chapter 2
3. Dr Peter Flood for his work on human primary colonic organoids in Fig 2.9 of Chapter 2, Fig 3.11 and Fig 3.12 of Chapter 3, and Fig 4.16 of Chapter 4
4. Dr Peter Flood and Dr Milan Bustamante-Garrido for generating data alongside me for Fig 3.3 and 3.4 of chapter 3
5. Dr Janaki Velmurugan for generating heat maps in Fig 3.3, 3.4, and 3.5 of Chapter 3
6. Dr Ciaran lee for generating HT-29 JAK-STAT CRISPR-cas9 knockouts used in Fig 2.7 of Chapter 2 and Fig 4.11 of Chapter 4
7. Dr Agnieszka Skowyra for generating DLD-1 JAK-STAT CRISPR-Cas9 knockouts used in Fig 4.14 of Chapter 4
8. Professor John Silke at Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia, for providing as a kind gift- parental, CASP8, CASP10, RIPK1, and RIPK3 knockout HT-29 cell lines used in Fig 4.8 and 4.12 of Chapter 4

Acknowledgement

ஶ்ரீ சித்தங்காத்தான் துணை. ஶ்ரீ பிரசன்ன மஹாகணபதயே நம:

Many people have contributed and supported me in the six years of work that resulted in this thesis. I take this wonderful opportunity to express my gratitude to them.

Firstly, to Dr Ken Nally for being a fantastic supervisor. Thank you for giving me the opportunity and showing confidence in me to be your PhD student. The interactions I have had with you in all these years have been very encouraging and have shaped me into the researcher I am today. With COVID-19 becoming a significant hurdle during the last phase, I appreciate the tremendous support you have shown me and making me believe that this PhD with you has been one of the best decisions of my life. Thank you.

Next, I would like to thank Dr Silvia Melgar for the guidance and valuable comments throughout my PhD course. I am glad to have started the mice experience with you and Valerio, which has given me the confidence to work with them in the future. I want to thank Dr Amanda Lohan for being our star project manager. This lab would not have been the same without you. Thank you for being a role model and showing me a career in project management. I want to express my gratitude to the Gut Inflammation group colleagues, Jerzy, Peter, Ciaran, Ana R, Janaki, Milan, Agnes and Karine, for their wonderful help and support. The collaborative work environment I have had has given me a wonderful time learning various skills. I want to thank AbbVie pharmaceuticals and Science Foundation Ireland for providing the funding for this project and APC Microbiome Ireland for the opportunity to showcase my work.

Friends and family play a significant role in a person's life, and during my PhD, I have had so many of them supporting me. A big thanks to Anjali for everything.

Words fall short of describing my gratitude to you. All I can say is I am glad to have been your housemate as that's when my life in Ireland changed. Another huge thank you to Nisha for all the help given, may it be with work or outside work. I could finish a lot of experiments on time because of your help over weekends. I also thank the people of lab 4.38, especially Vale, Sree, and Ana H. Thank you for making a work environment so fun and sharing stories from different parts of the world with me. I am thankful to my dance team 'Aatma' and Lekha, for being my stressbuster. I am grateful to my housemates for making the number 377 not just a number but an emotion. Thank you, 377, for not breaking down and standing tall till I finished my PhD. No place can beat you. And a big shout out to my friends in Dublin and my KV friends back home, who have stayed up at night to listen to my stories, who have constantly told me that they will always be there no matter where I am and have always made me realise that I can do it.

Last but not least, thanks to my entire first line of the family for constantly supporting and encouraging me. And finally, for my pillars of support, a huge thank you, father -Rajaram, mother-Shanthi, sister-Sreedevi, brother-in-law-Karthik Raghuram, and my niece-Shreshta. Without your support, I would have never made it this far. Thank you for believing in me and encouraging me to do what I wanted. Your blessings and prayers have helped me to cross the finish line today.

COVID-19 significantly affected the process of my PhD, but it greatly affected the lives of many. I would like to dedicate this thesis to all the lost lives and to the families significantly affected by the COVID-19 pandemic.

List of Poster Presentations and Publications

Poster Presentations

1. **Keystone Symposium- Why so many ways to die, 2019, Brazil**- *IFN-γ and TNF-α synergise through pathway cross-regulation to induce IFNGR1/2-JAK1/2-STAT1-mediated cell death of colonic epithelial cells.* **Subhasree Rajaram**, Vijaykumar Muley, Jerzy A. Woznicki, Peter Flood, Milán Bustamante-Garrido, Ciaran Lee, Fergus Shanahan, Syed Akbar Zulquernain, Jane McCarthy, Ken Nally
2. **APC symposium, 2019, Ireland-** *IFN-γ and TNF-α synergise through pathway cross-regulation to induce IFNGR1/2-JAK1/2-STAT1-mediated cell death of colonic epithelial cells.* **Subhasree Rajaram**, Vijaykumar Muley, Jerzy A. Woznicki, Peter Flood, Milán Bustamante-Garrido, Ciaran Lee, Fergus Shanahan, Syed Akbar Zulquernain, Jane McCarthy, Ken Nally

Lightning Oral Presentation

1. **APC symposium, 2019, Ireland- First Prize.** *IFN-γ and TNF-α synergise through pathway cross-regulation to induce IFNGR1/2-JAK1/2-STAT1-mediated cell death of colonic epithelial cells.* **Subhasree Rajaram**

Manuscripts (Published)

1. ***Cell Death & Disease 12, 864 (2021).***

   DOI: https://doi.org/10.1038/s41419-021-04151-3

   'TNF-α synergises with IFN-γ to induce caspase-8-JAK1/2-STAT1-dependent death of intestinal epithelial cells'- *Jerzy A.Woznicki, Nisha Saini, Peter Flood,* ***Subhasree Rajaram,*** *Ciaran M.Lee, Panagiota Stamou, Agnieszka Skowyra, Milan Bustamante-Garrido, Karine Regazzoni, Nyree Crawford, Simon S.McDade, Daniel B.Longley, Pedro Aza-Blanc, Fergus Shanahan, Syed A. Zulquernain, Jane McCarthy, Silvia Melgar, Bradford L.McRae and Ken Nally.*

Manuscripts (In Preparation)

1. **'IFN-γ and TNF-α Synergise through Pathway Cross-Regulation to Induce IFNGR1/2-JAK1/2-STAT1-mediated Cell Death of Colonic Epithelial Cells'**- ***Subhasree Rajaram****, Vijaykumar Muley, Peter Flood, Milán Bustamante-Garrido, Ciaran Lee, Fergus Shanahan, Syed A.Zulquernain, Jane McCarthy, Jerzy A. Woznicki, and Ken Nally*
2. **'IFN-γ and TRAIL synergise to induce Th1 chemokines and cell death in human colon cancer through a Casp8-JAK1/2-STAT1 pathway'**- ***Subhasree Rajaram****, Peter Flood, Ciaran Lee, Nisha Saini, Agnieszka Skowyra, Fergus Shanahan, Syed A.Zulquernain, Jane McCarthy, Silvia Melgar, Bradford L.McRae, and Ken Nally*

List of Figures

Figure 1.1: Cytokines in inflammatory bowel diseases 12

Figure 1.2: TNFSF signalling through their three types of receptors 22

Figure 1.3: Overview of the IFN-γ signalling 44

Figure 1.4: Molecular and cellular consequences of IFN-γ and TNF-α synergism in inflammatory bowel diseases 48

Figure 2.1: IFN-γ and TNF-α synergise to induce cell death in HT-29 cells 119

Figure 2.2: IFN-γ and TNF-α combined treatment has a different gene expression profile compared to either cytokine treatment alone 120

Figure 2.3: The combination of IFN-γ and TNF-α induces inflammatory cell death independent of canonical cell death pathways 125

Figure 2.4: WGCNA identifies fourteen highly co-expressed gene modules upon treatment with the combination of IFN-γ and TNF-α 129

Figure 2.5: IFN-γ and TNF-α cross-regulate each other's receptor mediated signalling pathways 133

Figure 2.6: Both STAT1 and NF-κB are required for the cross-regulation of signalling components induced by IFN-γ and TNF-α 136

Figure 2.7: IFN-γ and TNF-α induced cell death is dependent on STAT1 137

Figure 2.8: Cross regulation of the receptors and downstream signalling components by IFN-γ and TNF-α is observed in primary human healthy colonic organoids 140

Figure 2.9: Synergistic cell death induced by the combination of IFN-γ and TNF-α in organoids is dependent on the JAK1/2-STAT1 pathway 141

Figure 2.10: Model for cross-regulation of signalling pathways by IFN-γ and TNF-α leading to JAK1/2-STAT1 mediated synergistic cell death 142

Figure 3.1: TNFSF screen optimisation protocols 171

Figure 3.2: IFN-γ and TNF-α synergise to induce CXCR3 chemokine production and cell death 175

Figure 3.3: IFN-γ and TNF-α synergise to enhance inflammatory chemokine/cytokine production and induce cell death in HT-29 cells. 178
Figure 3.4: IFN-γ and TNF-α synergise to enhance inflammatory chemokine/cytokine production and induce cell death in DLD-1 cells 179
Figure 3.5: Inflammatory chemokine/cytokine production after 24 hrs of treatment with TNFSF ligands 181
Figure 3.6: TNFSF ligands did not affect cell viability of colonic epithelial cancer cell lines 182
Figure 3.7: Identification of TNFSF ligands with TNF-α-like properties based on synergistic regulation of CXCR3 chemokines in combination with IFN-γ 184
Figure 3.8: Identification of TNFSF ligands with TNF-α like property based on synergistic induction of cell death along with IFN-γ 185
Figure 3.9: Validation screen of seven selected TNFSF ligands alone and in combination with IFN-γ in HT-29 cells 188
Figure 3.10: Validation screening of seven selected TNFSF ligands alone and in combination with IFN-γ in DLD-1 cells 189
Figure 3.11: TNFSF ligands synergise with IFN-γ to induce Th1 chemokines in human healthy colonic organoids 192
Figure 3.12: TNFSF ligands synergise with IFN-γ to induce Th1 chemokines in human Crohn's disease colonic organoids 193
Figure 4.1: Colon cancer cell lines sensitive to TRAIL but resistant to IFN-γ at 24 hrs post-cytokine treatment 238
Figure 4.2: Colon cancer cell lines sensitive to TRAIL but resistant to IFN-γ at 72 hrs post-cytokine treatment 239
Figure 4.3: Colon cancer cell lines exhibit differential sensitivity to IFN-γ and TRAIL at 24 hrs post cytokine treatment 240
Figure 4.4: Colon cancer cell lines exhibit differential sensitivity to IFN-γ and TRAIL at 72 hrs post cytokine treatment 241

Figure 4.5: Baseline expression levels of components of the TRAIL and IFN-γ signalling pathways in a panel of colon cancer cell lines 242
Figure 4.6: IFN-γ and TRAIL induce different inflammatory chemokine signatures in a panel of colon cancer cell lines 245
Figure 4.7: IFN-γ and TRAIL induced synergistic cell death is independent of canonical apoptotic or necroptotic cell death pathways 249
Figure 4.8: IFN-γ and TRAIL induced synergistic cell death is partially dependent on caspase-8 253
Figure 4.9: JAK inhibitors rescue HT-29 cells from IFN-γ and TRAIL induced cell death 256
Figure 4.10: Knockdown of JAK1, JAK2 or STAT1 inhibits IFN-γ+TRAIL induced cell death and CXCL10 chemokine production 259
Figure 4.11: JAK1, JAK2 and STAT1 knockout cell lines are resistant to IFN-γ and TRAIL induced cell death 261
Figure 4.12: JAK inhibitors can block IFN-γ and TRAIL induced cell death and CXCL10 production in caspases and RIPK deficient HT-29 cells 263
Figure 4.13: Inhibition of both caspase and JAKs blocked IFN-γ and TRAIL mediated cell death in the TRAIL sensitive DLD-1 line 266
Figure 4.14: Caspase inhibition in JAK1, JAK2 and STAT1 knockout cell lines completely blocks IFN-γ+TRAIL DLD-1 cell death 268
Figure 4.15: IFN-γ and TRAIL resistant RKO cancer line has reduced activation of STAT1 upon treatment with IFN-γ+TRAIL 272
Figure 4.16: Colorectal cancer organoids are resistant to IFN-γ and TRAIL to IFN-γ and TRAIL-induced cell death. 274

List of Tables

Table 1.1: TNF Superfamily of Ligands 26

Table 2.1: ROCHE Universal Probe Library assays used in this study 114

Table 2.2: List of siRNAs used in this study 115

Table 4.1: crRNA sequence 225

Table 4.2 Seeding densities of cell lines used 228

Table 4.3 SMARTpool siRNA sequences 233

Table 4.4: Universal Probe Library Assays 234

List of Abbreviations

ADAM	A disintegrin and metalloproteinase
AICD	Activation-induced cell death
ANOVA	Analysis of variance
AP-1	Activating protein1
Apaf-1	Apoptotic protease activating factor-1
APC	Antigen presenting cells
APRIL	A proliferation-inducing ligand
5-ASA	Aminosalsilic Acids
ATF4	Activating transcription factor 4
ATG16L1	Autophagy related 16 like 1
ATP	Adenosine triphosphate
AZA	Azathiopurine
BAFF	B-cell activating factor
BCMA	B-cell maturation antigen
BMDMs	Bone marrow derived macrophages
BIM	BCL-2 interacting mediator of cell death
BSA	Bovine serum albumin
CD	Crohn's disease
CDH	Cadherin

cFLIP	Cellular FLICE-like inhibitory protein
cIAP	Cellular inhibitors of apoptosis proteins
CRC	Colorectal cancer
CTRL	Control
CTL	Cytotoxic T lymphocytes
CYLD	Cylindromatosis
CZP	Certolizumab pegol (anti-TNF)
DC	Dendritic cells
DcR	Decoy receptor
DD	Death domain
DED	Death-effector domain
DEGs	Differentially expressed genes
DISC	Death-inducing signalling complex
DMSO	Dimethyl sulphoxide
DR	Death receptor
DSS	Dextran sodium sulphate
EDA	Ectodysplasin A
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EMA	European medicines agency

ER	Endoplasmic reticulum
ETS	Erythroblast Transformation Specific
FADD	Fas-associated death domain
FDA	Food and Drug Administration
FERRO	Ferrostatin; 3-Amino-4-cyclohexylaminobenzoic acid ethyl ester
FBS	Fetal bovine serum
G'872	GSK-872; N-(6-(Isopropylsulfonyl)quinolin-4-yl)benzo[d]thiazol-5-amine
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GAS	Gamma-activated sequence
GBP1	GTPase guanylate binding protein
GI	Gastrointestinal tract
GITR	Glucocorticoid-induced TNFR-related protein
GO	Gene Ontology
GSA	Gene set analysis
GSDMD	Gasdermin D
GVHD	Graft versus host disease
GWAS	Genome-wide association studies
HNF	Human nuclear factor
HPRT	Hypoxanthine Phosphoribosyltransferase 1

HTRF	Homogeneous time-resolved fluorescence assay
HVEM	Herpes virus entry mediator
IBD	Inflammatory bowel diseases
IDO	Indoleamine-2,3-dioxygenase
IECs	Intestinal epithelial cells
IFN	Interferon
IFNGR	IFN-γ receptor
IgA	Immunoglobulin A
IκB	Inhibitor of NF-κB
IKK	IκB kinase
IL	Interleukins
ILCs	Innate lymphoid cells
IP3	Inositol-1,4,5-triphosphate
IRE1	Inositol requiring enzyme 1
IRF	Interferon regulatory factor
IRGM	Immunity-related GTPase family M protein
ISC	Intestinal stem cells
ISG	Interferon-stimulated genes
ISRE	Interferon-stimulated response element
IT	IFN-γ+TNF-α

ITr	IFN-γ+TRAIL
IκB	Inhibitor of kappa B
JAK	Janus kinases
JAM	Junction adhesion molecule
JNK	c-Jun N-terminal kinase
KD	Knock-downs
kDa	kilo Dalton
KO	Knockouts
LAMB1	Laminin beta 1
LIGHT	Homologous to **L**ymphotoxin, exhibits **I**nducible expression and competes with HSV **G**lycoprotein-D for **H**erpesvirus entry mediator, a receptor expressed by **T**-lymphocytes
LP	Lamina propria
LRP6	Lipoprotein receptor-related protein 6
LT	Lymphotoxin
LUBAC	Linear ubiquitin chain assembly complex
6-MP	Mercaptopurine
MAMP	Microbial-associated molecular pattern
MCP1	Monocyte chemoattractant protein
MHC	Major histocompatibility complex

MLCK	Myosin II regulatory light chain
MLKL	Mixed lineage kinase domain-like
MLNs	Mesenteric lymph nodes
MOMP	Mitochondrial outer membrane permeabilisation
MSCs	Mesenchymal stem cells
MSD	Meso scale discovery assay
mTOR	Mammalian target of rapamycin
MUC2	Mucin 2
NAC	N-Acetyl-L-cysteine
Nec1s	Necrostatin 2, 7-Cl-O-Nec-1; 5-((7-Cl-1H-indol-3-yl)methyl)-3-methylimidazolidine-2,4-dione)
NEMO	NF-κB essential modulator
NF-κB	Nuclear factor kappa B
NGF	Nerve growth factor
NIK	NF-κB inducing kinase
NK	Natural killer cells
NKT cells	Natural killer T-cells
NLR	NOD-like receptor
NLRP3	NLR family pyrin domain containing
NOD2	Nucleotide-binding oligomerisation domain-containing protein 2

NOS	Nitric oxide synthase
NSA	Necrosulfonamide
OPG	Osteoprotegrin
OSM	Oncostatin-M
PBS	Phosphate-buffered saline
PCC	Pearson correlation coefficient
PCR	Polymerase chain reaction
PDGF	Platelet-dervied growth factor
PERK	PKR like ER-kinase
PRDM5	PR domain containing protein 5
PRR	Pattern recognition receptor
qRT-PCR	Real-Time quantitative reverse transcription PCR
RANKL	Receptor activator of NF-κB ligand
RIPK	Receptor interacting serine/threonine-protein kinase
RNAi	RNA interference
ROS	Reactive oxygen species
RPL13A	Ribosomal Protein L13a
RT	Room temperature
SCID	Severe combined immunodeficiency
SERT	Serotonin transporter

SLC	Solute carrier protein
SNP	Single Nucleotide Polymorphisms
STAT	Signal transducer and activators of transcription
TACE	TNF-converting enzyme
TACI	Transmembrane activator and CAML interactor
TBS	Tris- buffered saline
TBS-T	TBS with 0.05-0.1% Tween-20.
TCR	T cell receptor
TGF	Transforming growth factor
Th Cells	T-helper cells
THD	TNF-homology domain
TJ	Tight junctions
TLR	Toll-Like receptor
TL1A	Tumor necrosis factor-like cytokine 1A
TME	Tumour micro-environment
TNBS	2,4,6-Trinitrobenzenesulfonic acid
TNF	Tumor necrosis factor
TNFR	TNF receptor
TNFSF	TNF superfamily
TO	Topological overlap

T reg	Regulatory T cells
TRADD	TNFR1- associated death domain protein
TRAF	TNF-receptor associated factor
TRAIL	TNF-related apoptosis-inducing ligand
TREM	triggering receptor expressed on myeloid cells
TRIF	TIR-domain-containing adapter-inducing interferon-β
TWEAK	TNF-related weak inducer of apoptosis
UC	Ulcerative colitis
UPR	Unfolded protein response
UT	Untreated
VEGF	Vascular endothelial growth factor
WB	Western blotting
WGCNA	Weighted gene correlation network analysis
WT	Wild-type
XBP	X-box binding protein
ZBP1	Z-DNA binding protein 1
z-VAD	N-Benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone

General Abstract

Inflammatory bowel diseases (IBD) are chronic diseases of the gut affecting a significant proportion of the population in the western world with increasing incidence globally. They are multifactorial diseases with susceptibility mediated by a combination of host genetics and environmental factors leading to altered gut barrier function with ongoing non-resolving innate and adaptive inflammatory responses to components of the gut microbiota. There are two major forms - Crohn's disease (CD) and Ulcerative colitis (UC) differing in their location along the gastrointestinal tract, their pathophysiology, and characteristics of the ongoing innate and adaptive immune responses. While CD is largely characterised by a mixed Th1/Th17 immune response, UC is characterised by a so-called Th2 immune response. As reviewed in Chapter1 of the thesis, various cytokines, particularly IFN-γ and TNF-α, produced by activated immune cells, play a significant role in the pathogenesis of IBD. TNF-α blocking antibodies, known collectively as anti-TNFs, revolutionised the treatment of IBD in the mid to late 1990s; however, about 60% of patients are classified as inadequate responders, experiencing either no response or losing the response over time to both these anti-TNF drugs and newer targeted therapies. Hence there is a large therapeutic gap in IBD, leading to a clinically unmet need for new potential therapeutic targets that can be targeted in addition to TNF-α to obtain maximum efficacy. In the first study (Chapter 2 of this thesis), we investigated potential mechanisms underpinning synergy between IFN-γ and TNF-α and their ability to induce cell death of intestinal epithelial cells. Through weighted gene co-expression network analysis (WGCNA) of microarray data from HT-29 cells treated with IFN-γ and TNF-α, we identified gene modules that suggested both cytokines can cross regulate components of each other's receptor-mediated signalling pathways. We confirmed these results through wet-lab

experiments and propose the activation of a positive feedback loop when cells are treated with both IFN-γ and TNF-α, leading to enhanced activation of the signalling pathway and cell death. Though genes associated with the TNFR-related apoptotic and necroptotic pathways are increased when cells are treated with IFN-γ+TNF-α, perturbation of these canonical cell death pathways using commercially available inhibitors could not block the cytokine-induced cell death. STAT1 was identified as the main component required for IFN-γ+TNF-α-induced cell death, and we propose that cytokine-mediated pathway cross-regulation drives a positive feedback signalling loop culminating in enhanced activation of STAT1 and STAT1-induced cell death. In the second study (Chapter 3 of this thesis), we identified TNF-α-like cytokines that can exhibit synergy with IFN-γ as an approach to finding new targets to overcome resistance to anti-TNFs. For this, we screened ligands of the TNF superfamily of which TNF-α is a member. We utilised IFN-γ+TNF-α induced cell death and chemokine production as phenotypic readouts to screen a library of recombinant TNFSF ligands. We identified three major hits - TRAIL, TWEAK and LIGHT that could synergise with IFN-γ to induce pro-inflammatory chemokines and cell death in colon cancer cell lines and primary human colonic organoids. In the last chapter (Chapter 4 of this thesis), we selected one of these hits- TRAIL and investigated its synergistic effects with IFN-γ. IFN-γ and TRAIL synergised to induce cell death and production of chemokines, particularly the Th1 chemokine CXCL10. Similar to IFN-γ+TNF-α synergism, the IFN-γ+TRAIL-induced synergistic cell death was independent of canonical TRAIL-R associated apoptosis and necroptosis and was regulated via a caspase-8-JAK1/2-STAT1 pathway with STAT1 activation playing a significant role. Our results show that TRAIL is a TNF-α like molecule from the TNF superfamily and is a potential target for the treatment of IBD. The Casp-8/JAK1/2-STAT1 pathway

plays a major role in modulating synergistic responses by the cytokine combinations. These results also explain, to an extent, the success of JAK inhibitors in the treatment of IBD. With further pre-clinical and clinical studies with antibodies targeting the hits from the screen, namely, TWEAK, LIGHT and TRAIL, either alone or in combination with anti-TNFs, new therapies can be developed for clinical trials in IBD.

Chapter 1 : Introduction

1.1. Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is a chronic inflammatory disease of the gastrointestinal tract (GI) associated with significant morbidity and low mortality rates. Clinically, patients present with abdominal pain, diarrhoea, fever, fatigue, weight loss, nausea and rectal bleeding, among other non-specific clinical features[1]. The onset of IBD is usually in the second or third decades of life; however, it can be diagnosed in infants and senior citizens too[2,3]. The incidence of IBD has grown exponentially in westernised countries, especially during the later years of the 20th century[4–6].

The two primary forms of IBD are ulcerative colitis (UC) and Crohn's disease (CD). Pathological UC and CD are very different- while in UC, inflammation is typically limited to the colon, in CD, the inflammation is patchy and segmented and can affect any part of the gastrointestinal tract[7]. However, a small subset of patients whose symptoms and pathology are not easily distinguishable are classified as IBD-undetermined type[8]. IBD is a multifactorial disease with complex interactions between environment, genetic, microbial and immune factors. Dysregulation of any of the factors leads to chronic inflammation and IBD. The role of the immune system and immunobiology in IBD immunoregulation and immunopathogenesis is discussed below.

1.1.1. Immunobiology and Immunopathogenesis of IBD

Immunopathogenesis of IBD is characterised by increased intestinal epithelial cell death, epithelial barrier disruption, infiltration by innate and adaptive immune cell populations, and increased levels of inflammatory mediators, including cytokines and chemokines[9–12]. The innate immune system in the gut consists of a single layer of

epithelial cells which forms a mucus-producing barrier, various resident phagocyte populations, including dendritic cells and macrophages, natural killer (NK) cells, innate lymphoid cell (ILC) populations, while the adaptive immune system consists of various T cell and B cell populations[13]. The inflammatory cytokine response is often derived from inflammatory macrophages and various $CD4^+$ T helper (Th) cell populations which contribute significantly to the inflammation in IBD[14]. The two primary forms of IBD, UC and CD, differ in terms of the type of Th cell responses – generally, a mixed Th1/Th17 response is associated with and drives CD, while it is proposed that a more Th2 like response drives UC, although there is evidence of Th17 responses in UC also[15].

1.1.1.1. Intestinal Barrier

The intestinal barrier comprises of the gut lumen facing mucous layer, a single intestinal epithelial cell layer (consisting of intestinal stem cells, enterocytes, goblet cells, enteroendocrine cells, and Paneth cells (small intestine) and the underlying lamina propria[16]. The mucous layer is formed by the polymerisation of mucins secreted from goblet cells in the intestinal epithelium[17]. Previously, it has been shown that in UC, there is a reduction in goblet cells and a decrease in mucin 2 (MUC2) secretion[18]. *MUC2*$^{-/-}$ mice develop spontaneous colitis, and this presents with cellular features similar to UC[19]. The next line of defence is mediated by antimicrobial peptides consisting of α-defensins, β-defensins and lectins[17]. The importance of innate immunity and antimicrobial peptides in IBD is shown by the fact that patients with NOD 2 (CARD15) mutations in CD present with a specific decrease in α-defensin production by Paneth cells[9,20]. The antibacterial lectin, RegIIIγ secreted by Paneth cells, is a key Myd88-dependent factor that limits bacterial colonisation of the intestinal epithelial surface and maintains spatial separation between the microbiota

and intestinal epithelium[21,22]. *RegIIIγ*$^{-/-}$ mice exhibited a marked increase in the number of mucosa-associated bacteria compared to wild-type and enhanced adaptive immune response[21]. Another anti-microbial peptide, resistin-like molecule β (RELMβ), limits contact of Gram-negative bacteria with the colonic epithelial surface[23]. But unlike RegIIIγ, RELMβ directly targets the bacteria and kills them via the formation of membrane-permeabilising pores that lyse the bacterial cells[23].

Tight junctions (TJ), which form links between neighbouring intestinal epithelial cells, play a significant role in maintaining the integrity of the epithelial cell layer, paracellular permeability and cellular polarity[24]. The TJ complex consists of occludins, junction adhesion molecules and the claudin family of proteins[24]. A genome-wide association studies (GWAS) study in 2009 identified three susceptible TJ genes for UC- *HNF4A* (human nuclear factor 4 α) regulating cell-cell junction, *CDH1* encoding E-cadherin, an important component of the adherens junction and key mediator of intercellular communication of the intestinal epithelium and *LAMB1* (laminin beta 1) that has a major role in the anchoring of single-layered epithelium[25,26].

Epithelial cells sense the intestinal microbes via the pattern recognition receptors (PRRs), such as NOD-like receptors (NLRs) and Toll-like receptors (TLRs), which recognise the microbial-associated molecular patterns (MAMPs)[13]. PRR signalling leads to the activation of the NF-κB (nuclear factor kappa B) family of transcription factors and increased expression of antimicrobial, immune regulatory and inflammatory genes[27]. Collectively, this transcriptional response results in an effective innate immune response against the gut microbiota and invading pathogens. The NLRs (NOD1 and NOD2) signal via recruitment of receptor-interacting protein kinase 2 (RIPK2), leading to activation of TAK1 and subsequent activation of the NF-κB family of transcriptional regulators resulting in the production of proinflammatory

cytokines and antimicrobial peptides[28]. *CARD15* encoding NOD2 was one of the first genes to be identified as a risk factor for CD[29]. NOD2 deficient mice displayed alterations in commensal microbiota, reduced Paneth cell-secreted α-defensins and increased susceptibility to colitis[30]. Another NLR protein, NLR family pyrin domain containing 3 (NLRP3), an initiator of inflammasome formation, has been implicated in the pathogenesis of IBD[31]. However, conflicting studies are in the literature linking NLRP3 to colitis. One report shows that *NLRP3*$^{-/-}$ mice have increased susceptibility to dextran sodium sulfate (DSS)- induced colitis with altered antimicrobial secretions compared to wild-type littermates[32], while other reports that *NLRP3*$^{-/-}$ mice developed less DSS-induced colitis with lower levels of proinflammatory chemokines compared to wild-type mice[33]. TLRs such as the TLR4 activate NF-κB through MyD88-dependent or MyD88 independent- TIR-domain-containing adapter-inducing interferon-β (TRIF)-dependent pathways[34]. Mice lacking specific TLR4 show increased susceptibility to colitis by inducing intestinal injury through downregulation of *IL6*, *CCL2* and *CSF3*[35]. Polymorphisms in TLR genes, especially the *TLR4* gene, have been associated with the development and progression of IBD[36].

Autophagy and ER-stress responses are mechanisms involved in maintaining the homeostasis of the intestinal barrier[37]. PRRs interact with both the pathways to coordinate bacterial handling and inflammatory response[38]. *ATG16L1*, an essential component of the autophagic pathway and *IRGM* (immunity-related GTPase family M protein), have been identified from IBD genome-wide association studies (GWAS) and have associated with increased susceptibility to CD[38]. Autophagic pathways have been shown to regulate inflammasome activation and inhibit pyroptosis[39]. ER stress is induced by the accumulation of misfolded or unfolded proteins[40]. Genetic studies have identified the *XBP1* gene, a gene involved in regulating unfolded protein response, as

a risk factor in IBD[41]. Alterations in *NOD2, ATG16L1 and XBP1* genes have also been linked to Paneth cell dysfunction resulting in antimicrobial response defects[42].

1.1.1.2. Role of T cells in IBD

T cells are key players of the adaptive immune response and play a critical role in driving ongoing inflammation and immunopathogenesis of IBD[13]. Persistent dysregulated T cell responses result in excessive release of cytokines, leading to recruitment and activation of innate immune cell populations such as inflammatory monocytes and macrophages, chronic inflammation, damage to the epithelial barrier and immunopathogenesis[9,10,13]. CD and UC appear to differ in terms of the different activated T cell subsets driving the ongoing inflammation. While CD presents a mixed Th1/Th17 subtype, UC presents with a Th2 response though recent studies have suggested a presence of Th17 response in UC[15]. High levels of IL-12 and IL-18 induce differentiation of Th1 cells[13]. Th1 cells secrete IL-2, interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α), which are vital for immunity against intracellular viral and bacterial infections[43]. Upon stimulation with IL-4, $CD4^+$ T cells differentiate into Th2 cells leading to their full activation and release of cytokines such as IL-4, IL-5, IL-13 and IL-23 required to eliminate extracellular parasites[13,43]. In response to transforming growth factor-β (TGF-β) and IL-6, naïve $CD4^+$ T cells differentiate into Th17 cells producing IL-17 isoforms and IL-21[44]. Studies have found that the IL-23 treatment in mouse models drives Th17 differentiation, expansion and cytokine production resulting in colitis[45,46]. High levels of IL-17 mRNA, particularly IL-17A, IL-23 mRNA, particularly IL23p19, and a higher number of IL-17 producing T cells have been detected in CD and UC[47,48]. GWAS studies have identified more than 20 single nucleotide polymorphisms (SNPs) associated with the IL-23/Th17 pathway indicating a significant role of Th17 cells in the pathogenesis of IBD[49].

Macrophages are antigen presenting cells (APCs) that are essential for bacterial recognition and elimination, the polarisation of innate and adaptive immunity, and maintaining homeostasis[50]. Intestinal macrophages may contribute to the maintenance of T cells – FoxP3^{+} Treg cells through the production of IL-10 and development of Th17 through IL-1β production[51]. Macrophages from *IL-10*$^{-/-}$ mice that develop spontaneous colitis produce high levels of the inflammatory cytokine IL12p70, which promotes a Th1 cytokine bias, as seen in CD[52]. In IBD, there is high infiltration of CD14^{hi}HLA-DR^{dim}macrophages which produce high levels of IL-12 and IL-23 under microbial stimulations[49]. Other APCs, such as dendritic cells, are present throughout the gut and present antigens to naïve T cells in the mesenteric lymph nodes (MLNs). Dendritic cells (DCs) in MLNs of Crohn's patients promote Th1 and Th17 immune responses[43,49].

1.1.1.3. The role of cytokines in IBD

Cytokines play a major role in maintaining homeostasis and driving inflammation in the gastrointestinal tract and mediate inter-cellular communication between epithelial cells, stromal cell types, tissue-resident and tissue infiltrating immune cell populations[53]. Depending on the stage of inflammation, cytokines may exert context-dependent immunoregulatory or inflammatory effects; hence, it is crucial to understand the cytokine network for clinical targeting[53,54]. GWAS studies have identified several IBD susceptible loci that encode components of cytokines and chemokine receptor signalling cascades[55].

Tumor necrosis factor-alpha (TNF-α) is a critical cytokine contributing to the pathogenesis of IBD. Macrophages, adipocytes and T cells are key producers of TNF-α. TNF-α exerts its pro-inflammatory effects largely through activation of NF-κB and transcription of NF-κB regulated inflammatory genes such as chemokines and

cytokines downstream of its signalling pathway[56]. TNF-α can also provide pro-survival signalling to activated $CD4^+$ T cells and can induce epithelial cell death and disruption of epithelial barrier integrity through apoptotic and necroptotic pathways[57]. TNF-α is highly produced in colons of mice with experimental colitis[58] and is also found in high levels in serum of patients with IBD[59]. The importance of TNF-α in IBD pathogenesis is clear because of the clinical success of anti-TNF therapies for the treatment of IBD over the past 25 years[60–63] (discussed more in sections 1.2.2.1 and 1.4.1).

Another important cytokine in human IBD pathogenesis and in murine models of intestinal inflammation and colitis is interferon-γ (IFN-γ). IFN-γ is a pleiotropic cytokine with pro-inflammatory activities, anti-viral, and anti-bacterial activities mediated primarily through its direct effect on immune cells such as macrophages, NK cells and T cells[64]. IFN-γ is predominantly produced by the Th1 cells and NK cells[65]. IFN-γ compromises the integrity of the intestinal barrier by triggering myosin II-dependent internalisation of key tight junction proteins[66]. The crucial role of IFN-γ in the pathogenesis of IBD was established from studies that show *IFN-γ*$^{-/-}$ mice have reduced DSS- induced colitis[66]. IFN-γ can synergise with TNF-α to disrupt the tight junctions and barrier functions by distinct regulation of β-catenin pathways[67,68]. IFN-γ compromises the integrity of the intestinal barrier by triggering myosin II-dependent internalisation of key tight junction proteins[69]. IFN-γ has also been shown to induce Paneth cell death and impaired regeneration of intestinal epithelium[70]. More about IFN-γ and its role in IBD is covered in section 1.5.1.

Increased levels of IL-1 family members have been identified in intestinal tissues and in the peripheral circulation of patients with both UC and CD[71]. The IL-1 family of cytokines consists of IL-1α, IL-1β, IL-18, IL-33, IL-36 isoforms- IL-36α,

IL-36β, IL-36γ and IL-36 receptor antagonist (IL-36Ra), IL-37 and IL-38, are released from APCs and epithelial cells to initiate inflammation upon local injury[72,73]. Most IL-1 family members perform dual functions depending on the phase of the disease (initiation vs sustaining of inflammation)[74]. Perturbation of inflammasome complex (NLRPs) activation, required for activation of IL-1β and IL-18, and perturbation of components of the IL-1β or IL-18 signalling pathways have been shown to be protective in murine models of colitis[73]. However, treatment with anti-IL-1β antibodies or knocking out IL-18 in mice makes them susceptible to DSS induced colitis[74]. Similarly with IL-33, though elevated levels are observed in patients with active IBD, IL-33 deficient mice are highly susceptible to colitis and colorectal cancer[75]. Further understanding of the regulation of IL-1 members and their context-dependent contribution to intestinal homeostasis, infection and inflammation may provide future opportunities for targeting them in human IBD.

IL-12, IL-23, IL-27 and IL-35 belong to the IL-12 family of heterodimeric cytokines with key roles in the development of Th1 and Th17 cells[76]. IL-12 and IL-23 are pro-inflammatory cytokines, while IL-27 and IL-35 act as immunoregulators or negative regulators of cytokine production[76]. IL-12 induces differentiation of naïve T cells into IFN-γ producing Th1 cells and induces IFN-γ production in ILCs[77]. IL-23 induces differentiation of naïve T cells into Th17 cells and activates memory T cells to produce Th17 cytokines such as IL-17A, IL-17F and IL-22[78]. Variants of IL23 receptor (IL23R) have been identified as risk loci for IBD[79]. Also, high levels of IL-12 and IL-23 have been found in the serum of patients with IBD and in mouse models of intestinal inflammation and colitis[79]. Targeting the IL-12/IL-23 pathway has been effective as a therapeutic strategy in IBD (see 1.2.2.2 below).

Th17 cells produce several cytokines, including IL-17A, IL-17F and IL-22. IL-17A and IL-17F promote neutrophil recruitment and act as pro-inflammatory stimuli[80]. IL-17A can synergise with other cytokines such as TNF-α and IFN-γ to prolong inflammatory responses[81]. However, conflicting results have been obtained from murine inflammation models where the IL-17 pathway has been investigated. In DSS-induced colitis, IL-17F or IL-17 receptor (IL-17R) deficiency showed a protective effect, while in a T cell-induced colitis model, IL-17A protected mice by inhibiting IFN-γ dependent inflammation[82]. IL-22 is an anti-inflammatory cytokine and is critical for maintaining intestinal barrier function and homeostasis[83]. There is a marked reduction of IL-22 in the intestinal tissue of patients with UC[53].

IL-10 and TGF-β are major anti-inflammatory cytokines produced by regulatory T cells (Treg cells) that suppress effector T cells and inflammatory macrophage responses. IL-10 inhibits the expression and production of the shared p40 subunit of the IL-12 and IL-23 cytokines in myeloid cells and controls Th1/Th17 cell differentiation[83]. Mice that lack IL-10 develop spontaneous colitis, and *IL10*$^{-/-}$ mice are widely used as colitis models to study the disease-modifying effects of other immune cell types, cytokines, chemokines, and environmental factors such as diet and the microbiome in intestinal inflammation[83]. Mutations in IL-10 receptor genes *IL10RA* and *IL10RB* have been found in patients with severe early-onset IBD, and it is now thought that the major role of IL-10 is to suppress macrophage inflammatory phenotypes in the gut[84].

IL-6 plays a crucial role in the differentiation of DC from leukemic precursor cells, promoting Th2 response by producing IL-4 and IL-13 and inhibiting Th1 cell differentiation[85]. IL-6 exhibit its pro-inflammatory effect through the IL-6- IL-6R (IL-6 receptor) pathway leading to STAT3 activation and prevention of programmed

apoptosis in mucosal T cells[53]. IL-6 levels were elevated in patients with CD, and blocking IL-6 using monoclonal antibodies in experimental models prevented intestinal inflammation[86].

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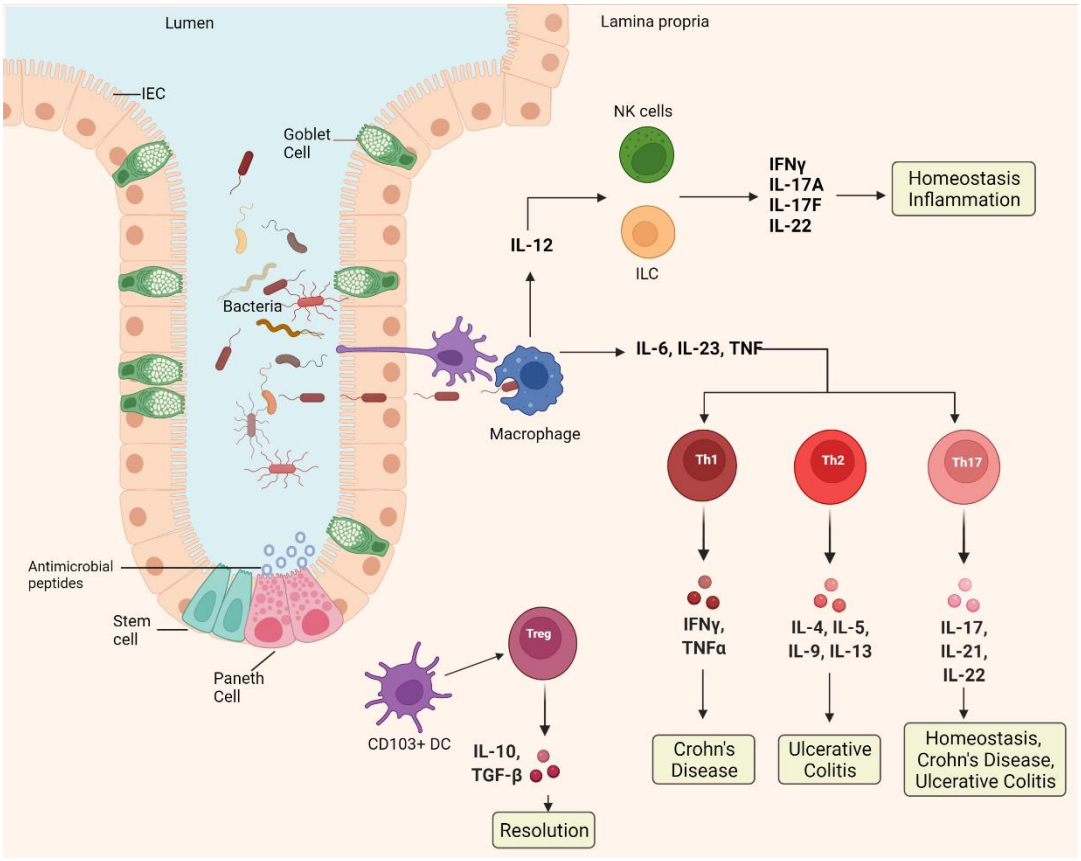


Figure 1.1: Cytokines in inflammatory bowel diseases

Various cells of the gut mucosal immune system, in particular, intestinal epithelial cells (IEC), dendritic cells (DCs), macrophages, natural killer (NK) cells, effector T cells (Th1, Th2 and Th17), regulatory T cells (Tregs) and innate lymphoid cells (ILCs) produce pro-inflammatory and anti-inflammatory cytokines in response to triggers such as microbes and microbial-derived molecules and stimuli. The key cytokines released in the inflamed mucosa of IBD are shown. Figure adapted from Neurath, 2014[53] and created in BioRender.com

1.2. IBD therapeutics- success, failures and novelties

As we have reviewed above, IBD is a multifactorial disease, and no particular cause has been identified to date. This section of the introduction discusses various targeted therapies that have been developed and assessed in both pre-clinical and clinical studies over the last few decades, success, failures, and the constant need to find new therapeutic targets.

1.2.1. Classic anti-inflammatory and immunosuppressive drugs

Despite the development and clinical utility of successful targeted therapies for IBD, a persistent therapeutic gap of around 60-70% across all therapies still exists for this group of diseases and older conventional treatments are still important and widely used to treat IBD[87,88]. Aminosalicylates (5-ASA), an anti-inflammatory drug initially developed for rheumatoid arthritis, effectively maintain remission in UC[87]. It acts by inhibiting IL-1, TNF-α, lipoxygenase pathways and NF-κB signalling[87]. Corticosteroids such as hydrocortisone and prednisolone are highly utilised drugs for IBD[87]. However, only 40% of patients respond and will still be in remission when steroidal drug use is reduced. Of the rest, 20% do not respond to steroids, and the remaining are dependent on steroids completely as they relapse when the steroid dependency is reduced[87]. Immunosuppressants such as azathioprine (AZA) and mercaptopurine (6-MP) are used on patients that are steroid-resistant or steroid-dependent[89]. These drugs impair purine biosynthesis and affect cell proliferation. They are effective in induction and maintenance of remission in CD[90]. Another immunosuppressive drug is methotrexate, which inhibits dihydrofolate reductase involved in purine and pyrimidine biosynthesis, thereby blocking DNA synthesis[90].

Patients refractory to these agents are treated with other biological therapies alone or in combination with these less targeted therapies to maintain long term remissions[91,92].

1.2.2. Anti-cytokine therapies

As discussed in section 1.1.1.3, numerous cytokines and complex networks are involved in the induction and progression of inflammatory bowel diseases. Targeting these cytokines through monoclonal antibodies and biologics has been both successful and not in IBD treatment.

1.2.2.1. Anti-TNF-α

Targeting TNF-α using monoclonal antibodies represented a revolution in the field of cytokine biology and targeted cytokine therapies for IBD and a range of other chronic inflammatory and autoimmune diseases[62,63,93]. The first monoclonal antibody targeting TNF-α - Infliximab was approved by the Food and Drug Administration (FDA) to treat CD in 1998[94]. Infliximab was then approved for the treatment of UC in 2005[62]. A fully humanised anti-TNF antibody, Adalimumab, was approved in 2007 to treat CD[62]. Certolizumab pegol (CZP), a PEGylated-Fab portion of a recombinant humanised antibody, and Golimumab were additional anti-TNFs subsequently approved for the treatment of CD[63]. All these anti-TNF-α biologics bind to either membrane or soluble TNF, thus blocking TNF-α mediated activation of TNFR (TNF-receptors)[63]. Anti-TNF drugs have been demonstrated to decrease pro-inflammatory cytokine production and induction of apoptosis of activated T-lymphocytes by blockade of TNF-α-mediated pro-survival signalling from $CD14^+$ monocytes[95,96]. Anti-TNFs can also exert their effect by induction of reverse signalling effect via cell surface TNF-α itself[97], induction of antibody-dependent cell-mediated cytotoxicity[98], and modulating the immune system by inducing IL-22 in T cells[99], inducing IL-10

production[100] and inhibiting IL-12/IL-23 expression in macrophages[101]. Although anti-TNF-α drugs demonstrate impressive efficacy in IBD, long-term remission is only achieved in about 40% of patients. Other patients with IBD administered anti-TNFs either do not respond (30% of patients) or lose response to this therapy over time (30-50% of patients)[61,93,102]. Hence, there is a need to identify novel therapeutics to achieve sustained remission in IBD.

1.2.2.2. Other anti-cytokine therapies

Ustekinumab, a humanised monoclonal antibody against the shared p40 subunit of IL-12/IL23, was approved by Food and Drug Administration (FDA) and European Medicines Agency (EMA) in 2016 for patients with CD who are refractory to anti-TNF or conventional therapies[103]. However, ustekinumab had higher rates of response and remission in TNF-α inhibitor-naïve patients compared to patients who were refractory to anti-TNF[104]. The FDA approved ustekinumab to treat moderate to severely active UC in 2019[105]. Other monoclonal antibodies targeting the p19 subunit of IL-23 (Risankizumab and Brazikumab) are currently in phase 2/3 trials[106,107].

PF-04236921 is the only antibody developed against IL-6. It is at phase 2 trials with initial results showing induction of clinical response and remission in patients with moderate-severe CD and refractory to anti-TNF[108]. So far, no other antibodies towards IL-6 have been assessed in clinical trials, even though several have been developed and investigated in pre-clinical studies[109].

1.2.3. Anti-adhesion therapies

IBD is characterised by increased infiltration of leukocytes into the intestinal mucosa leading to prolonged inflammation[10]. Anti-adhesion therapies target the interaction of integrins with their ligands and inhibit leukocyte trafficking. Natalizumab is an

antibody that targets the α4 subunit of α4β7 integrin. It was approved by the FDA in 2008, but only for moderate-severe CD patients who do not respond to anti-TNF therapy[110]. Natalizumab can also target α4β1 integrin and prevent leukocytes from binding to vascular cell adhesion molecule 1 (VCAM-1) on vascular endothelial cells in the central nervous system (CNS) as well as in the intestine[110]. Reduced T cell trafficking in the brain can lead to multifocal leukoencephalopathy (PML) due to the reactivation of the John Cunningham (JC) virus[111]. Another antibody, Vedolizumab, is specific for targeting the α4β7 and not α4β1, thereby reducing the occurrence of PML[110]. Vedolizumab was approved by the FDA in 2014 for moderate to severe UC or CD patients who do not respond to conventional or TNF-α inhibitor therapy[112]. Etrolizumab, an antibody targeting the β7 subunit present in α4β7 and αEβ7 integrins, is now in phase 3 clinical trials for UC[113,114].

1.2.4. Small molecule inhibitors

1.2.4.1. JAK inhibitors

Janus kinases (JAKs) are members of intracellular non-receptor tyrosine-protein kinases that transduce extracellular stimuli to modulate a wide range of cellular processes, including cell growth and survival, differentiation, proliferation, and migration[115]. They are components of the receptor-mediated signal transduction cascades of many inflammatory cytokines, including interferons, epidermal growth factor (EGF), interleukin 2, 3 and 6 families, and growth hormones[116]. JAK family consists of four proteins- JAK1, JAK2, JAK3 and Tyk2[117]. Ligand binding to the receptor induces auto-activation by transphosphorylation of the JAKs[118]. Activated JAK phosphorylates the intracellular domain of the receptor to make docking sites for signal transducer and activator of transcription (STAT)[117]. STATs are then phosphorylated by JAK, resulting in STAT dimerization and translocation to the

nucleus, where STATs drive the expression of target genes[119]. JAK/STAT pathway regulates the signalling of most of the cytokines implicated in the pathogenesis of IBD[120].

Tofacitinib is the first JAK inhibitor targeting JAK1 and JAK3 to be approved by the FDA in 2018 for the treatment of patients with moderate to severe UC[121]. Recently in Mar 2022, Upadacitinib, a selective JAK1 inhibitor, was approved by the FDA for the treatment of adult moderately to severely active UC[122]. Upadacitnib is also in phase 3 trials for the treatment of CD. Filgotinib, a novel oral JAK1-selective small-molecule inhibitor, has been evaluated in a Phase II trial in patients with CD[123]. A phase 2b/3 trial also shows filgotinib effective in induction and maintenance of remission of treatment against moderately to severely active UC[124]. Peficitinib is an oral JAK inhibitor with selectivity for JAK3. A phase 2 trial demonstrated efficacy in moderate to severe-UC only at higher doses of the compond[125].

1.2.4.2. Other small molecule inhibitors

Ozanimod is a novel oral selective small-molecule immunomodulatory agonist mainly for G protein-coupled Sphingosine-1-phosphate (S1P) receptors 1 and 5 (S1P1 and S1P5) and blocks lymphocyte egress from lymph nodes into the systemic circulation[126]. Stimulation of S1P1 on lymphocytes results in the internalisation and degradation of the target receptor, thus preventing the migration of lymphocytes along a chemotactic gradient of S1P from lymphoid organs into the circulation and the gut[127]. Ozanimod has recently entered Phase III trials as an induction and/or maintenance therapy in UC and Phase II trials for moderate and severe CD[126].

Mongersen is an orally delivered anti-sense oligonucleotide inhibiting the production and activity of SMAD7 by facilitating RNase H-mediated degradation of

SMAD7 mRNA[127]. SMAD7 is an endogenous inhibitor of TGF-β and is elevated in IBD. Though mongersen showed high efficacy in phase 2 clinical trials for CD, a recent phase 3 trial failed to identify the same effectiveness and had to be terminated[128]. It is now in phase 2 clinical trial for UC[126].

1.2.5. Challenges in IBD therapeutic success

One of the main challenges in identifying therapeutic targets within cytokine networks for the treatment of IBD is the fact that cytokines often elicit context-dependent functions and effects on target cells dependent on the homeostatic and inflammatory state of the tissue microenvironment[53,54]. Many cytokines such as TNF family members, IL-17 isoforms, IL-33 and IL-22 have dual roles as inflammatory and immunoregulatory signals for cells within the same tissue[129]. For example, IFN-γ levels are elevated in gut mucosal tissue of patients with IBD, similar to TNF-α and on paper, it is an excellent target for therapeutic intervention. However, an antibody targeting IFN-γ (Fontolizumab) did not provide significant efficacy in the phase 2 trial for CD[130]. The failure has been attributed to the anti-inflammatory properties of IFN-γ, although the full mechanistic reasons why this approach did not work are still not clear[131]. Targeting IL-13 with anrukinzumab monoclonal antibody did demonstrate any significant therapeutic effect in patients with UC compared to placebo groups[132]. Conflicting results from pre-clinical murine experimental models of intestinal inflammation and colitis also show that IL-13 and many other cytokines have complex roles and may not necessarily represent good therapeutic targets for humans[53].

Another challenge is using the relative tissue expression of cytokine as a major criterion for target validation. For example, IL-17A mRNA and protein expression were high in patients with IBD. Anti-IL17A treatments have been successful in inflammatory diseases such as psoriasis and ankylosing spondylitis. However, the

therapeutic targeting of IL-17A using the monoclonal antibody Secukinumab for moderate to severe CD failed[133]. Patients treated with Secukinumab had elevated levels of inflammatory markers compared to placebo groups, and more patients experienced adverse events leading to discontinuation of the study[131].

Another major challenge is the lack of response and the development of resistance to current therapeutic approaches such as steroids, anti-TNFs and other targeted therapies[102]. In response to anti-TNFs, only 40% of patients with IBD experience a full, long-lasting therapeutic response, and the remainder of patients lose response to these drugs over time[61,93,102]. Various studies are now focused on investigating the potential cellular and molecular mechanisms underpinning this resistance phenomenon. Overall, these groups are using various omics approaches to try and stratify patients into responders and non-responders based on microbial, immune cell and molecular signatures that can help predict resistance to anti-TNF therapies[102]. A meta-analysis study identified two cell subsets of macrophages and plasma cells that were significantly higher in non-responders than responders[134]. The increase in inflammatory macrophages in non-responders was attributed to the upregulation of CCL7 and TREM-1 receptors (triggering receptor expressed on myeloid cells 1)[134]. Another study analysing 200 patients identified oncostatin M (OSM) as a major factor in predicting resistance to anti-TNF. The study found high *OSM and OSMR* mRNA expression in biopsies of patients with IBD, and this increased expression was associated with poor response to anti-TNF[135]. Anti-TNF responders also presented with higher expression of TNFR2 on T cells than non-responders before anti-TNF treatment[136]. These cells exhibited an increase of IL-23 receptor (IL23R) on the $TNFR2^+$ T cells in response to anti-TNF therapy rendering patients resistant to anti-TNF therapy. IL-23/IL-23R signalling activation increased

Th1 and Th17 cytokines through the STAT3 pathway[136]. Recently, through the application of single-cell genomics and bioinformatic analysis, various immune cell modules have been associated with anti-TNF resistance. Single-cell analysis of inflamed tissues from patients with Crohn's disease identified a module of heterogenous cells consisting of IgG plasma cells, inflammatory mononuclear phagocytes, activated T cells and stromal cells, named the GIMATS module, which was associated with failure to anti-TNF therapy[137]. A recent study also identified two modules (denoted M4/M5) from a transcriptomic dataset derived from inflamed CD or UC compared to non-inflamed patient tissue[138]. These modules M4 and M5 highlight IL-1R as a key driver to recruit inflammatory fibroblast/neutrophils and contribute to nonresponse to anti-TNF in IBD[138].

All of these factors highlight the need for a greater understanding of the complexity and redundancy of the biology underpinning immune cell, stromal cell and cytokine driven chronic inflammation and the failure of normal resolution mechanisms.

1.3. The TNF superfamily

The TNF superfamily (TNFSF) consists of 19 ligands and 29 receptors expressed on various cells of the immune system (See Table 1.1). TNFSF ligand-receptor pairs signal for proliferation, differentiation, cell survival and apoptosis. The TNFSF ligands are type II transmembrane proteins characterised by a conserved C-terminal TNF-homology domain (THD)[139]. The THD is responsible for trimerising the ligands facilitating the binding of the ligands to their respective receptors[140]. TNFSF ligands are produced as a cell membrane-associated form but undergo regulated proteolysis by distinct proteases to release soluble forms[141]. Solubilisation is essential for most of the ligands to function and exert their full biological activities, but for some – such as FasL - it may inhibit their function[142]. TNFSF ligands with similar structures can share common receptors but bind with different binding affinities; for example, RANKL and TRAIL share the same receptor OPG; TNF-α and lymphotoxin share TNFRSF1A and TNFRSF1B[143].

The TNF receptor (TNFR) superfamily is characterised by the presence of cysteine-rich domains in their extracellular domains[142]. The receptors are divided into three categories- (1) death receptors activating death pathways such as apoptosis and necroptosis, (2) TRAF-interacting receptors activating pathways associated with survival and proliferation and (3) decoy receptors inhibit the signalling of ligands through the other two receptors (Fig 1.2).

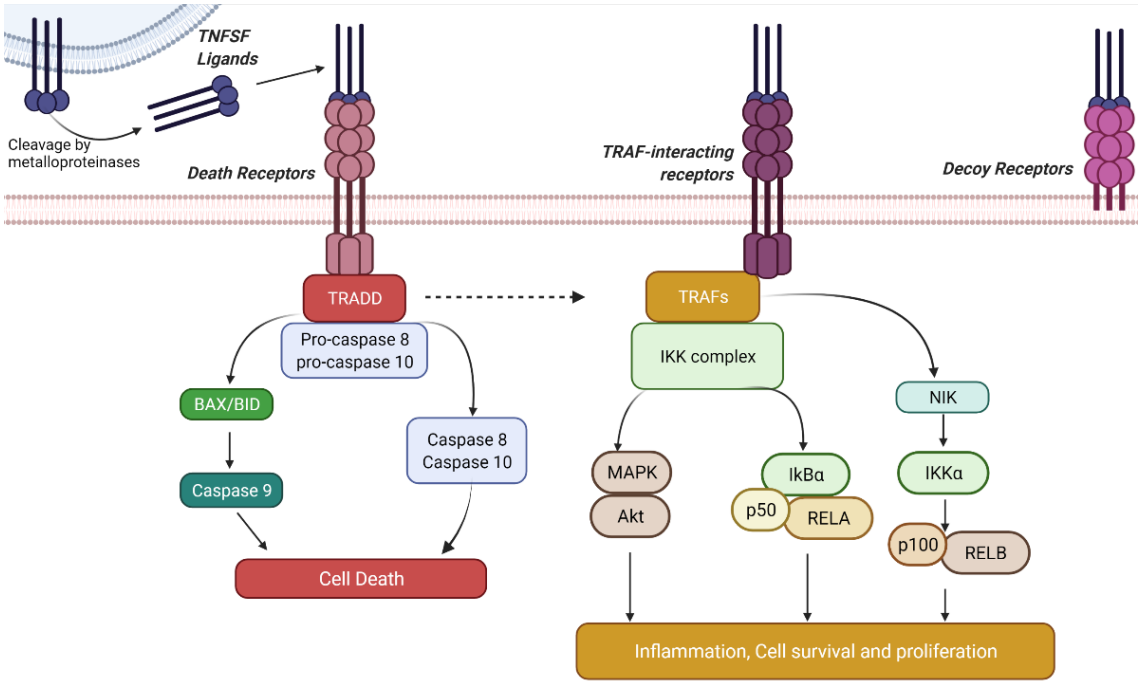


Figure 1.2: TNFSF signalling through their three types of receptors

TNF superfamily (TNFSF) ligands are type II transmembrane proteins characterised by a conserved C-terminal TNF-homology domain. They are released to their soluble form through proteolytic cleavage by metalloproteinases. TNFSF ligands interact with TNFRSF receptors to exert their biological effects. The TNFRSF are classified into three groups according to functional similarities in downstream signalling - the death receptors, TRAF-interacting receptors and decoy receptors. Death receptors contain death domains that interact with TRADD/FADD and trigger cell death pathways. The TRAF-interacting receptors bind to TRAFs and activate NF-κB and AP-1 transcription factors leading to survival or inflammatory pathway activation. Decoy receptors lack cytoplasmic domains, and hence there is no activation of downstream signalling. Some death receptors such as TNFR and TRAIL are also TRAF-interacting receptors. Figure adapted from Wajant, 2015[161] and made in BioRender.com.

There are six death receptors - TNFR1, Apo-1/Fas/CD95, DR3, DR4/TRAIL-R1 (TNF-related apoptosis-inducing ligand receptor), DR5/TRAIL-R2 and DR6. These receptors consist of a death domain (DD) located on their intracellular C-terminal region that facilitates interaction with death-domain-containing adaptor proteins - FADD (Fas-associated death domain) and TRADD (TNFR- associated death domain)[144]. The binding of ligands typically leads to trimerisation of receptors, receptor activation and binding of FADD. FADD contains another DD like domain called the death-effector domain (DED). Caspase-8 binds to the DED of FADD, thus forming the death-inducing signalling complex (DISC)[145]. Caspase-8 is activated in the DISC, and matured caspase-8 cleaves and activates effector caspases such as caspase-3 and caspase-7 to induce apoptosis[146]. The activation of caspase-8 is inhibited by cellular FLICE-like inhibitory protein (c-FLIP) that are also recruited to the DISC through DED interactions[147].

TNF receptor-associated factor (TRAF)-interacting receptors can activate multiple signalling pathways that mediate cellular activities like survival, proliferation, differentiation, and migration. All TRAF family members consist of a receptor binding C-terminal domain (TRAF-domain, except for TRAF7), various numbers of zinc finger domains, and an N-terminal RING domain with associated E3-ubiquitin ligase activity (except TRAF1)[148]. Receptors with DD bind to TRAFs indirectly via TRADD, while receptors lacking DD bind directly through a cytoplasmic TRAF-interacting motif (TIM)[149]. Ligand binding activates receptors and induces oligomerisation of TRAFs. Oligomerised TRAFs can bind to various signalling proteins, ultimately activating nuclear factor-κB (NF-κB) and activating protein 1 (AP-1) transcription factors[150].

The NF-κB family of transcription factors consists of RelA/p65, RelB, NF-κB1/p105, NF-κB2/p100 and c-Rel subunits which can heterodimerise and homodimerize with each other[151]. There are two types of NF-κB activation - canonical and non-canonical. In the canonical pathway, NF-κB is maintained in an inactivate state in the cytoplasm by IκB- inhibitory proteins (IκBα and IκBβ), which are phosphorylated by the IκB-kinase (IKK) complex consisting of IKKα, IKKβ and regulatory subunit NEMO leading to their K63 ubiquitination and degradation by the proteasome leading in turn to the relocation of NF-κB from the cytoplasm into the nucleus and activation of gene expression[152]. Phosphorylation and ubiquitination of IκB through the IKK complex results in the release of NF-κB subunits that translocate to the nucleus and activate transcription of target genes[152]. Activation of the non-canonical NF-κB pathway involves the processing of the p100 precursor. The p100 precursor consists of an IκB domain and is thus sequestered in the cytosol[149]. The IκB domain is phosphorylated by IKKα containing NF-κB inducing kinase (NIK) complex and induces p100 processing resulting in the production of the p52 subunit. p52 interacts with RelB, and the p52/RelB dimers translocate to the nucleus to induce transcription[151].

The decoy receptors of the TNF superfamily of receptors are the OPG (osteoprotegerin), TRAIL-R3 (DcR1), TRAIL-R4(DcR2) and DcR3[153]. They act as inhibitors rather than inducers of signalling pathways[144]. The DcR1 and DcR2 are cell-surface proteins that bind to TRAIL with the same affinity as DR4 and DR5 death receptors. DcR1 lacks the cytoplasmic death domain, while DcR2 has a partially deleted death domain. Hence, the two receptors are incapable of inducing apoptosis[154]. OPG is a secreted protein that can bind with RANKL as well as TRAIL but with a lower affinity compared to other receptors. DcR3 is a soluble receptor that binds with

FasL, LIGHT and TL1A, thereby inhibiting signalling through receptors Fas, HVEM, LTβR and DR3[155].

Most TNFSF members are expressed in cells of the immune system and play an important role in maintaining T cell-mediated immune response[156]. Various TNFSF are involved in autoimmune and inflammatory disorders such as asthma, diabetes, multiple sclerosis, systemic lupus erythematosus, arthritis, cancer and inflammatory bowel diseases[157–160]. The following sections cover the role of individual TNFSF members in the pathogenesis of inflammatory bowel diseases.

Table 1.1: TNF Superfamily of Ligands

Gene	Ligand	TNFSF ligand producing cells	Receptors
TNFSF1	LT-α, TNF-β	NK, T and B cells	TNFR1, TNFR2, LTβR, HVEM
TNFSF2	TNF-α	Mϕ, NK, T and B cells	TNFR1, TNFR2
TNFSF3	LT-β	DCs, Mϕ, T, B and NK cells	LTβR, HVEM
TNFSF4	OX40L	T and B cells, endothelial and smooth muscle cells	OX-40
TNFSF5	CD40L, CD154	T and B cells, granulocytes	CD40
TNFSF6	FasL	Cytotoxic T cells, activated T and NK cells, monocytes, Paneth cells	Fas(CD95), DcR3
TNFSF7	CD27L, CD70	NK, T and B cells	CD27
TNFSF8	CD30L, CD153	T cells and monocytes	CD30
TNFSF9	4-1BBL	B cells, DCs and Mϕ	4-1BB
TNFSF10	TRAIL	T and NK cells, DCs, Epithelium	TRAIL-R1 (DR4), TRAIL-R2 (DR5), TRAIL-R3 (DcR1), TRAIL-R4 (DcR2), OPG
TNFSF11	RANKL, TRANCE	Activated T cells and Osteoblasts	RANK, OPG
TNFSF12	TWEAK, APO3L	T cells, Mϕ, DCs, monocytes	Fn14
TNFSF13	APRIL	Mϕ, lymphoid cells	BCMA, TACI
TNFSF13B	BAFF	T cells, monocytes, DCs, Mϕ	BAFFR, BCMA, TACI
TNFSF14	LIGHT	T cells, monocytes, immature DCs, granulocytes	HVEM, LTβR, DcR3
TNFSF15	TL1A, VEGI	Endothelial cells, monocytes, activated lymphocytes, DCs	DR3, DcR3
TNFSF18	GITRL	T_{reg} cells, NK cells and neutrophils	GITR
EDA	EDA-A1, EDA-A2	Ectodermal origin of keratinocytes, liver, etc.	EDAR, EDA2R
NGF	β-NGF	Mast cells, activated $CD4^+$ T cells	NGFR

NK- Natural killer cells, Mϕ- Macrophages, DC- dendritic cells

1.4. TNFSF superfamily in Inflammatory Bowel Diseases

We have reviewed before that TNF plays an important role in the pathogenesis of IBD. Apart from TNF, other members of TNFSF such as LIGHT, TRAIL, TWEAK, TL1A, FasL, Lymphotoxin and RANKL are recently reported to contribute to the pathogenesis of IBD either through enhancing the pro-inflammatory function of immune cells or by inducing cell death of epithelial cells resulting in barrier disruption.

1.4.1. Role of TNF-α in IBD

Tumor necrosis factor-α (TNF-α) is one of the most studied cytokines in terms of its role in innate and adaptive immunity, cell death, chronic inflammatory diseases, autoimmune disease and IBD pathogenesis[56,162]. TNF-α was identified almost 47 years ago as a factor that induces tumour cell death and is now described as a key player in various processes such as cell death, cell differentiation, and pro-inflammatory responses[56]. TNF-α is initially expressed as a cell membrane protein which is then cleaved in a regulated way by the metalloproteinase TNF-α-converting enzyme (TACE or ADAM17) to release soluble TNF-α[163]. Soluble TNF-α can bind to two receptors - TNFR1 and TNFR2 that differ from each other by the presence of a death domain in the cytoplasmic region of TNFR1[149]. TNFR1 is expressed by virtually all cell types, but TNFR2 is expressed selectively by certain immune cells such as T cells and by endothelial cells[164]. The intracellular signalling pathways activated upon binding of TNF-α to its receptors are complex and can lead to activation of both apoptosis and survival/inflammatory pathways[162]. The binding of TNF-α to TNFR1 leads to the formation of different protein complexes. The formation of Complex I involves the recruitment of TRADD to receptors which serve as an assembly platform for the binding of RIPK1 and TRAF2[165,166]. cIAP1/2 are further recruited to the

complex through their interaction with TRAF2 and polyubiquitylate RIPK1[167]. Linear ubiquitin chain assembly complex (LUBAC) is recruited to the TNFR1 complex via cIAP generated ubiquitin chains[168]. LUBAC ubiquitylate the NF-κB essential modulator (NEMO), which is required for efficient activation of NF-κB subsequent NF-κB translocation to the nucleus to activate transcription of target genes involved in cell survival, immune regulation, inflammation and proliferation[162]. When RIPK1 is not ubiquitylated, it dissociates from the complex and assembles in the cytosol with TRADD, FADD, pro-caspase-8, and FLIP to form complex IIa[169]. Once complex IIa is assembled, pro-caspase-8 undergoes autocatalytic cleavage leading to its full activation and execution of caspase-8 dependent apoptosis[162]. Under conditions where cIAPs are depleted before, during or subsequent to the formation of complex I there is a reduction in RIPK1 ubiquitylation, leading to the assembly of RIPK1 with RIPK3[170]. This triggers the formation of apoptotic complex IIb also known as the ripoptosome consisting of RIPK1, RIPK3, pro-caspase-8 and FLIP[171]. TNF-α/TNFR1 interaction can also lead to necroptosis when caspase-8 activation is blocked or perturbed in some way[56]. Under conditions of capsase-8 perturbation, RIPK1 interacts with RIPK3 to form the complex IIc also known as the necrosome[172]. This complex further activates mixed lineage kinase domain like pseudokinase (MLKL) to trigger the necroptotic pathway and necropoptosis[173]. The binding of TNF-α to TNFR2 results in NF-κB activation through TRAF2. TNFR2 pathway acts as a co-stimulatory signal for T cells and expansion and suppression of T_{reg} cells[174].

In murine models of inflammatory bowel diseases, DSS-induced colitis[175], TNBS-induced model[58], and T Cell transfer models[176], TNF-α mRNA and protein levels are upregulated. Treatment with anti-TNF-α targeting antibodies ameliorated disease in all of these models[58,175,176]. TNF-α can target multiple cell types and exerts

different phenotypic effects on different cell types, including activation of inflammatory gene expression programs. As a well-known death-inducing ligand, TNF-α induces IEC death, which is thought to be a significant contributor to the overall immunopathogenesis observed in IBD. Studies dating back to 1998 show that extrinsic TNF-α administration leads to excessive enterocyte death at the villi and crypt of the colon[177]. Several mechanisms have been proposed for TNF-α-induced IEC death. Selective deletion of NEMO in IECs spontaneously led to chronic intestinal inflammation in mice with increased IEC apoptosis and bacterial translocation into the mucosa[178]. Elevated expression of A20, a negative regulator of NF-κB, promoted TNF-α dependent IEC death by stabilising the ripoptosome complex leading to RIPK1 dependent apoptosis[179]. Our group recently showed that TNF-α synergises with IFN-γ to induce IEC death through a caspase-8-JAK-STAT pathway[180]. Though TNF-α/caspase-8 dependent apoptosis was predominantly seen in IEC death, conditional depletion of caspase-8 in the intestinal epithelium led to inflammatory lesions in the ileum and susceptibility to colitis[181]. This phenotype was prevented by using a necroptotic inhibitor targeting RIPK1/RIPK3. Depleting FADD resulted in spontaneous colitis with epithelial cell necrosis, which was prevented by the inhibition of RIPK3[182]. A recent study identified Z-DNA binding protein 1 (ZBP1) together with the TNFR1 as triggers for colitis in FADD deficient mice, and FADD inhibited necroptosis and gasdermin D (GSDMD)-dependent pyroptosis-like death of epithelial cells[183]. All these studies indicate that multiple modes of IEC cell death pathways are possible and activated dependent on context. In addition to killing intestinal epithelial cells, TNF-α also affects the expression and cellular distribution and localization of tight-junction proteins leading to a decrease in intestinal epithelial barrier integrity[57]. Loss of junction proteins such as occludin, claudin, cadherin, junction adhesion

molecule (JAM), or catenins by IFN-γ, are much more enhanced in the presence of TNF-α[184–186]. TNF-α induces expression of myosin II regulatory light chain (MLCK), which activates myosin II and leads to barrier dysfunction[187,188].

TNF-α can also induce inflammatory cytokines and chemokines through its NF-κB signalling arm. TNF-α increases levels of CXCL1-3 in mouse intestinal organoids as well as colonic cancer cell line models[189]. TNF-α induces mRNA of CXCR3 ligands CXCL 10 and CXCL11 and CCR5 ligand CCL5, which drive T cell recruitment to the inflammatory site, and CXCR2 ligands CXCL1, CXCL6 and CXCL8 involved in neutrophil migration, resulting in immune cell trafficking and tissue inflammation[190,191]. Our group identified the Bcl-G protein as a regulator of a select group of chemokines - especially CCL5 and CCL20, which are found to be upregulated by TNF-α in combination with IFN-γ[192].

While targeting TNF-α has been proven successful in the clinic, recent and current research efforts focus on identifying mechanisms underpinning resistance to anti-TNFs and the potential biological redundancy and synergism of TNF-α with other cytokines, such as IFN-γ in the pathogenesis of IBD.

1.4.2. FasL

Fas ligand (FasL or CD95L) is an apoptosis-inducing ligand of the TNFSF expressed as a cell membrane-associated form, and metalloproteinases can cleave FasL to its soluble form[193]. Membrane-bound FasL induces apoptosis in target cells via its receptor CD95/Fas, while the soluble form is involved in non-apoptotic activities such as promotion of inflammation, autoimmunity and tumorigenesis[194,195]. FasL is expressed by immune cells such as cytotoxic T lymphocytes, activated T cells, NK cells and monocytes and is tightly regulated[196]. In the GI tract, Paneth cells are the

only type of epithelial cell that constitutively express FasL[197]. FasL is the only member of the TNFSF that differs in its intracellular region compared to other TNFSF ligands. It possesses a conserved proline-rich domain allowing interactions with SH3 and WW-domain containing cytosolic proteins and potential tyrosine phosphorylation sites[193]. It binds to the receptor Fas/CD95, which is expressed constitutively or is highly inducible in various cell types, including lamina propria T-lymphocytes and intestinal epithelial cells[198]. The FasL/Fas signalling pathway maintains immune homeostasis through activation-induced cell death (AICD) of T cells and prevents T cell hyperactivation[193].

The role of FasL/Fas in the pathogenesis of IBD centres around the induction of apoptosis in IECs. IECs constitutively express Fas and can be subjected to apoptosis through FasL present on the immune cells. In active UC, apoptosis of crypt epithelium is observed, and apoptosis is mediated through FasL expressed by lamina propria lymphocytes[199,200]. There are reports showing that IFN-γ can induce expression of Fas and FasL on epithelial lines, thus increasing their sensitivity to FasL mediated apoptosis[201–203]. FasL/Fas cross-talk with TLRs in IECs and augments TLR-4 and TLR-5 induced cytokine and chemokine production[204]. In Crohn's disease, there is excessive T-cell infiltration into the lamina propria and defective Fas-mediated T-cell apoptosis[205,206]. An increase in the expression of the anti-apoptotic protein Bcl-2 decreased expression of Bax, and increased levels of FLIP resulted in excessive T-cell activation and survival, while such mechanisms are not observed in UC[205–207]. However, studies on FasL/Fas in murine models have revealed inconsistent and contrasting results. While the transfer of $CD4^+$ T cells expressing FasL to SCID mice induced colitis, transplantation of bone marrow cells from *gld* mice (mice that are unable to express Fas ligand and deficient in Fas signalling) into T-cell and NK-cell

deficient Tg26 mice did not prevent colitis[208,209]. Mouse models lacking expression or activity of Fas on IECs were still susceptible to DSS induced colitis[210].

Increased FasL transcripts were found in the affected mucosal region of patients with active UC but not in regions of patients with UC in remission, active CD or healthy subjects[211]. It was also observed that the soluble form of Fas, which lacks a transmembrane region and acts as an inhibitor to FasL/Fas induced apoptosis, was lower in serum from patients with active UC than those in remission or in healthy non-IBD controls[212]. More research is required focussing on the cross-talk abilities of FasL/Fas as well as their role in different models of colitis to evaluate the contribution of FasL/Fas to the pathogenesis of IBD.

1.4.3. TRAIL

TNF-α related apoptosis-inducing ligand (TRAIL) is another member of the TNFSF family that is known to preferentially induce apoptotic cell death in a variety of tumour cells but not in normal untransformed cells[213,214]. TRAIL is expressed as a cell membrane-associated form and is cleaved by metalloproteinases to its soluble form[215]. Soluble TRAIL can bind to five receptors - TRAIL-R1 (DR4), TRAIL-R2 (DR5), TRAIL-R3 (DcR1), TRAIL-R4 (DcR2) and OPG, with the highest affinity for TRAIL-R2[216]. DR4 and DR5 are death-inducing receptors, and the interaction of TRAIL with these receptors rapidly induces apoptosis through caspase-dependent pathways in sensitive cells[217]. The decoy receptors, DcR1 and DcR2, inhibit apoptosis induction by reducing the concentration of available TRAIL to bind to the death receptors[218]. In certain cell lines, TRAIL has also been shown to induce RIPK1/RIPK3-mediated necroptosis in the context of caspase-8 inhibition similar to TNF-α[219,220]. TRAIL can also activate NF-κB, Akt and MAPK pathways through its interaction with DR4, DR5 and DcR2[217,221]. TRAIL also induces NF-κB-dependent

expression of pro-inflammatory chemokines and cytokines through a FADDosome complex comprising pro-caspase-8, RIPK1 and FADD[222].

In human IECs, TRAIL has been shown to induce expression of the chemoattractant cytokine CXCL8 and the PRR *NOD2*/*CARD15*, a risk allele for IBD via the NF-κB pathway[223]. TRAIL also appears to exhibit a significant immunoregulatory role in murine models of colitis. Administration of TRAIL to mice with DSS-induced colitis resulted in reduced severity of gut inflammation[224]. Furthermore, TRAIL-R (that mimics human DR4 and DR5) deficiency in mice increased susceptibility to DSS-induced colitis, with increased epithelial damage, loss of colonic crypts and inflammatory cell infiltration[225].

Elevated levels of TRAIL mRNA were found in the inflamed colonic and ileal tissues in patients with active CD and UC compared to normal control tissue[223]. Reenaers *et al.* (2008) reported that in active intestinal sections of CD, TRAIL and TRAIL-R2 were up-regulated in the intestinal epithelial cells compared to that in normal tissues[226]. However, in contrast, Brost *et al*. (2010) reported TRAIL and TRAIL-R2 mRNA was down-regulated in the surface epithelium and upregulated in the enterocytes of the crypt epithelium[227]. In a population study on Chinese Han people, increased levels of soluble TRAIL were found in the plasma of patients with UC compared to the controls[228]. Also, the frequency distribution of three genetic mutations in *TRAIL* (G1525A/G1588A/C1595T) in the 3'UTR was significantly lower in the UC cohort than in healthy cohorts[228]. The expression of apoptosis-inducing receptors DR4 and DR5 is found to be lower in T cells from inflamed mucosa than that of normal non-IBD mucosa, resulting in lower apoptosis of T cells in inflamed regions of patients with IBD[229]. The exact role of TRAIL in the pathogenesis of IBD

still remains undefined, with conflicting findings from murine and human studies to date.

1.4.4. TWEAK

TNF-related weak inducer of apoptosis (TWEAK) is mainly expressed on T cells, macrophages and dendritic cells. It is synthesised as a transmembrane protein and is cleaved by furin proteases to release soluble TWEAK[230]. TWEAK binds to the fibroblast growth factor-inducible-14 (Fn14) receptor, which lacks a death domain. Fn14 gene expression is highly regulated and is inducible on epithelial, mesenchymal, and endothelial cells during inflammation[231]. Fn-14 can bind to TRAF 1,2,3 and 5 but importantly, TRAF-2 and TRAF-5, and activate NF-κB signalling[231]. Fn14 is usually expressed at a low level in normal cells but is highly induced by growth factors such as fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF) in injured and diseased tissues of DSS-induced colitis, vasculature after balloon catheter injury, the spinal cord of mice with experimental autoimmune encephalomyelitis (EAE), kidneys of diseased mouse models of systemic lupus erythematosus (SLE), and in many tumours[232]. TWEAK-Fn14 signalling results in several cell biological processes, including cell survival, proliferation, angiogenesis and induction of inflammatory cytokines and chemokines, all of which regulate tissue regeneration following injury[233]. TWEAK can also induce apoptosis in conjunction with other cytokines such as IFN-γ, TNF-α, or TRAIL on tumour cell lines, monocytes as well as epithelial cell lines[234–237]. Since the receptor lacks a death domain, various hypotheses have been proposed regarding the signalling pathway employed by TWEAK to induce cell death in target cells. These mechanisms include activation of a TNFR-1 mediated pathway, NF-κB dependent cell death, or c-Jun N-terminal kinase signalling[238].

Studies in murine models of intestinal inflammation and colitis show that TWEAK can play various context-dependent roles depending on the method of colitis induction and the phase and progression of the disease. TWEAK induces IEC death after γ-irradiation in mice, and TWEAK/Fn14 is indispensable for IL-13 induced IEC death after γ-irradiation[239]. In a TNBS-induced colitis model, both knock out of TWEAK and treatment with anti-TWEAK antibody offered protection with reduced inflammatory cell infiltrates and epithelial damage after the induction[240]. Combined treatment with anti-TWEAK and anti-TNF-α antibodies shows that TNBS-colitis is mediated through canonical NF-κB signalling and is protected to a greater effect with combinatorial treatments[241,242]. TWEAK/Fn14 pathway was also shown to promote upregulation of Th2 cytokines in TNBS-induced colitis and fibrosis[243]. Knockout of Fn14 protected mice from colitis in the TNBS-induced colitis model with a reduction in crypt loss, leukocyte infiltration, and collagen accumulation[243]. Fn14 knockout also reduced Th1 and Th2 mediated immunity in SAMP ileitis model mice[244]. However, Fn14 knockout increased susceptibility to colitis and colitis-associated cancer in the DSS-induced model[245]. These results suggest that the TWEAK/Fn14 pathway might have a complex context-dependent role in the regulation of intestinal mucosal immune responses, intestinal injury, and mucosal healing.

In human studies, mucosal samples from patients with UC had increased TWEAK and Fn14 expression, which correlated with disease activity[239]. Increased expression of TWEAK and Fn14 at the mRNA and protein levels in the ilea of patients with CD compared to healthy controls was also reported[244]. Another study found that serum soluble TWEAK levels were higher in patients with IBD than in healthy non-IBD controls, and the levels positively correlated with disease scores[246]. However, a

study from Norway on serum TWEAK levels in newly identified IBD cases between 2012 and 2015 found no significant difference between IBD and healthy cohorts[247].

1.4.5. LIGHT

LIGHT (homologous to **l**ymphotoxin, exhibits **I**nducible expression, and competes with HSV **G**lycoprotein-D for **H**erpesvirus entry mediator, a receptor expressed by **T**-lymphocytes) also known as TNF superfamily member 14 (TNFSF14) is a protein expressed by activated T cells, NK cells and immature DCs[248,249]. LIGHT binds to the TRAF-interacting receptors - herpesvirus entry mediator receptor (HVEM) expressed mainly on lymphocytes and lymphotoxin β receptor (LTβR) expressed on stromal and myeloid cells. LIGHT can also bind to the soluble decoy receptor - DcR3. LIGHT-HVEM signals through TRAF-2 mediated NF-κB/p50 components induce survival and inflammatory gene expression[250] and act as a co-stimulatory signal for T cells leading to proliferation, activation and survival of T cells, particularly $CD8^+$ T cells[251,252]. LIGHT binding to LT-βR can also induce apoptosis through signalling via the LT-βR-TRAF3 pathway[253]. Binding of LIGHT to decoy receptor DcR3 results in inhibition of LIGHT signalling resulting in decreased T cell activation[254].

Although LIGHT's direct or specific role in IBD has not been investigated as intensively as some other members of the TNFSF, animal models of colitis suggest a possible role of LIGHT in the pathogenesis of IBD. Transgenic mice with overexpression of LIGHT on T cells spontaneously develop intestinal inflammation with high inflammatory cell infiltrate into the lamina propria leading to tissue damage[255,256]. In an adoptive transfer model with *RAG*$^{-/-}$ mice, transfer of T cells expressing LIGHT, high levels of IFN-γ and TNF-α were found in the colon and IFN-γ- producing $CD4^+$ T cells levels increased[257]. Loss of LIGHT or HVEM in DSS induced colitis model mice exhibit lower weight loss and reduced intestinal

inflammation with low leukocyte trafficking, crypt destruction, and epithelial cell death compared to wild type control[258]. However, in animal trials by Krause *et al.* LIGHT- deficient mice had exaggerated DSS-induced colitis compared to wild type mice[259], and this finding was in direct conflict with previous work showing that neutralization of LIGHT ameliorated disease in a DSS model[260]. These discrepancies could be due to various reasons, including differences in the microbiota, the DSS model of colitis used, which is affected by both the % DSS used given either mild or severe colitis and a lot to lot variation of DSS[259]. Further studies are required to investigate the role of LIGHT in murine models of intestinal inflammation.

The human LIGHT gene is located on chromosome 19p13.3, in a known susceptible region for Crohn's disease[261]. LIGHT mRNA was significantly upregulated in colonic mucosal biopsies and is also increased in the intestines of patients with active CD compared to patients that had an inactive CD or were normal[249,261]. Recently, LIGHT was found to be elevated in a population of stromal cells that were expanded in UC compared to healthy non-IBD individuals[262]. In addition, LIGHT was shown to promote inflammatory gene expression and down-regulate homeostatic factors in fibroblasts, which have been found to have a role in the pathogenesis of IBD[263]. Treatment of patients with CD who have previously failed treatment with anti-TNF with an anti-LIGHT antibody CERC-002 has given positive phase1 trial results[264]. This antibody is also in trials for COVID-19-associated pneumonia and acute respiratory distress syndrome (ARDS)[265].

1.4.6. TL1A

TNF like molecule 1A (TL1A) was identified in 2002 as a member of the TNF superfamily and is encoded by the *TNFSF15* gene. TL1A exists in a membrane-bound form and is cleaved by metalloproteinase to a fully functional soluble protein. TL1A

is mainly found expressed on endothelial cells, dendritic cells, monocytes and activated lymphocytes, particularly gut-specific $CCR9^+$ lymphocytes[266]. IL-1 and TNF-α are the main inducers of TL1A on T cells and endothelial cells[267–269]. The primary receptor for TL1A is death receptor 3 (DR3). DR3 is expressed highly on activated lymphocytes, Foxp3+ Tregs, stimulated NK cells, ILCs and NKT cells[266,269]. Though DR3 contains a death domain, only a few studies focus on TL1A/DR3 pathway induced cell death[267,270,271]. Most of the studies investigating the role of the TL1A/DR3 pathway have focused on the co-stimulatory, proliferative and pro-inflammatory effects on the activated immune cells in several autoimmune and inflammatory diseases, including rheumatoid arthritis, psoriasis, asthma and IBD[272].

Various studies using mouse models have also established the role of TL1A in intestinal inflammation[273–275]. Knockout mice for TL1A, though not present with physical abnormalities compared to their wild type littermate controls, significantly differed in the immune parameters. DCs from *TL1A*$^{-/-}$ mice failed to support the differentiation and proliferation of Th17 cells[276]. *TL1A*$^{-/-}$ mice also had decreased $TCR\gamma\delta^+$ and $CD8^+$ lymphocytes with altered gut microbiota compared to wild type[277]. In contrast, a study by Jia *et al.*, 2016 showed that *TL1A*$^{-/-}$ or *DR3*$^{-/-}$ mice were more susceptible to DSS-induced colitis with increased IL-17A expression and decreased $CD25^+$ $Foxp3^+$ Treg cells compared to the wildtype[278]. These studies show the importance of the TL1A/DR-3 pathway in gut homeostasis and the opposing role of TL1A in acute vs chronic inflammation. Another study showed that while high TL1A expressing $Foxp3^+$ Tregs cells couldn't suppress mucosal inflammation, low expression of TL1A maintained $Foxp3^+$ Tregs cells and controlled colitis[279]. Studies in SAMP1/YitFc mice, a spontaneous model of ileitis resembling human CD, have shown increased mucosal TL1A mRNA expression with high mucosal expression of

IL-13 and IL-5[273]. Activation of DR3 signalling through an agonist antibody in the SAMP1/YitFc mice worsened ileitis development[280]. There was increased CD25-Foxp3$^+$ Treg cells and ILC1s, which increased Th1/Th2 response in the mice resulting in accelerated inflammation.

In DSS-induced colitis model, TL1A and DR3 mRNA were upregulated in MLN and colon region[274]. Neutralising TL1A using a monoclonal antibody in this model reduced mucosal inflammation along with a decrease in IFN-γ, IL-17 and IL-6 production by mucosal T cells[274]. Mice with constitutive expression of TL1A on myeloid cells were more susceptible to DSS-induced colitis[281]. These mice had lower TJ proteins and increased TNF-α mRNA, resulting in increased permeability and impaired intestinal barrier[281]. A recent study found that DCs obtained from such TL1A transgenic mice expressed high levels of co-stimulatory molecules with increased IL-1β, TNF-α and IL-12/IL-23 p40[282]. Transgenic mice expressing TL1A developed goblet cell hyperplasia in the ileum with elevated levels of IL-13 and IL-17 and presented with enhanced CD4$^+$ T cell activation[283,284]. Overexpression of TL1A on T cells in DSS and T cell transfer models resulted in higher intestinal inflammation, increased mononuclear cell infiltration of lamina propria, and increased fibrostenosis - all hallmarks of severe CD in humans[284,285]. Studies show that blocking or neutralising TL1A by anti-TL1A antibodies can ameliorate intestinal fibrosis and significantly improve disease pathology[286,287]. An anti-TL1A antibody, PF-0648005 is currently in Phase 2 clinical trials for the treatment of UC[288].

The importance of TL1A in the pathogenesis of human IBD stems from studies that reported significant associations of TNFSF15 gene variants to IBD susceptibility. Yamazaki et al., in 2005, were the first to report that genetic variations in *TNFSF15* were associated with susceptibility to IBD in Japanese and European cohorts[289].

Furthermore, TL1A and DR3 expression were high in patients with IBD, particularly in the intestinal lamina propria, and the expression of the proteins correlated with the disease severity[290–292]. Patients with the active disease were detected with TL1A and DR3 in their serum[293]. Lamina propria DR-3 expressing T cells were found in a higher proportion of patients with IBD than healthy subjects[290,294]. Additionally, TL1A was found to be a strong co-stimulatory and inducer of IFN-γ secretion by several cells, including $CD4^+$ T cells, NK cells, and gut-specific $CCR9^+$ $CD4^+$ cells[290,294–296]. TL1A can also synergise with IL-12, IL-18 and IL-23 to induce pro-inflammatory cytokine secretion resulting in chronic inflammation[274,297]. Thus, TL1A/DR3 pathway may yet represent a very promising candidate target for clinical therapies for IBD.

1.5. IFN-γ and TNF superfamily ligands

1.5.1. Interferon-gamma (IFN-γ)

IFN-γ is another important cytokine having an essential role in the pathogenesis of IBD. It is the sole member of the type II interferons. IFN-γ, initially identified as an anti-viral protein, has an important role in tissue homeostasis, immune and inflammatory responses and tumour surveillance. It is primarily produced by immune cells such as NK cells, Th1 cells, antigen-presenting cells, $CD8^+$ cytotoxic T lymphocytes (CTLs), and innate lymphoid cells (ILCs)[298]. While IFN-γ produced by NK cells contributes to early host defence, IFN-γ released by T-lymphocytes contributes to the adaptive immune response and boosts innate immunity further[299]. IFN-γ signals through the IFN-γ receptor, which consists of two subunits, IFNGR1 and IFNGR2. The binding of IFN-γ to the receptor activates the canonical JAK-STAT signalling pathway[300]. Ligand binding induces JAK2, bound to IFNGR2, autophosphorylation and JAK1, bound to IFNGR1, transphosphorylation. Activated JAKs phosphorylate the tyrosine residue 440 of IFNGR1 to create a docking site for STAT1[300]. The STAT1, phosphorylated by JAKs at the tyrosine 701 residue, forms dimers, translocate to the nucleus and regulates gene expression by binding to gamma-activated sites (GAS) elements in the promoter region of interferon-stimulated genes (ISG)s[300]. IRF-1, one of the primary response genes activated by STAT1, can activate secondary response genes or the interferon-stimulated response element (ISRE)[298].

In experimental models of inflammation, such as DSS-induced colitis, robust production of IFN-γ is observed[67]. DSS-induced inflammation is reduced to a greater extent in IFN-γ-/- mice than in wildtype, implicating a significant role for IFN-γ in experimental colitis[67,69]. In support of this, a neutralisation antibody against IFN-γ

significantly ameliorated inflammation and disease in the DSS-induced colitis model as well as T cell-mediated colitis in SCID mice[176,301]. GWAS studies show that *IFNG* and its receptor gene *IFNGR2* are associated with risk loci identified for IBD[302]. There are increased levels of IFN-γ secretion by $CD4^+$ T cells from the lamina propria from inflamed mucosa of patients with CD compared to healthy subjects[303–305]. IFN-γ mRNA expression levels are also increased in patients with mild and severe UC compared to controls[306].

The pathophysiological effects of IFN-γ have been attributed to its direct effects on epithelial cells, endothelial cells, vascular permeability, and barrier integrity either alone or in synergy with TNF-α. It is well documented that IFN-γ can induce IEC death through Fas-mediated apoptosis[307]. IFN-γ has also been reported to induce cell death of HT-29 cells, an epithelial cell line model, through upregulation of caspase-1 and downregulation of anti-apoptotic Bcl2[203]. However, several other mechanisms through which IEC are disrupted, leading to impaired barrier function, have been reported. IFN-γ induces secretion of Wnt inhibitor Dkk1, which degrades low-density lipoprotein receptor-related protein 6 (LRP6) during sustained inflammation and results in reduced IEC proliferation[69]. This effect is exacerbated in the presence of TNF-α[69]. IFN-γ increases the expression of the PR domain containing 5 PFM2 (PRDM5) which is a transcriptional regulator involved in tissue differentiation and may promote IEC apoptosis in CD[308]. Tight Junctions (TJ), important to maintaining epithelial barrier integrity, are disrupted in IBD[24]. IFN-γ induced rapid internalisation and endocytosis of TJ proteins- occludin, JAM-A, and claudin-1 resulting in epithelial cell permeability and impaired barrier function[185,309]. IFN-γ also reduced β_1-integrin-containing vesicles resulting in decreased focal adhesion, preventing epithelial cell proliferation following tissue injury[310]. Loss of

adherens junction protein VE-cadherin by IFN-γ also increases barrier permeability[311]. IFN-γ-induced cell death of Paneth cells via mTOR pathway suppression resulted in decreased anti-microbial factor release and increased microbiota- and T cell-dependent intestinal inflammation[70,312–314]. The reduction of the intestinal stem cell (ISC) niche by apoptosis through the JAK/STAT pathway is activated by IFN-γ produced by T cells[315]. Neutralizing antibodies or knockout of *IFNG or IFNGR* prevented ISC loss and maintained intestinal homeostasis. Vascular endothelium has been recognised as a second barrier in the gut, and recent studies focus on the effects of IFN-γ on angiogenesis and vascular permeability in intestinal diseases[316,317]. One mechanism identified is that IFN-γ induces GTPase guanylate binding protein (GBP-1) expression in murine models, inhibiting epithelial cell proliferation and invasiveness, thereby increasing vascular permeability[305,318]. IFN-γ can also exert anti-inflammatory effects and prevent epithelial cell death or colitis. During early inflammation, IFN-γ can induce epithelial proliferation by increasing the Wnt/β-catenin pathway[69]. In a T cell adoptive transfer model of colitis, IFN-γ protected mice from colitis by upregulation of MHCII expression on intestinal epithelial cells, promoting Treg-cell mediated tolerance[319]. These studies show that IFN-γ has anti-inflammatory properties and provides potential mechanistic explanations for the failure of anti-IFN-γ treatments in IBD clinical trials.

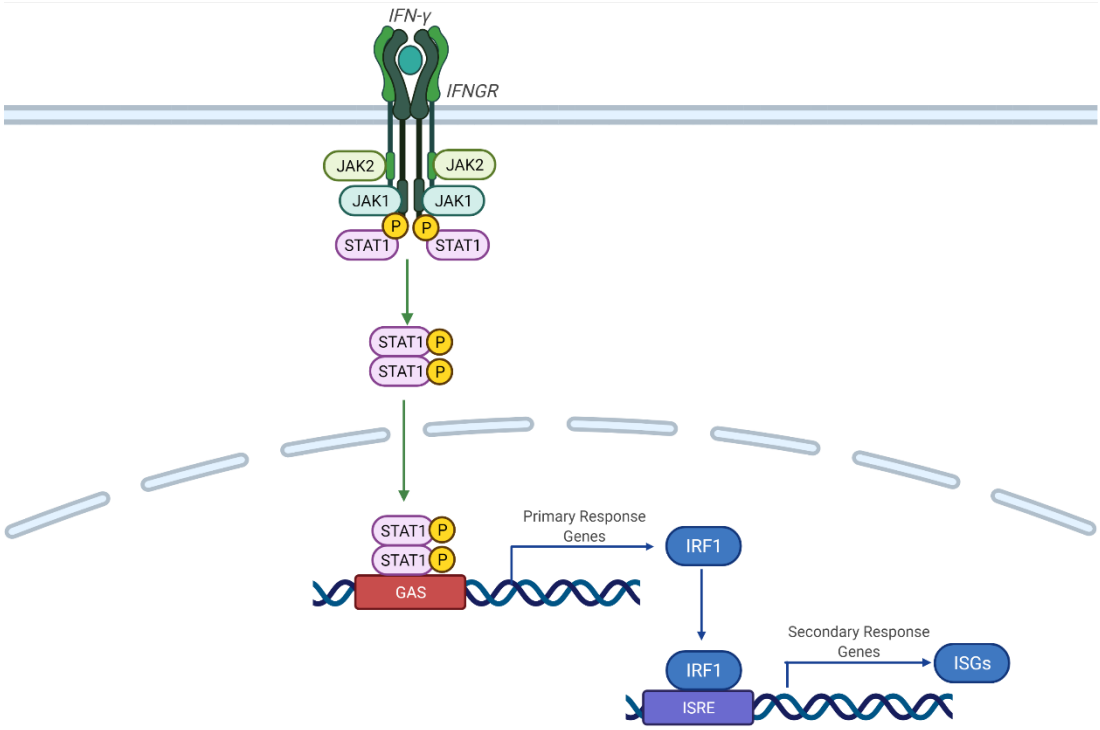


Figure 1.3: Overview of the IFN-γ signalling

Interferon-γ (IFN-γ) binds to its receptor IFNGR, consisting of subunits IFNGR1 and IFNGR2. The binding of IFN-γ to IFNGR induces phosphorylation of Janus kinases (JAK1 and JAK2). The JAKs further phosphorylate signal transduction and activator of transcription (STAT1) at tyrosine 701 residue. Phosphorylated STAT1 forms dimers, translocate to the nucleus and binds to gamma-activated sequence (GAS). This leads to the transcription of primary response genes, particularly interferon regulatory factor 1 (IRF1). IRF1 acts as a transcription factor to bind to the interferon-stimulated response element (ISRE), leading to the activation of secondary response genes to IFN-γ. Figure made in BioRender.com.

1.5.2. IFN-γ synergism with TNF-α

Synergism between IFN-γ and TNF-α has been studied for a long time in various contexts and diseases, including viral infection, cancer, ischemia, diabetes, and IBD. With a particular focus on their roles in IBD, the two Th1 cytokines are associated with increased IEC death and barrier function disruption. Our group identified that the two cytokines together induced death in primary organoids cultured from biopsies of patients with CD[192].

Multiple mechanisms have been proposed for cytokine-induced synergistic cell death depending on the tested cell lines and types. While in most epithelial cell lines, cell death was apoptosis[320,321], in others, apoptotic inhibition led to TNF-α dependent necroptosis through RIPK1/3[322]. Studies on pancreatic β-cell models show that the two cytokines induced ROS-dependent cell death and loss of mitochondrial integrity[323,324]. IFN-γ and TNF-α upregulate Fas expression in IECs, thereby leading to Fas-induced caspase-8 dependent apoptotic cell death[325]. A group in the United States recently reported that in human colon cancer cell lines and in murine bone-marrow-derived macrophages (BMDMs), the type of cell death induced by the combination of IFN-γ and TNF-α was PANoptosis, which consists of pyroptosis, apoptosis and necroptosis pathways being activated simultaneously[326,327]. The authors also identified STAT1 as a central player in cytokine-induced cell death. In support of this, a recent study from our lab identified that JAK inhibitors blocking the JAK/STAT pathway could rescue colonic epithelial cell lines and CD organoids from IFN-γ and TNF-α induced cell death. However, our group identified that cell death was neither apoptotic, necroptotic, nor ferroptotic and therefore could not be classified as PANoptosis. The cell death reported by our lab occurred through the JAK1/2/STAT-1 pathway and was dependent on a non-enzymatic scaffold function of caspase 8[180].

The two cytokines also reduce barrier integrity through non-apoptotic pathways. TNF-α increases the effect of IFN-γ inhibition of the β-catenin/wnt pathway, reducing epithelial cell proliferation and decreasing barrier function[69]. Loss of junction proteins such as occludin, claudin, cadherin, junction adhesion molecule (JAM), or catenins by IFN-γ, are much more enhanced in the presence of TNF-α[184–186]. TNF-α induces expression of myosin II regulatory light chain (MLCK) via the IFN-γ induced TNFR2 pathway[187,188]. MLCK1, in turn, phosphorylates MLC resulting in tight junction disassembly, including loss of occludins and barrier dysfunction. MLCK expression is elevated in IBD and colitis models, and inhibition of MLCK by oligomycin or ML-7 restores barrier function[328–330]. Loss of autophagy genes such as *ATG16L1* (a CD risk allele) increases the sensitivity of myeloid cells to TNF-α-mediated IFN-γ cell death resulting in impaired intestinal homeostasis and fatal systemic responses[331]. The cytokines also regulate NOD2, a risk allele for IBD, whose overexpression sensitised IEC to LPS and secretion of chemotactic CXCL8 [332]. IFN-γ and TNF-α can synergise to induce multiple inflammatory chemokines[332]. CXCL10, an inflammatory chemokine, is produced in THP1 cells by cross-talk of IFN-γ and TNF-α signalling pathways[333]. CXCL10 and its receptor CXCR3 mRNA are increased in colonic mucosal biopsies from active UC and CD[334].

Experimental colitis models of IBD - DSS, T cell transfer model or *IL-10*$^{-/-}$, all have elevated levels of IFN-γ and TNF-α. Administration of anti-IFN and anti-TNF reduces the incidence of severe disease in all of these models[176]. In DSS-induced colitis, IFN-γ and TNF-α exert their effects by inducing excessive amounts of nitric oxide (NO) synthase (iNOS) that contribute to tissue inflammation and tissue damage[301]. CX3CL1-CX3CR1 axis regulates iNOS in DSS colitis model[335]. However, in the cell line model for HT-29, the cell death was found to be independent of NOS[336].

Anti-inflammatory protein Klotho, which protects endothelium from NO-induced dysfunction, is downregulated by IFN-γ and TNF-α. Experimental models of colitis present with significant downregulation of Klotho[337]. Mice treated with IFN-γ and TNF-α reduce heat shock protein levels in the colonic mucosa, which is required to protect intestinal epithelial cells from infectious agents and maintain intestinal homeostasis[338]. This phenomenon of reduced levels of heat shock protein is also seen in inflamed colonic tissue of *IL-10*$^{-/-}$ mice and in patients with Crohn's disease. A recent study reported that IFN-γ and TNF-α inhibited the repair ability of mesenchymal stem cells (MSCs) and resulted in increased colitis with the secretion of inflammatory factors IL-17, TNF-α, IL-1β, IFN-γ and the anti-inflammatory IL-10[339]. IFN-γ and TNF-α altered serotonin transporter (SERT) expression in Caco2 cells, resulting in altered motility, secretion, and sensation in the gut. SERT levels are reduced in UC and TNBS-induced colitis[340].

The two cytokines can also induce anti-inflammatory effects. Secretion of IFN-γ and TNF-α by Th1 cells promotes the secretion of monocyte chemoattractant protein 1 (MCP-1) from Goblet cells. MCP-1 restricted homing of plasmacytoid DCs (pDCs) and prevented IFN-γ sensitised TNF-α induced apoptosis of intestinal epithelium[341]. In epithelial cells, TNF-α enhances IFN-γ-induced indoleamine-2,3-dioxygenase (IDO) through STAT1/IRF1 pathway[342]. IDO promotes the conversion of inflammatory T cells to Treg cells[343], and its expression is increased in lesions from UC and CD[344].

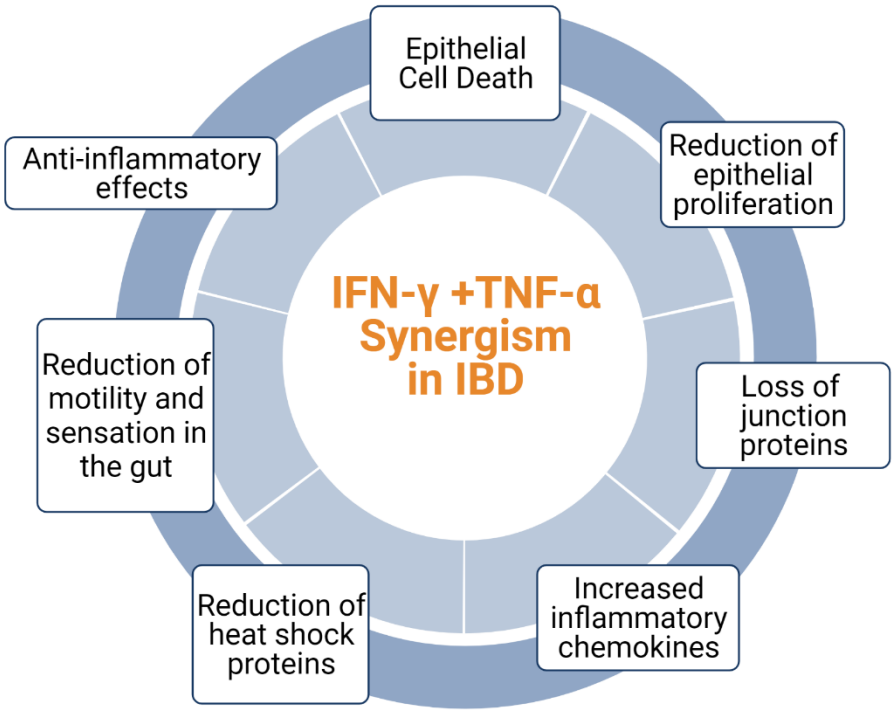


Figure 1.4: Molecular and cellular consequences of IFN-γ and TNF-α synergism in inflammatory bowel diseases

Various *in-vitro* and *in-vivo* studies have described different mechanisms by which IFN-γ and TNF-α synergise to induce significant effects on multiple cell types in the gut in the context of inflammatory bowel diseases. They have a major impact on barrier integrity by increasing epithelial cell death, decreasing epithelial cell proliferation, and disrupting the integrity of tight junctions. They also exacerbate inflammation by driving the infiltration of activated immune cells due to increased production of inflammatory chemokines. Figure made in BioRender.com.

1.5.3. IFN-γ and other TNFSF

IFN-γ can synergise with various TNFSF to induce inflammatory signals. Except for TNF-α, the synergism between IFN-γ and other TNFSF is not widely studied in the context of IBD. However, studies in other inflammatory diseases such as cancer, psoriasis, fibrosis, and others, have reported cross-talk between IFN-γ and TNFSF ligands, mainly TRAIL, TWEAK, and LIGHT have been reported. This section covers mechanisms of crosstalk reported that might play a plausible role in IBD.

IFN-γ is widely known for sensitising cancer cells to TRAIL-induced cell death. However, their synergism in IBD is yet to be explored. One might reason that IFN-γ and TRAIL can induce intestinal epithelial cell death, as seen in several cancer epithelial cell lines leading to epithelial barrier disruption. Begue *et al*. found that TRAIL-induced IEC apoptosis requires an active caspase cascade induction[223]. Interestingly, studies on various cancer lines show that IFN-γ upregulates caspase-8 in TRAIL-resistant lines and makes them sensitive to TRAIL-induced apoptosis[345–347]. Inhibition of caspase-8[348] or impairment in the activation of caspase-8[349] renders cells resistant to TRAIL even in the presence of IFN-γ. Another mechanism identifies that IFN-γ can block the upregulation of cIAP-2 by TRAIL and sensitise HeLa cells to TRAIL-induced cell death[350]. IFN-γ and TRAIL synergise with TNF-α to induce cell death in colon carcinoma by repressing Bcl-xL expression[351]. IFN-γ regulates TRAIL and its receptors selectively depending on cell types. IFN-γ upregulated TRAIL and TRAIL-R (DR4/DR5) in tumour cells and not normal cells, resulting in paracrine and autocrine TRAIL-induced cell death[345,352–354]. In anti-viral defence, IFN-γ upregulates TRAIL and downregulates TRAIL-R in uninfected cells, increasing TRAIL-induced cell death on infected cells only[355]. IFN-γ regulated TRAIL levels in NK cells, augmenting their cytotoxic effects against tumour cells[356,357]. In IBD, we

find differential expression of TRAIL and its receptors on the epithelium and T cells[227,229].

TWEAK can promote cell proliferation or cell death depending on cell types and physiological context. TWEAK, in combination with IFN-γ, induces apoptosis in mesangial cells[358], keratinocytes[359], and colonic epithelial cells[360]. IFN-γ induces TWEAK expression on monocytes leading to monocyte-induced cytotoxicity against cancer cell lines[234]. Mechanistically, IFN-γ and TWEAK induce apoptotic cell death via caspase-8. However, in colonic carcinoma line HT-29, caspase-8 inhibition led to necrosis through cathepsin-B[360]. Cytotoxic effects of IFN-γ on squamous carcinoma line or erythroblasts could be completely rescued with the combination of antibodies targeting TWEAK, TRAIL and FasL, suggesting co-operating cell death signalling from the two death ligand[234,237]. Recently, a positive correlation between levels of TWEAK and IFN-γ was identified in patients with psoriasis[361]. Combined treatment with TWEAK and IFN-γ produces various inflammatory chemokines such as CCL2, CCL5, CCL20, CXCL1, IL-6, and IL-1[236,358,362]. miR -149 is identified as a mediator between IFN-γ and TWEAK signalling crosstalk[236]. In the context of IBD, IFN-γ's role in modulating TWEAK responses for cell death and production of inflammatory chemokines needs to be further explored.

LIGHT enhances IFN-γ production by acting as a co-stimulatory signal for T-cell activation and increases cytotoxic activity of T-lymphocytes in several diseases[251,363,364]. LIGHT-HVEM signalling regulates IFN-γ production by ILC cells in the small intestine in response to bacteria *Yersinia enterocolitica* infection[365]. IFN-γ can synergise with LIGHT to induce apoptosis in cells. One of the main mechanisms suggested through work with different cell lines such as hepatocellular carcinoma - Hep3BT2[366] and HepG2[367], breast cancel line - MDA-MB-231[368], and pancreatic β-

cells - MIN6 cells[369] is the downregulation of anti-apoptotic protein Bcl-2. In the colonic cancer line HT-29, downregulation of anti-apoptotic protein Bcl-X_L and upregulation of pro-apoptotic molecules Bak and Bad triggered the activation of cell death by IFN-γ and LIGHT[370]. Another study evaluating the effects of IFN-γ and LIGHT on HT-29 cells found that both apoptosis and necrosis were activated in HT-29 cells[371]. In this study, cell death was rescued by inhibiting IFN-signalling using a non-selective inhibitor, Genistein, while inhibition of p38 MAPK partially rescued the cells. Free radical scavenger could completely inhibit IFN-γ and LIGHT induced cell death of Hep3BT2 cells, indicating a role of ROS in the synergistic cell death[366,372]. In all these studies, while apoptosis was predominantly dependent on caspase-3, determined by its increased activation, inhibition of caspases only partially rescued the cells, suggesting that other mechanisms might be at play and require further investigation.

1.6. Aims of the thesis

The inflammatory cytokines TNF-α and IFN-γ play a critical role in driving the immunopathogenesis of Inflammatory Bowel Diseases (IBD). Notably, both cytokines can synergise with each other to increase the expression of inflammatory genes and induce cell death in intestinal epithelial cells. Targeting pro-inflammatory cytokines, such as TNF-α, has greatly benefitted patients with IBD. Anti-TNF biologics revolutionised the treatment of IBD, but only 30-40% of patients respond initially to these drugs. Remaining patients with IBD are either non-responders or lose response to anti-TNFs over time. This might be due to the pleiotropic function of cytokines, complex cytokine networks, multiple factor regulation including environment, genetic and microbiome factors as well as biological redundancy built into cytokine and chemokine networks and the activation of redundant cytokine or other ligand-receptor pathways. Based on the fact that TNF-α and IFN-γ occupy highly connected nodes in cytokine networks and can synergize with each other and with other pathways we hypothesized that the ability to synergize with other cytokines is one of the defining characteristics of mediators that occupy highly connected nodes in disease-associated cytokine networks. These mediators might then represent good therapeutic targets for IBD. On this basis, we investigated the molecular mechanisms underpinning TNF-α and IFN-γ synergism and used synergy with IFN-γ as selection criteria to identify other members of the TNF superfamily of ligands which might be redundant with TNF-α in driving inflammation in IBD.

The main aim of Chapter 2 was to investigate and identify potential mechanisms underpinning synergism between TNF-α and IFN-γ in terms of gene regulation and cell death of intestinal epithelial cells. In this Chapter we report the

identification of critical genes and pathways activated when the cytokines are combined, which can be targeted to block cytokine-induced synergistic cell death.

The aim of Chapter 3 was to identify cytokines which are redundant with TNF-α in terms of their ability to synergise with IFN-γ. In particular, we focussed on the TNF superfamily members of which TNF-α is also a member.

The aim of Chapter 4 was to investigate in more detail the mechanisms underpinning the ability of TRAIL – one of the TNF-like cytokines identified in Chapter 3 – to synergise with IFN-γ both in terms of induction of inflammatory chemokine production and cell death.

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Chapter 2 : IFN-γ and TNF-α synergise to induce STAT1 dependent cell death through pathway cross regulation

2.1. Abstract

IFN-γ and TNF-α are Th1 cytokines that synergise to induce inflammatory gene expression and cell death in a wide variety of cell types. In the gut, they can trigger disruption of the epithelial barrier, a common manifestation seen in inflammatory bowel diseases (IBD). The mechanisms underpinning synergism of IFN-γ and TNF-α for both gene regulation and induction of cell death are poorly understood. We investigated this phenomenon in the human colonic epithelial cell line HT-29 and human primary colonic organoids using a combination of gene expression profiling, weighted gene co-expression network analysis (WGCNA), RNAi, RT-qPCR, western blotting and HTRF STAT1 phosphorylation assays. Gene expression and gene ontology enrichment analysis showed that IFN-γ+TNF-α (IT) induced cell death was associated with enrichment of terms for multiple cell death pathways such as apoptosis, necroptosis and also mitochondrial metabolism. However, inhibitors of these canonical cell death pathways did not block IT-induced cell death, suggesting other mechanisms or redundancy in cell death pathways activated by this cytokine combination. WGCNA of global gene expression identified 14 highly correlated modules of co-regulated genes, of which two, the magenta and yellow modules, were selected for further evaluation. Genes in the magenta module induced by IFN-γ treatment encoded proteins belonging to the TNF-α signalling pathway (*TNFRSF1*A, *TNFRSF1B*), while yellow module genes induced upon TNF-α treatment encoded proteins from the IFN-γ signalling pathway (*IFNGR1, IFNGR2, JAK1*). IT treatment resulted in a two-fold increase in overall module gene expression compared to single treatments. RNAi perturbation confirmed that the upregulation of TNF-α receptors by IFN-γ was dependent on STAT1, and the upregulation of IFN-γ receptors by TNF-α was NF-κB dependent. STAT-1 knockdown but not NF-κB knockdown rescued cells

from IT induced cell death. Also, priming of cells with TNF-α resulted in enhanced activation of STAT1 upon subsequent treatment with IFN-γ. Pathway cross-regulation was also observed in cytokine treated human primary colonic organoids undergoing synergistic cell death. Collectively, we identified a possible molecular mechanism through which IFN-γ and TNF-α synergise with one another. Our data support a model whereby each cytokine signalling pathway cross-regulates genes for components of the co-operating cytokine signalling pathway, thereby boosting the response of cells to both cytokines over time.

2.2. Introduction

The intestinal tract is constantly challenged with both food-borne antigens and microbial antigens. A single layer of epithelial cells serves as a critical barrier preventing excessive contact of these antigens with underlying immune cells in the lamina propria[1,2]. In inflammatory bowel diseases, the epithelial barrier is often compromised with altered barrier function contributing to the initiation of inappropriate inflammatory responses that can lead to intestinal epithelial cell (IEC) death[3,4]. In Crohn's disease (CD), a form of IBD, this inflammatory response is associated with Th1 type immunity characterized by elevated levels of the inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ)[5,6].

TNF-α is a pleiotropic cytokine with both pro-survival and pro-death effects on multiple cell types[7]. TNF-α binding to TNF receptor 1 (TNFR1) results in the formation of signalling complex I consisting of TNFR1-associated death domain protein (TRADD), receptor-interacting protein kinase 1 (RIPK1), E3 ubiquitin ligases-cellular inhibitor of apoptosis proteins (cIAP1/2) and the linear ubiquitin chain assembly complex (LUBAC)[8]. Ubiquitinated RIPK1 in complex I recruit transforming growth factor-β (TGFβ)-activated kinase 1 (TAK1)[9]. TAK1 further activates signalling mediated by Jun N-terminal kinase (JNK) and p38 as well as the IκB kinase (IKK) complex. The IKK complex activates nuclear factor κB (NF-κB), which regulates the transcription of pro-survival and inflammatory genes. The binding of TNF-α to its receptor can also lead to different forms of cell death signalling. Ubiquitylation of RIPK1 acts as a central switch determining if TNF-α signals for cell survival or cell death[10]. Deubiquitylation of RIPK1 by enzymes A20 or

cylindromatosis (CYLD) or due to the depletion of E3-ubiquitin ligases cIAP1/2 results in the dissociation of RIPK1 from complex I into the cytosol. Complex IIa is formed through the interaction of cytosolic RIPK1 with TRADD, FAS-associated death domain protein (FADD), pro-caspase-8, and long isoform of FLICE-like inhibitory protein ($FLIP_L$)[10]. Dissociation of RIPK1 can also lead to complex IIb formation where RIPK1 assembles with RIPK3 rather than TRADD[11]. Both complex IIa and IIb activate caspase-8 and elicit classical apoptotic cell death[12]. When caspase-8 activation is inhibited, RIPK1 and RIPK3 form the necrosome complex (complex IIc), which recruits the pseudokinase mixed lineage kinase domain-like (MLKL) and stimulates the necroptotic cell death pathway[13]. TNF-α can signal to multiple cells through TNFR1 and exerts both immunoregulatory and inflammatory effects in the gut. Patients with IBD show high levels of TNF-α produced by lamina propria (LP) mononuclear cells such as inflammatory macrophages[14]. Anti-TNF antibodies are highly efficient in inducing and maintaining clinical remission of IBD and have been used in the clinic for more than 25 years[15,16]. However, anti-TNFs have been ineffective in the treatment of about 20-30% of patients with CD, and 30-50% of patients lose response over the course of the therapy[17–19]. Though the reason for the failure of anti-TNFs in these patients is still under investigation, a potential explanation might be that additional TNF-like cytokines are redundant with TNF-α in the pathogenesis of IBD[19–21].

IFN-γ is a pleiotropic cytokine with major roles in the inflammatory response, tissue homeostasis and tumour surveillance[22]. IFN-γ binds to its receptor IFNGR consisting of two ligand-binding IFNGR1 chains and two signal-transducing IFNGR2 chains[23]. IFNGR1 is bound by Janus kinase 1 (JAK1) at its intracellular domain and IFNGR2 by JAK2[24]. The binding of IFN-γ to the receptor brings JAK1 and JAK2 in

close proximity and facilitates the autophosphorylation and transphosphorylation of JAKs, thus activating them[25]. Activated JAK1 phosphorylate IFNGR1 to provide a docking site for signal transducer and activator of transcription 1 (STAT1). STAT1 gets phosphorylated at the tyrosine 701 (Tyr 701) residue and serine 727 (Ser 727) residue by JAKs[23]. Phosphorylated STAT1 forms homodimers and translocates to the nucleus, and binds to IFN-γ activated site (GAS) to induce the expression of target genes, including the transcription factor interferon regulatory factor 1 (IRF1)[26]. Like TNF-α, IFN-γ can also signal for apoptosis and necroptosis. IFN-γ exerts apoptotic effects through upregulating caspase-8 expression and activation through a STAT1/IRF1 pathway in tumour cells[27]. IFN-γ also upregulates the expression of death-inducing ligands such as FasL or TRAIL, which activates caspase-8 through their signalling pathway[28–30]. IFN-γ induces necroptosis when effector molecules, such as caspase-8[31], NF-κB[32], FADD[33] or RIPK1[34], are inhibited. Necroptosis in these contexts occurs through the JAK-STAT-IRF1 pathway and involves Z-form nucleic acid-binding protein 1 (ZBP1). ZBP1 interacts with RIPK3 through its C-terminal RIP homotypic interaction motif (RHIM) and activates necroptosis[35]. RIPK1, FADD and caspase-8 form an inhibitory complex and suppress IFN-induced necroptosis by cleaving RIPK3[35]. Patients with CD have high levels of IFN-γ secreted by $CD4^+$ T cells in lamina propria of inflamed mucosa compared with healthy subjects[36]. Patients with UC also express high levels of IFN-γ mRNA compared to control subjects[37]. Also, *IFNG* and *IFNGR2* genes are present in a loci identified as a risk factor for IBD through genome-wide association studies (GWAS) studies[38].

IFN-γ and TNF-α can co-operate to promote intestinal damage by inducing synergistic cell death of IECs[39–42]. We recently reported that IFN-γ and TNF-α synergistically kill intestinal epithelial cells through a caspase-8-JAK1/2-STAT1

pathway, with STAT1 as one of the key effectors[43]. Blocking STAT1 activation with JAK inhibitors proved effective in rescuing cells from IFN-γ and TNF-α induced cell death. However, the mechanism resulting in enhanced activation of STAT1 is still under investigation. In this study, we investigated the mechanisms underpinning IFN-γ+TNF-α synergism and STAT1 activation through microarray data profiling. We use weighted gene co-expression network analysis to analyse the data and identify highly correlated module genes. We also use primary human colonic organoids to translate results identified in the cell line model and propose a feedback mechanism loop activated through IFN-γ+TNF-α combinatorial treatment.

2.3. Materials and Methods

2.3.1. Cell culture

Human colorectal adenocarcinoma cell line HT-29 was obtained from American Type Culture Collection (#HTB-38). HT-29 parental and the CRISPR/Cas9 *STAT1* knockout clones were cultured in McCoy's 5A medium (#M9309, Sigma- Aldrich) supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin, and 0.1 mg/mL streptomycin (#P4333, Sigma-Aldrich). The cell lines were incubated at 37ºC supplemented with 5% CO_2. The lines were passaged 1-2 times per week and routinely checked for mycoplasma contamination using MycoAlert Mycoplasma Detection Kit (#LT07-118, Lonza).

2.3.2. Generation of CRISPR/Cas9-mediated gene knockout line

HT-29 cells were electroporated with gRNA-Cas9 RNP complexes using the Neon Transfection System (#MPK10025, Invitrogen). Alt-R CRISPR-Cas9 crRNA targeting exon 10 of *STAT1* (TGTGATAGGGTCATGTTCGT) and Alt-R CRISPR-Cas9 tracrRNA labelled with ATTO 550 (#1075928) were purchased from IDT. Briefly, 100 µM gRNA duplex was prepared by mixing equimolar amounts of crRNA and tracrRNA in Duplex Buffer (#11-01-03-01, IDT), followed by incubation for 5 mins at 95 °C. gRNA-Cas9 RNP complexes were prepared by mixing 1.2 µL of gRNA duplex and 1.7 µL of Atl-R Cas9 nuclease (#1081058, IDT) with 2.1 µL of phosphate-buffered saline (PBS) (#D8537, Sigma-Aldrich) for 20 mins at room temperature (RT). For electroporation, HT-29 cells were harvested by trypsinisation (#T3924, Sigma-Aldrich), washed with PBS, and resuspended in PBS at a concentration of

$1.0*10^7$ cells/mL. For each electroporation, 10 µL of cells were gently mixed with 2.5 µL of the gRNA-Cas9 RNP complexes and electroporated using the Neon Transfection System with the following parameters: 1650 V, 10 ms, 4 pulses. Immediately after electroporation, cells were transferred to 0.5 mL of prewarmed McCoys 5A (10% FBS, antibiotic-free) and incubated overnight in a 24-well plate. The next day cells were trypsin-harvested and single-cell sorted using FACSAria Fusion (BD Biosciences). Expanded clones were screened for STAT1 knockout by Sanger sequencing with validation by western blotting.

2.3.3. Human studies

The collection and use of colonic pinch biopsies were ethically approved by the Clinical Research Ethics Committee of the Cork Teaching Hospitals (CREC). All patients provided informed consent in agreement with the Declaration of Helsinki. The biopsies were collected from consented adult non-IBD patients at Cork University Hospital and Mercy University Hospital. All non-IBD patients were comprised of otherwise healthy individuals undergoing investigation for symptoms such as weight loss, anaemia or altered bowel habit.

2.3.4. Primary human colonic organoid culture

Protocols for crypt isolation and organoid culture were adapted from previously described methods[44,45]. Colonic biopsies from non-IBD patients were collected and stored at 4 °C in collection media [Advanced DMEM/F12 (#12634-010, Gibco)] supplemented with 10% FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 10 mM HEPES (#15630-122, Gibco), 2 mM Glutamax (#35050061, Gibco), 1X

Amphotericin B (#SV300.78.01, HyCloneTM) and 100 µg/mL gentamicin (G1397, Sigma-Aldrich). Biopsies were washed in cold 1X PBS supplemented with 1X Amphotericin B and 100 µg/mL gentamicin. They were dissociated using Gentle Cell Dissociation Reagent (#07174, Stem Cell Technologies) supplemented with 1X Amphotericin B and 200 µg/mL gentamicin for 20 mins at room temperature with shaking at 180 rpm. Reagent activity was quenched using cold Wash Medium (Advanced DMEM/F12 supplemented with 10% FBS, 100 IU/mL penicillin, 0.1 mg/mL streptomycin, 10 mM HEPES, and 2 mM Glutamax) and the suspension was passed through a 70 micron filter. The filtered solution was centrifuged at 200 rcf for 5 mins and the supernatant discarded. The pellet was re-suspended in Wash Medium and transferred to a 1.5 mL tube and centrifuged at 400 rcf for 3 mins. The supernatant was removed and the pellet containing the isolated crypts was resuspended in Reduced Growth Factor Type 2 BME (#3533-010-02, AMS Biotechnology). In 48-well plates, 20 µl domes of crypt/BME solution were seeded and placed in a cell culture incubator for 20 mins. Once polymerised, the domes were overlaid with 300 µL of Proliferation Medium. Proliferation media was prepared as follows: serum-free media (Advanced DMEM/F12 supplemented with 10 mM HEPES, 2 mM Glutamax, 1X N2 supplement (#17502001, Gibco), 1X B27 supplement (#17504044, Gibco), 1 mM N-acetylcysteine (#A9165, Sigma-Aldrich) and 50 ng/mL human recombinant EGF (#100-47, PeproTech) was combined 1:1 with L-WRN conditioned media[46]. This 50 % L-WRN media was supplemented with 10 mM Nicotinamide (#N0636, Sigma-Aldrich), 10 µM Y-27632 (#S1049, Selleckchem), 10 µM SB-202190 (#S7067, Sigma-Aldrich), 5 µM CHIR-99021 (#SML1046, Sigma-Aldrich) and 500 nM A-83-01 (SML0788, Sigma-Aldrich), and filtered using the Steriflip vacuum system (Merck-Millipore). Proliferation media was replenished twice a week. For passaging,

organoids were removed from BME and dissociated using Tryple Express (#12604, Gibco) supplemented with 10 µM Y-27632, which was then quenched with Wash Medium. The cell solution was centrifuged at 400 rcf for 3 mins and the supernatant was removed. The pellet was re-suspended in fresh BME and seeded. Passaging was performed every 1–2 weeks, using a 1:3 split ratio. For all experiments, organoids were used in an undifferentiated state by seeding them in the Proliferation medium. For live phase-contrast light microscopy, following treatments as indicated in the figures, images were acquired using the Evos FL microscope (ThermoFisher). Acquired image as processed using ImageJ software with cropping around the region of interest, brightness/contrast adjustment using enhanced local contrast (CLAHE), and insertion of scale bars.

2.3.5. Recombinant proteins, reagents and antibodies

Recombinant human IFN-γ (#300-02) and TNF-α (#300-01A) were purchased from Peprotech. 25 ng/mL IFN-γ and 50 ng/mL TNF-α were used for treatments throughout unless different concentrations are specified. The following inhibitors were used; apoptosis and necroptosis were inhibited using 20 µM Z-VAD-FMK (Z-VAD) (#14463, Cayman Chemicals) and 10 µM of 7-Cl-O-Nec-1 (Nec1s) (#20924, Cayman Chemicals), ferropotosis was inhibited using 5 µM Ferrostatin (FERRO) (A4371, ApexBio) and ROS was quenched using the anti-oxidant N-acetyl cysteine (NAC) at 10 mM. Cells were treated with inhibitors for 30 mins prior to cytokine treatments. For western blotting the following antibodies were used: anti-caspase 8 (#9746), anti-caspase 3 (#9662), anti-cleaved caspase 3 (#9661), anti-TNFR1 (#3736), anti-TNFR2 (#3727), anti-NFκB p65 (#3034), anti-IκBα (#4812), anti-JAK1 (#3332), anti-JAK2 (#3230), anti-STAT1 (#9172), anti-p-STAT1 Tyr701(#9167), anti-p-STAT1 Ser727

(#9177) and, anti-IRF1 (#8478); all from Cell Signaling Technology and diluted at a ratio 1:1000; anti-RIPK1 (0.25 µg/mL, #610459, BD bioscience), anti-IFNGR1 (0.1 µg/mL, #sc-12755, Santa Cruz), anti-IFNGR2 (0.58 µg/mL, #ab171081, Abcam), anti-β-actin (1:10000, #A1978, Sigma-Aldrich), anti-GAPDH (1:80000, #am4300, Ambion), and the secondary antibodies - goat anti- Rabbit (1:10000, #A6154, Sigma-Aldrich) and goat anti-mouse (1:10000, #A2554, Sigma-Aldrich). All antibodies were diluted in 5% bovine serum albumin (BSA) in Tris-buffered saline supplemented with 0.1% Tween-20 (TBS-T).

2.3.6. Microarray gene expression profiling

HT-29 cells were treated in triplicate with either IFN-γ or TNF-α or both (denoted as IT) for 8 hrs, and untreated cells were used as controls (denoted as UT). Total RNA was extracted using the RNeasy Mini kit (Cat no. 74106, Qiagen) according to the manufacturer's protocol. The RNA was labelled and hybridised to Affymetrix microarray (GeneChip Type - Human Genome U133 Plus 2.0) according to the manufacturer's instructions. Raw microarray data (.CEL files) were pre-processed in R using the "expresso" function of the "affy" Bioconductor package, with "mas" algorithm for background correction and no normalisation, followed by scaling of arrays to the same average intensity (i.e. 200). The resulting expression values for 54,675 probes were highly similar among arrays, with an average expression correlation of 0.964. The arrays were normalised by a quantile method available in "preprocessCore" Bioconductor package. A total of 29,856 probes that were present in three or more arrays were selected. The maximum expression value of multiple probes for the same gene was used as a representative expression in an array. The resulting total of 13,055 genes was analysed further. The significance of differential

gene expression in each treatment compared to UT was assessed with the limma package[47]. Genes with a *P*-value of 0.05 or less and an absolute fold change of 0.5 were considered differentially expressed.

2.3.7. Transcriptional regulator prediction

PWMEnrich Bioconductor package in R was used to identify the transcription factor binding motifs in the promoter regions of all differentially expressed genes, and those that were up-regulated and down-regulated separately in the combined treatment set. The human genomic sequences and transcription start sites of the differentially expressed genes were obtained from BSgenome.Hsapiens.UCSC.hg19 and TxDb.Hsapiens.UCSC.hg19.known gene Bioconductor packages, respectively. The promoter sequences spanning from upstream 2000 and downstream 300 nucleotide bases from the transcription start sites of the genes were used for the identification of transcription factor binding motifs using motifEnrichment function. Motifs with affinity scores above 3 and *P*-value of 0.05 or less were considered as significantly enriched in the promoter sequences of differentially expressed genes.

2.3.8. Functional enrichment analysis

Piano Bioconductor package was used for running gene set analysis (GSA)[48]. First, Gene Ontology (GO) biological process terms corresponding to all the analysed genes were extracted from "org.Hs.eg.db" human gene annotation package and subsequently converted into GO sets. GO annotations derived from electronic annotation transfer were not considered for analysis. The GO sets were then subjected to runGSA function of the Piano package to test their differential enrichment in treated samples compared

to UT using seven methods. The number of genes in the set was set between 10 to 300, and for the significance assessment, 10,000 permutations were used for gene sampling. Consensus scores of seven methods were obtained using the consensusHeatmap function.

2.3.9. Identification of gene modules using WGCNA

Weighted gene correlation network analysis (WGCNA) was performed to identify co-regulated genes and cluster these genes into modules associated with the different treatments or traits[49]. First, we selected the top 8000 genes with variable expression patterns across all conditions, which eliminated genes that do not change expression in the sample groups studied and also simplified computation. The correlation matrix was obtained by calculating the Pearson correlations across all samples' genes. Next, the adjacency matrix was calculated by raising the absolute values of the correlation matrix to a power ($\beta = 12$)[49]. For ease in computation, the gene list was further restricted by omitting genes with low connectivity (k) (the summation of connection strengths for each gene with all other genes). 4000 genes were selected with connectivity above the median connectivity value of 0.227. Topological overlap (TO) was then calculated, taking into account the correlation signs, as described previously[49,50]. Next, the genes were hierarchically clustered using 1-TO as the distance measure, and modules were determined using a dynamic tree-cutting algorithm, with a deep split value of 2 and a minimum module size of 60. The selected 4000 genes were grouped into 14 modules with gene numbers ranging from 82 to 666 per module. Once modules were identified, the module eigengene was calculated using all genes in each module. The GO term functional enrichment analysis of modules was performed by clusterProfiler Bioconductor package. We plotted the

distribution of expression fold changes computed between treated groups compared to controls. The significance of the differential fold changes of module genes was assessed using Kruskal Wallis non-parametric multi-group comparison test.

2.3.10. Endpoint cell viability and cell death assays

$2*10^4$ cells/well of HT-29 cells ($1*10^4$ in case of organoids) were seeded in 96-well plates and incubated overnight in media containing antibiotics and 10% FBS until they reached 80% confluency. Cells/organoids were treated with either IFN-γ, TNF-α or both for durations indicated in the figures. Cell viability was measured using CellTiter-Glo® assay (Cat no. G7573, Promega) following the manufacturer's instructions and as previously described by Woznicki *et al*. (2020)[41]. The luminescence reading was captured using a Synergy2 microplate reader (BioTek).

For crystal violet staining, HT-29 cells, after cytokine treatments, were washed twice with phosphate-buffered saline (PBS) at the endpoint and fixed using 100% methanol for 10 mins at -20 ºC. Cells were stained using crystal violet solution (0.5% crystal violet solution (Cat no. V5265, Sigma- Aldrich) in 20% methanol) for 15 mins. The cells were washed in PBS and left to dry overnight. The plates were scanned using the Odyssey 9120 imaging system (LI-COR), and digital images were processed in ImageJ software.

2.3.11. Cell cycle analysis

$3*10^4$ cells/well of HT-29 cells were seeded in 96-well plates and incubated overnight in media containing antibiotics and 10% FBS until they reached 80% confluency. Cells were treated with either IFN-γ, TNF-α or both for durations indicated in the

figures and trypsinised. Following trypsinisation, the cells were washed with 2 mM EDTA PBS. They were fixed in 70% ethanol for 2 hrs and washed twice with PBS containing 0.1% Triton X-100 (PBS-T). The cells were stained using 5 µg/mL propium iodide, 33 µg/mL RNase A in PBS-T for 1 hr at 37 ºC, and analysed using BD Accuri C6 flow cytometer (BD Bioscience). The data acquired were processed using the ModFit LT software (Verity Software House).

2.3.12. RNA isolation and RT-qPCR

$3*10^5$ cells/well of HT-29 cells were seeded in 12-well plates and incubated overnight in media containing antibiotics and 10% FBS until they reached 80% confluency. HT-29 cells were treated with cytokines for durations as indicated in the figures, and total RNA was extracted using the RNeasy Mini kit and on-column DNA digested (#79526, Qiagen) according to the manufacturer's protocol. Organoids were seeded in 48-well plates, overlaid with proliferation medium and left to recover for 3 days before cytokine treatment. For lysis of organoids, they were washed twice with cold 1X PBS and 300 µL of Culturex Organoid Harvesting Solution (#3700-100-01, Bio-Techne) was added. BME domes were disrupted and transferred to 1.5 mL tubes. After 1 hr incubation at 4 °C with shaking at 180 RPM, organoids were centrifuged, washed twice in cold 1X PBS and re-suspended in 350 µL of buffer RLT supplemented with β-mercaptoethanol. RNA isolation was performed using the RNeasy Micro kit (#74004, Qiagen) following the manufacturer's instructions. 500 ng of total RNA from HT-29 cell line or 100 ng from organoids were reverse transcribed using transcriptor reverse transcriptase (#3531287001, Roche) and random primer (#11034731001, Roche). RT-qPCR was performed on 2.5-10 ng of cDNA with 1X Sensi Mix II probe kit (#BIO-83005, Bioline), 500 nM forward and reverse primers (see Table 2.1 for

sequences) (designed using Probe Library System Assay Design platform (Roche) and purchased from Eurofins genomics) and 250 nM Probe (Roche) in a Light Cycler 480 instrument (Roche). The $2^{-\Delta\Delta CT}$ method was used to calculate fold changes in mRNA expression relative to the geometric mean of the two housekeeping genes- *β-actin and GAPDH* for HT-29 cell line and *HPRT1 and RPL13A* for organoids[51,52].

2.3.13. Western blotting

For protein extraction, HT-29 cells were seeded in 60 mm dishes at a density of 1.5 $*10^6$ cells/dish and incubated overnight until 70-80% confluence was reached. Following cytokine treatments as specified and for the indicated durations in the figure legends, HT-29 cells were washed with PBS and proteins extracted using RIPA lysis buffer containing 50 mM Tris (pH 8.0), 150 mM sodium chloride, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1X HALT phosphatase and protease inhibitor cocktail (#78440, Thermo Scientific) and 0.25 mM AEBSF-HCl serine protease (#S7378, Selleckchem). Cell debris was removed by high-speed centrifugation for 20 mins at 4°C. The protein concentration was determined using Pierce® BCA assay (#23225, Thermo Scientific) according to the manufacturer's protocol. 20-30 µg of protein was denatured in 1X Bolt LDS Sample Buffer (#B0007, Invitrogen) supplemented with 1X Bolt Sample Reducing Agent (#B0009, Invitrogen) by heating for 10 mins at 70 °C. The samples were separated in Bolt® 4-12% Bis-Tris gels (#NW04125BOX, Invitrogen). After proteins were transferred to a 0.45-micron PVDF membrane (#IPVH00010, Millipore), the membranes were blocked for 1 hr with 5% BSA (#A7906, Sigma-Aldrich) dissolved in TBS-T. Primary antibodies, diluted in 5% BSA in TBS-T, were added to the membranes and left rocking overnight at 4 ºC. Membranes were washed thrice with TBS-T before adding the secondary antibodies

for 1 hr at RT. The blots were developed using WesternBright Quantum HRP substrate (#K-12042-D10, Advansta) following the manufacturer's instructions. The images were captured using a LAS-3000 imager (Fujifilm). Where required, the brightness/contrast was adjusted for the entire image using ImageJ-win64 software.

2.3.14. RNAi mediated gene knockdown

HT-29 cells were reverse transfected with siRNA (25 nM) for 48 hrs using Lipofectamine RNAi Max transfection reagent (#13778150, Invitrogen). The following siRNAs were obtained from Dharmacon as ON-TARGETplus SMARTpool reagents: ON-TARGETplus non-targeting pool- siCTRL (#D-001810-10-20), si*STAT1* (#L-003543-00-005) or si*NF-κB RELA/P65* (#L-003533-00-005) (See table 2.2). siRNA-Lipofectamine transfection complex was prepared by mixing equal volumes of 0.25 μM siRNA solution in Opti-MEM and Lipofectamine RNAi Max transfection reagent diluted 33.33 fold in OptiMEM. The resulting transfection complex (20 μL for 96-well and 60 μL for 48-well plates) was added onto the plates and incubated for 20 mins at RT. Meanwhile, HT-29 cells were harvested by trypsinisation and resuspended in antibiotic-free media containing 10% FBS. Cell suspensions (5,000 cells/80 μL for 96-well and 15,000 cells/300 μL for 48-well) were added to the wells containing the transfection complexes. After 48 hrs, cells were treated with cytokines as indicated in the figures and figure legends. Knock-down efficacy was measured at 48 hours post-transfection by RT-qPCR and western blotting.

2.3.15. Homogeneous Time Resolved Fluorescence (HTRF) STAT1 and NF-κB Phosphorylation Assays

HTRF assays is a FRET (fluorescence resonance energy transfer)-based technology with time-resolved measurement of fluorescence for quantification of proteins. The assay was performed according to the manufacturer's instructions to detect the following proteins: total STAT1 (#63ADK096PEG, CisBio), phospho-STAT1 Tyr701 (#63ADK026PEG, CisBio), total NF-κB (#64NFTPEG, CisBio), phospho-NF-κB Ser536 (#64NFBPET, CisBio). HT-29 cells ($2*10^5$ cells/mL) were seeded in 96 wells and incubated for 24 hrs in 100 µL of media before cytokine treatments. After cytokine treatments, the supernatants were removed, 50 µL of supplemented lysis buffer was added to each well, and the cells were lysed for 30 mins with shaking at room temperature. 16 µL of the lysed sample was transferred to a 384-well plate and mixed with 4 µL of antibody solution mix (containing an equal ratio of provided cryptate and a d2 STAT1 antibody). After overnight incubation of 16 hrs, the plate was read for fluorescence at two different wavelengths- 665 nM and 620 nM using a Synergy plate reader (BioTek). The normalised values were calculated by the following method:

- HTRF Ratio= (Signal at 665 nm * 10^4)/signal at 620 nm
- Normalized value= (phospho-Protein HTRF Ratio) *100/(Total Protein HTRF Ratio)

2.3.16. Statistics

Statistical analysis was carried out using GraphPad Prism version 5 as described in the figure legends. $P < 0.05$, $P < 0.01$, and $P < 0.001$ were considered to be statistically significant.

Table 2.1: ROCHE Universal Probe Library assays used in this study

Gene	**Gene ID**	**Probe #**	**Forward Primer**	**Reverse Primer**
β-Actin	60	11	ATTGGCAATGAGCGGTTC	CGTGGATGCCACAGGACT
GAPDH	2597	60	AGCCACATCGCTCAGACAC	GCCCAATACGACCAAATCC
TNFR1	7132	5	TCCAAATGCCGAAAGGAA	CAATAATGCCGGTACTGGTTC
TNFR2	7133	23	CTCCTTCCTGCTCCCAATG	CACACCCACAATCAGTCCAA
NF-κB-RELA/p65	5970	47	ACCGCTGCATCCACAGTT	GATGCGCTGACTGATAGCC
IκBα	4792	38	GTCAAGGAGCTGCAGGAGAT	GATGGCCAAGTGCAGGAA
RIPK1	8737	25	GTACCTTCAAGCCGGTCAAA	CAGGGAAGGAGCAAACCAG
IFNGR1	3459	57	CATGCAGGGTGTGAGCAG	AACATTAGTTGGTGTAGGCACTGA
IFNGR2	3460	70	GCTGCTGCTCGGAGTCTT	GGTCTCCAAAGTTAGCTGTGCT
JAK1	3716	72	CAGTTCATGCTGCTGAAAACC	TCTCTACTTTCCAAAGCTACTTCAGAG
JAK2	3717	50	ACCTGGTGAAAGTCCCATATTC	AGGCCACAGAAAACTTGCTC
STAT1	6772	74	GGATCAGCTGCAGAACTGGT	TTTCTGTTCCAATTCCTCCAA
IRF1	3659	36	GAGCTGGGCCATTCACAC	AGTTGGCCTTCCACGTCTT

Table 2.2: List of siRNAs used in this study

siRNA	**Gene ID**	**Gene Accession**	**siRNA sequence**
siCTRL	-	-	Non-targeting pool. #D-001810-10-20, Dharmacon
si*STAT1*	6772	NM_007315.3	• AGAAAGAGCUUGACAGUAA • GAACCUGACUUCCAUGCGG • CUACGAACAUGACCCUAUC • GCACGAUGGGCUCAGCUUU
si*NF-κB-RELA/p65*	5970	NM_021975.3	• CCACACAACUGAGCCCAUG • GGCUAUAACUCGCCUAGUG • CCCACGAGCUUGUAGGAAA • GGAUUGAGGAGAAACGUAA

2.4. Results

2.4.1. IFN-γ and TNF-α synergise to induce cell cycle arrest and cell death

Previous studies have demonstrated that IFN-γ and TNF-α can synergise to induce cell death in intestinal epithelial cell line models[40,41,43]. In this study, we confirm these findings by showing that IFN-γ and TNF-α (IT) synergised to induce cell death in HT-29 cells in a time-dependent fashion inducing about 80% cell death after 48 hrs compared to their individual effects (Fig 2.1A). Cell death was accompanied by the activation of caspase-8 and caspase-3 after 24 hrs and 48 hrs of IT treatment (Fig 2.1B). Cell death (about 30%) and caspase activation were induced upon stimulation with IFN-γ alone over 48 hrs but not with TNF-α. IFN-γ treatment resulted in cell cycle progression over time with higher percentage (35%) of cells in the S-phase. However, TNF-α and IT treatment induced G1-S cell cycle arrest at 12 hrs, evident with a higher percentage of cells in G1 phase and a lower percentage in the S-phase compared to untreated cells (UT) (Fig 2.1C).

2.4.2. IFN-γ and TNF-α treatment differentially regulates a higher proportion of genes compared to individual cytokine treatment

To better understand mechanisms underpinning the synergistic killing of HT-29 cells by the two cytokines, we performed genome-wide gene expression profiling of HT-29 cells treated with IFN-γ or TNF-α alone or the combination of IFN-γ and TNF-α (IT) for 8 hrs and compared them to untreated cells (UT). Cells treated separately with IFN-γ and TNF-α individually treated cells had an expression correlation of 0.97 and 0.96

against UT samples, respectively. Whereas IT had a lower expression correlation of 0.88 against UT. Principal component analysis also indicates a high degree of similarity in the changes in gene expression in HT-29 cells in response to stimulation with either IFN-γ or TNF-α alone compared to UT samples (Fig 2.2A). In contrast, cells treated with the IT combination differed substantially. The first two principal components captured 97.4% of the variance in the expression profiles.

Of the 6,759 differentially expressed genes (DEGs) identified using the limma package in R, only 2-4% of the DEGs were unique to IFN-γ (185) or TNF-α (303) treatments, respectively, whereas the combined treatment with IT resulted in differential expression of about 50% of the genes (Fig 2.2B). The fraction of upregulated and downregulated genes were similar in the IFN-γ treatment, while TNF-α and IT treatments had a greater fraction of differentially expressed genes that were downregulated compared to upregulated (Fig 2.2C). TNF-α and IT treatments suppressed the expression of more than 22% of the genes compared to the IFN-γ treatment alone. We then analysed the enrichment of transcription factor binding motifs in the promoter regions of these DEGs. IFN-γ regulated genes had motifs enriched for STAT and IRF family binding (Fig 2.2D). Interestingly, the same motifs were found in genes up-regulated by IT treatment (Fig 2.2D IT-up). Genes regulated by TNF-α were also enriched in transcription factor binding motifs belonging to the STAT1/2 and IRF families. IT down-regulated genes had motifs enriched for binding to the ETS (Erythroblast Transformation Specific) family of transcription factors (ELK1, ETV6, ETS1) (Fig 2.2D IT-down). Chemokine genes such as *CXCL-1,3,8,9,10, CCL-2,20,22, CX3CL1*; Guanylate binding protein genes (*GBP- 1, 1P1, 5*); genes belonging to the complement system (*C1S, C3, CFB*); genes associated with major histocompatibility complex (*DRA, DPA1, DOB, DRB4, TAP1, CD74*); and

interferon-induced genes (*IFI16, IFITM1, IFI44*) were all increased in expression by IT treatment compared to IFN-γ or TNF-α treatment alone (Fig 2.2E). Genes whose expression was decreased by IT treatment included adhesion molecules (*MUC1, ICAM4*); solute carrier family (*SLC2A6, SLC6A14*), long non-coding RNAs (*HOTAIR, LINC00261, LINC00920, MNX1-AS1*); zinc finger proteins (*ZNF585A, ZNF488, RTP4*).

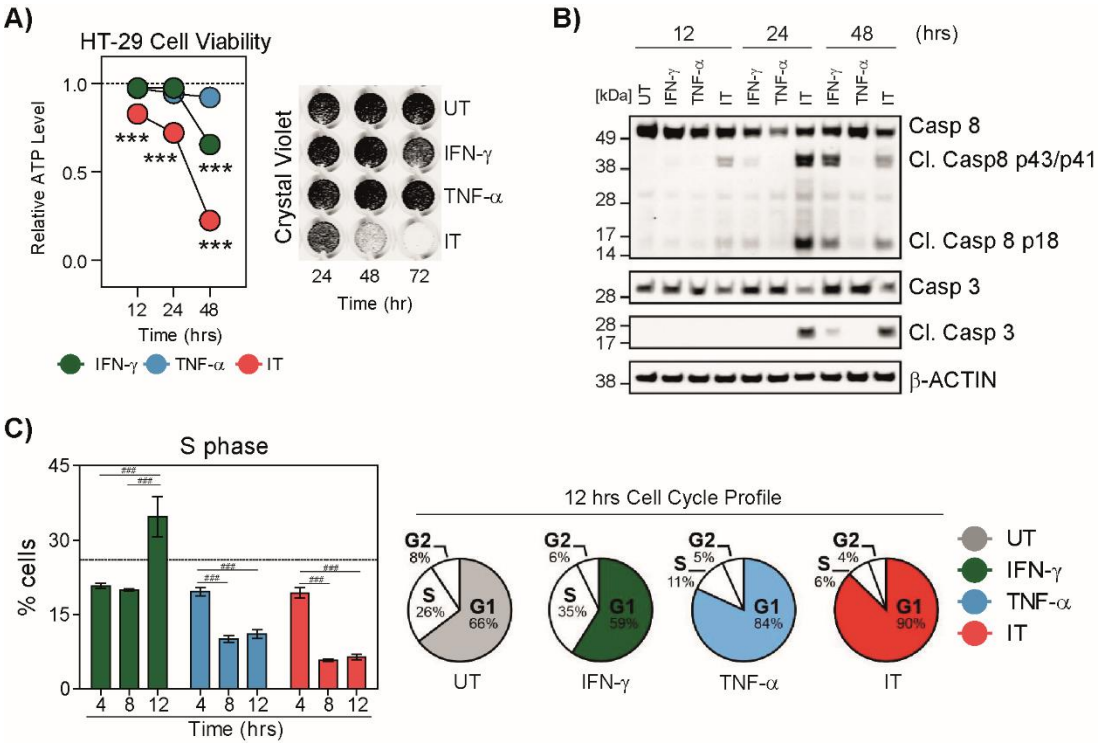


Figure 2.1: IFN-γ and TNF-α synergise to induce cell death in HT-29 cells

HT-29 cells were stimulated with IFN-γ, TNF-α, or both cytokines for the indicated times. (**A**) Cell viability was analysed by CellTitre-Glo® (left panel) and cell death by crystal violet staining (right panel). Data were normalised to the UT samples. (**B**) Western blots for cleaved caspase-3 and caspase-8 confirm caspase activation. Blots are representative of three independent experiments. (**C**) Cell cycle profile of cells analysed by flow cytometry. Data are normalised to respective untreated samples. Data are represented as mean ± SEM from three independent experiments. *p<0.05, ** p <0.01, *** p <0.001, student's t-test compared to UT, #- two-way ANOVA with Bonferroni posthoc test compared to 4 hrs treatment.

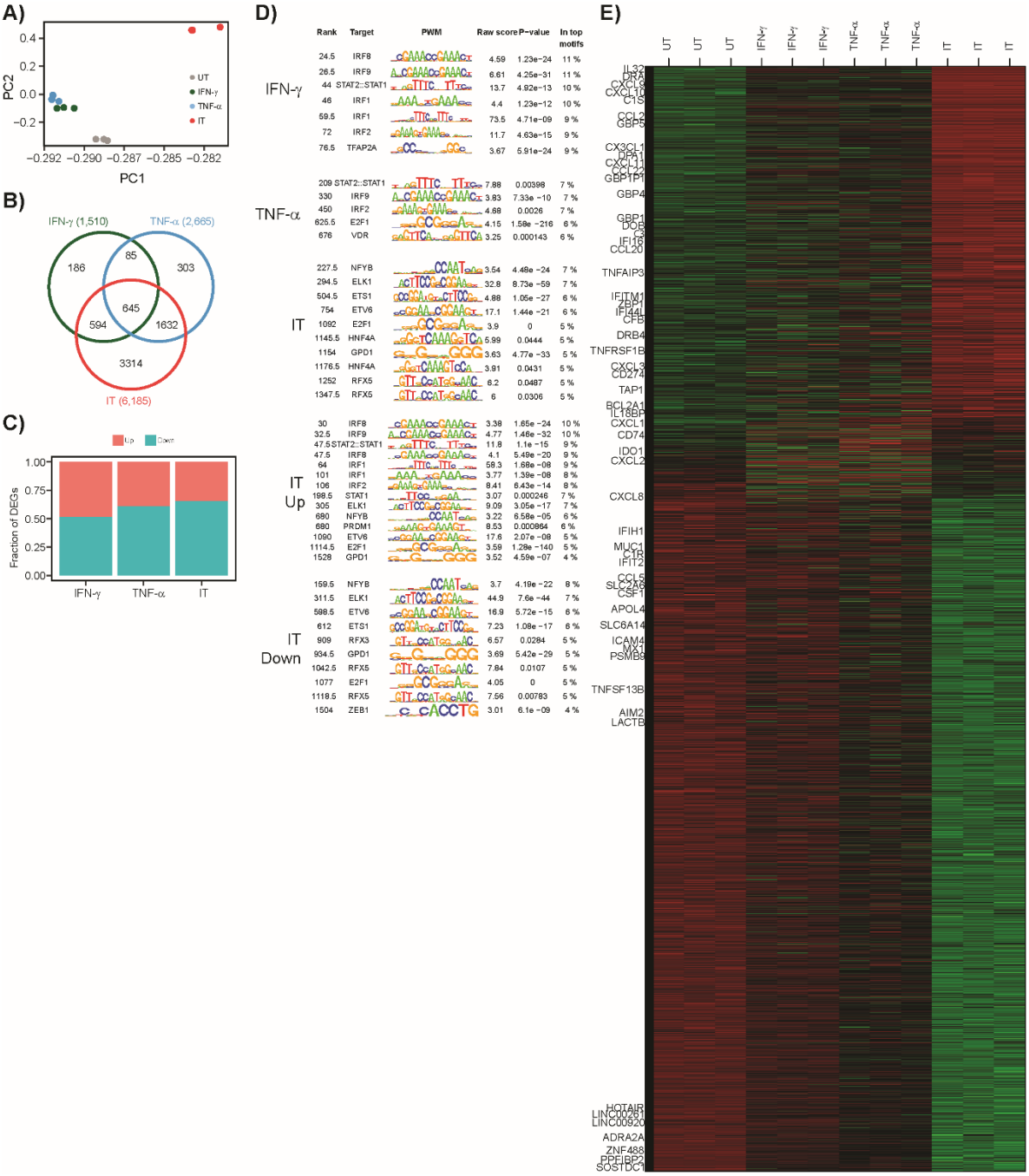


Figure 2.2: **IFN-γ and TNF-α combined treatment has a different gene expression profile compared to either cytokine treatment alone**

(**A**) PCA plot representing the expression relationship between samples from genome-wide gene expression profiling of HT-29 cells treated with IFN-γ, TNF-α or a combination of both cytokines (IT) for 8 hrs. (**B**) Venn diagram showing the number of differentially expressed genes in each of the treatment conditions compared to the untreated (UT) samples and (**C**) the fraction of differentially expressed genes. (**D**)Transcription factor binding motifs in the

promoter regions of differentially expressed genes identified through the PWMEnrich Bioconductor package. (**E**) Heatmap of all the differentially expressed genes compared to UT samples. Select genes coding for chemokines, guanylate binding proteins, proteins associated with complement systems, major histocompatibility complex, adhesion molecules, solute carrier family and zinc finger proteins are mentioned on the left of the heatmap.

2.4.3. IFN-γ and TNF-α regulate genes associated with multiple cell death pathways, DNA replication and repair and immune response

To identify and understand the biological processes associated with differentially expressed genes induced by cytokine treatments, we derived Gene Ontology (GO) sets through the Piano package in R (Fig 2.3A). Focussing first on the GO terms associated with mitochondrial functions, while IFN-γ did not have a significant effect, TNF-α downregulated genes that were enriched in mitochondrial electron transport and mitochondrial respiratory chain complex I assembly. Genes associated with mitochondrial translational elongation and mitochondrial translation termination were unique to the IT-down enrichment. In terms of GO terms for DNA replication and repair, IFN-γ and TNF-α had opposing effects. IFN-γ upregulated genes enriched for DNA replication, DNA synthesis involved in DNA repair, strand displacement, telomere maintenance and G1/S transition of mitotic cell cycle, while TNF-α downregulated all GO terms associated with DNA replication and repair. IT had a similar effect to that of TNF-α in that DNA replication and repair GO terms were enriched in IT-down. These results correlate with our earlier finding (Fig 2.1C) of the presence of a higher percentage of cells in the S-phase when HT-29 cells were treated with IFN-γ and a lower percentage of cells in the S-phase when cells were treated with TNF-α or IT. As expected, TNF-α treatment and IT treatment upregulated genes with enrichment for NF-kappaB/TNF signalling- I-kappaB kinase/NF-kappa B signalling, NIK/NF-Kappa-B signalling, positive regulation of NF-kappa B transcription factor activity, cellular response to tumour necrosis factor, and tumour necrosis factor-mediated signalling pathways. We also identified that these processes were also

enriched in the IFN-up group. IFN-γ also enriched for GO terms involved in interferon signalling, such as interferon-gamma mediated signalling, response to interferon-gamma, along with response to interferon-alpha, positive regulation of type-I interferon production. Interestingly, TNF-α and IT- treatments also upregulated genes with enrichment for interferon signalling. All cytokine treatments, individual or combination, led to significant enrichment of biological processes involved in immune responses and cytokine signalling, such as inflammatory response, innate immune response, response to lipopolysaccharide, defense response to virus, T-cell receptor signalling pathway, chemokine-mediated signalling and cytokine-mediated signalling. ER-unfolded protein response (UPR) GO terms were associated with TNF-α-up and IT-up groups. These responses include the process of IRE-1- mediated UPR, PERK-mediated UPR, ER-golgi vesicle-mediated transport. Lastly, focusing on cell death, GO terms for apoptotic process, necroptotic process, regulation of extrinsic apoptotic signalling pathway via death domain receptors as well as negative regulation of extrinsic apoptotic signalling pathway via death domain receptors, were associated with all three IFN-γ-up, TNF-α-up and IT-up groups.

2.4.4. IFN-γ and TNF-α induced inflammatory cell death is independent of canonical death pathways

Since cell death GO terms- apoptosis process and necroptosis process- were enriched in IFN-γ-up, TNF-α-up and IT up treatments, we perturbed key effector molecules in these pathways using small molecule inhibitor to pinpoint the mode of IT-induced cell death. Apoptosis induced by TNFR1 death receptor complexes involves downstream activation of caspases[7]. Inhibiting apoptosis with a pan-caspase inhibitor (z-VAD) failed to block IT induced cell death (Fig 2.3C). TNFR-1 can also signal for RIPK1

dependent caspase-8 apoptosis[7]. However, inhibiting RIPK-1 with Nec1s alone could not inhibit IT-induced cell death. Caspase inhibition, particularly caspase-8, leads to RIPK1-dependent necroptosis[53,54]. Treating cells with z-VAD and Nec1s only marginally increased cell viability (7%) after the IT-treatment. Inhibiting ferroptosis through ferrostatin, an iron-dependent form of non-apoptotic cell death[55] also did not block cells from IT-induced cell death. Ferrostatin, in combination with z-VAD and Nec1s, could only increase the cell viability by 10% after the IT-treatments. It has been demonstrated that reactive oxygen species (ROS) can drive apoptosis and TNF-α induced necroptosis[56,57]. However, eliminating ROS using the antioxidant N-acetyl cysteine (NAC) did not block IT-induced cell death. Surprisingly, even the combination of all the inhibitors to simultaneously block all these inhibitors of cell death pathways could not rescue HT-29 cells from IT-induced cell death. However, as reported by our lab in a recent paper, these inhibitors could rescue cells from classical sensitisers such as cycloheximide (translational inhibitor), BV-6 (SMAC mimetic) or IKK-16 (IKK inhibitor) and TNF-α-induced killing of HT29 cells[43]. These results indicate the existence and activation of alternate or additional redundant cell death pathways induced by the combination of IFN-γ and TNF-α in intestinal epithelial cells.

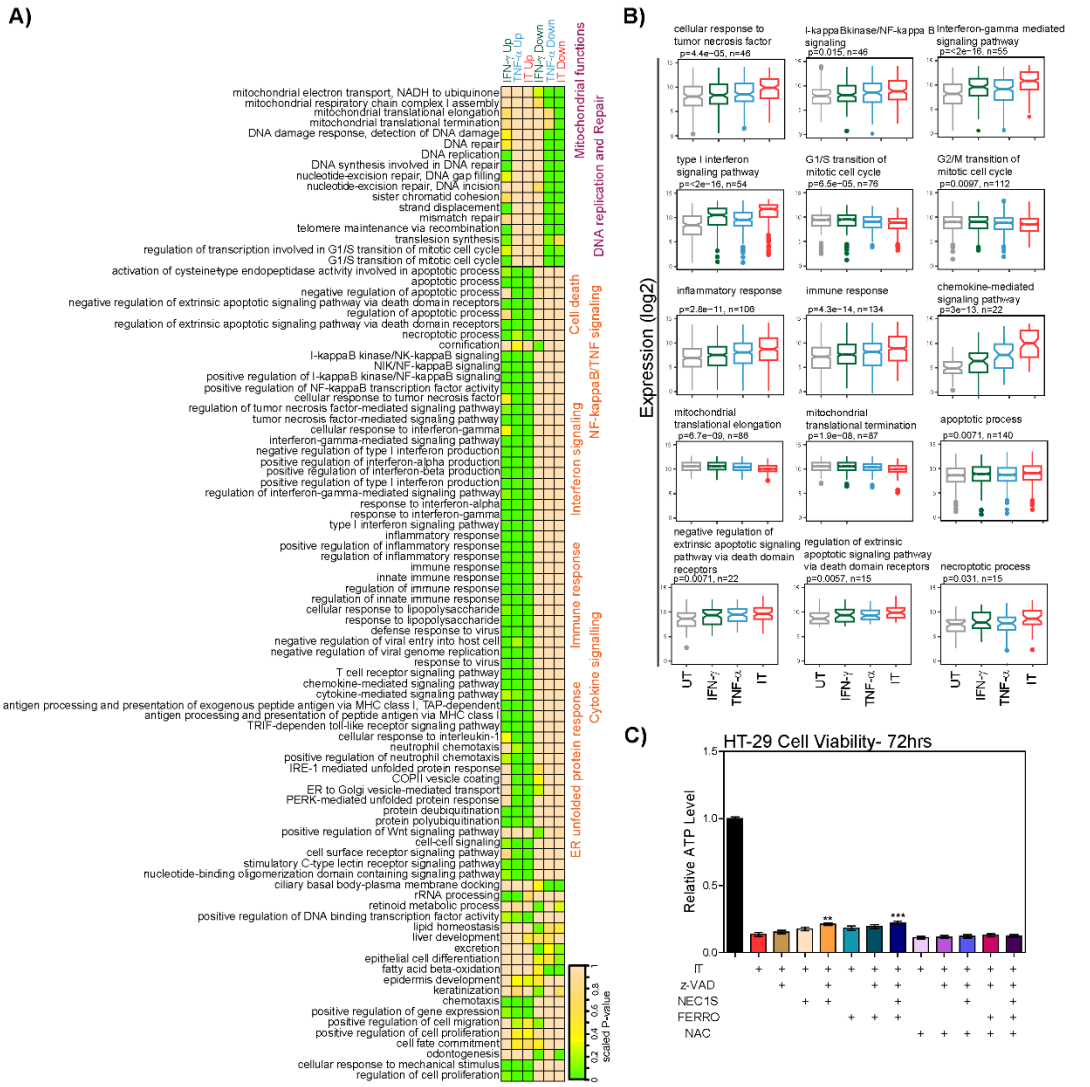


Figure 2.3: The combination of IFN-γ and TNF-α induces inflammatory cell death independent of canonical cell death pathways

(A) Enrichment of Gene Ontology (GO) biological process terms identified through the Piano Bioconductor Package **(B)** Box plots of log fold change for genes belonging to selected GO terms. **(C)** Cell Viability of HT-29 cells pre-treated with either 20 µM inhibitor of apoptosis (z-VAD), 10 µM necroptosis (nec1s), 5 µM ferroptosis-ferrostatin (FERRO) or 10 mM an antioxidant- N-acetylcysteine (NAC) and their combinations and treated with IT for 72 hrs. Data are represented as mean ± SEM from three independent experiments in triplicates. *p<0.05, **p<0.01, ***p<0.001, one-way ANOVA with Tukey's multiple comparison test with IT.

2.4.5. WGCNA identifies fourteen highly co-expressed gene modules associated with individual and combination cytokine treatments

Weighted gene correlation network analysis (WGCNA) was performed on 4000 highly differentially expressed genes to identify groups of genes or gene modules that were either positively or negatively associated with different cytokine treatments (IFN-γ alone, TNF-α alone or the combination of IFN-γ and TNF-α). Each module consisted of genes that are considered to be highly related in terms of enrichment for similar biological pathways or functions. Fourteen highly correlated modules were obtained, with turquoise, blue and brown modules containing a higher percentage of genes than other modules (Fig 2.4A). The distribution of expression fold changes was plotted and computed between treated groups compared to UT for module genes (Fig 2.4B/C). GO term enrichment analysis of module genes belonged to four major categories, which include cell division (replication and nucleotide biosynthesis), unfolded protein response, vesicle trafficking, and signalling involved in immune response. Overall, the association of the modules, either positively or negatively, were higher with IT treatment compared to individual cytokine (IFN-γ or TNF-α) treatment (Fig 2.4B). The magenta module genes were upregulated with IFN-γ treatment and IT treatment. This module was enriched for interferon signalling processes- response to interferon-gamma, response to type I interferon, and type I interferon signalling pathway, immune response- defense response to virus, and regulation of T-cell activation. The genes belonging to the red module were significantly downregulated with IFN-γ and IT treatment but did not have any enrichment of GO terms in the four categories analysed (Fig 2.4C). Salmon and yellow modules were positively

associated with TNF-α treatment and IT-treatment and contained genes that were enriched for unfolded protein responses process such as response to unfolded protein, endoplasmic reticulum unfolded protein response, response to topologically incorrect protein, and response to endoplasmic reticulum stress. Salmon module had a higher gene ratio associated with these processes compared to the yellow module. The yellow module had a higher gene ratio for the enrichment of immune responses such as response to lipopolysaccharide and response to molecules of bacterial origin. The yellow module is also enriched for GO terms associated with protein transportation, such as Golgi vesicle budding, vesicle coating, and vesicle budding from membrane. TNF-α and IT treatment downregulated genes in the cyan, green, pink and turquoise modules. Except for cyan, all the other three modules contained genes that enriched for GO terms of DNA replication process like DNA replication, DNA-dependent DNA replication, nuclear DNA replication, cell cycle DNA replication, mitotic nuclear division. The pink module had genes with higher significance and a higher gene ratio enriched for these processes, while the green module had a lower significance of enrichment. Cyan module did not enrich for the GO terms analysed. The greenyellow and purple modules were positively associated only with IT treatment and not the cytokine treatment alone. These modules also had lower significance for genes enriching for GO terms such as regulation of T-cell activation, regulation of lipid kinase activity, regulation of antigen-receptor mediated signalling pathway, and positive regulation of transcription from RNA polymerase II for the purple module; and response to ER, Golgi vesicle budding, vesicle coating, and vesicle budding from membrane for the greenyellow module. Blue, black and brown modules were associated negatively with IT treatment. The blue module was enriched for nephron epithelium development, nephron tubule morphogenesis, nephron tubule development

and kidney morphogenesis. The black module was enriched for glutamine metabolic process, nucleotide biosynthetic process, glycosyl compound biosynthetic process. Both these modules had a lower significance and gene ratio associating with these processes. The brown module did not enrich for the pathways investigated in this study. Of all 14 modules, only the tan module had genes that were significantly upregulated by both IFN-γ and TNF-α either alone or in combination. A higher gene ratio with high significance was enriched for interferon signalling- response to interferon-gamma, response to type I interferon, type I interferon signalling pathway in this module. The tan module also positively enriched genes involved in response to ER stress and response to lipopolysaccharide.

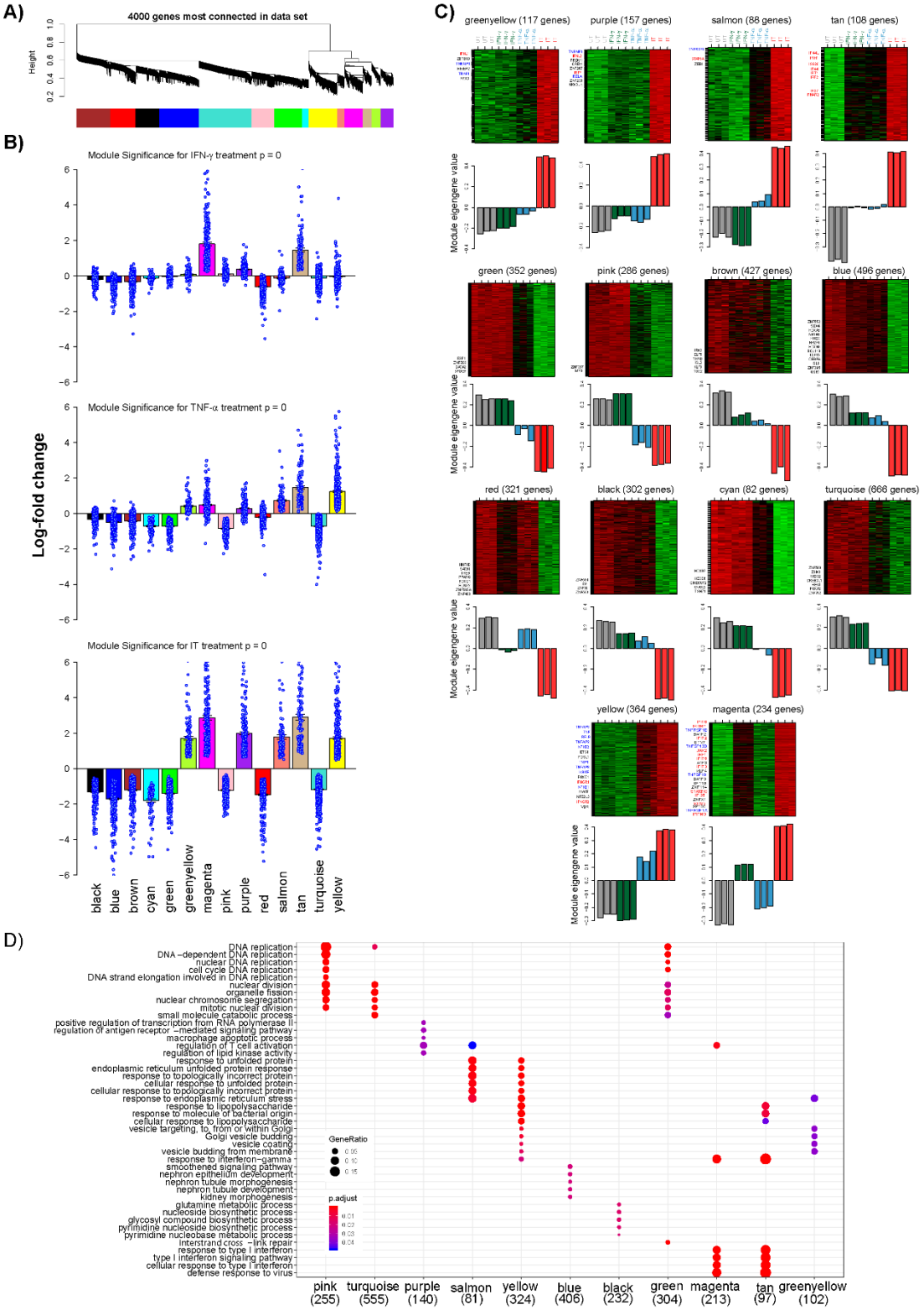


Figure 2.4: WGCNA identifies fourteen highly co-expressed gene modules upon treatment with the combination of IFN-γ and TNF-α

(**A**) Fourteen modules were identified by weighted gene co-expression network analysis (WGCNA). (**B**) Module significance of each treatment compared to untreated (UT) for each

of the modules identified. (**C**) Heat maps and bar plots of module eigengene representing the summarised expression of genes in each module. (**D**) Enriched GO terms identified by clusterProfiler Bioconductor package for genes of each module except brown, cyan and red modules.

2.4.6. IFN-γ and TNF-α cross regulate each other's receptors and signalling components

An intriguing finding from WGCNA was the identification of two modules - the yellow and magenta modules – which contained genes coding for components of both co-operating cytokine pathways. The yellow module contained genes that were upregulated by TNF-α and further increased by IT treatment. These genes included genes coding for IFN-γ receptors (*IFNGR1* and *IFNGR2*), *NFKB1, NFKB2,* and *RELB* (Fig 2.4C-Yellow). The magenta module had genes upregulated by IFN-γ alone and enhanced by IT treatment. These included genes coding for the TNF receptors- *TNFR1* and *TNFR2, JAK2,* and *IRF1* (Fig 2.4C-Magenta). To validate these findings, we performed a time-course experiment on HT-29 cells treated with either IFN-γ, TNF-α or the combination of both. At the mRNA level (Fig 2.5A), we identified that IFN-γ upregulated the expression of components of its own signalling pathway - *JAK2, STAT1 and IRF1* in a time-dependent manner. In addition, it also upregulated TNF-α pathway genes such as the TNF-receptors *TNFR1 and TNFR2* in a time-dependent fashion. Treatment with TNF-α increased expression of *NF-κB RELA/p65, IκBα, and RIPK1* belonging to the TNF-α signalling pathway up to 4 hrs. TNF-α also upregulated the expression of *IFNGR1, IFNGR2 and JAK1* belonging to the IFN-γ signalling pathway at 4 hrs, and there was a reduction of the expression at 8 hrs. IT treatments resulted in increased and sustained expression of all these selected genes over the course of 8 hrs.

At the protein level (Fig 2.5B), expression of the TNF-α receptors (TNFR1 and TNFR2) increased upon IFN-γ treatment and IT treatment. TNF-α treatment increased the expression of IFNGR1 and IT treatment further increased the expression of

IFNGR1. Interestingly, no effects on the protein levels of IFNGR2 were observed at 4 or 8 hrs with any of the treatments. While IκBα protein levels were increased upon IFN-γ treatment, no change was observed at the mRNA level. We also looked at the levels of two phosphorylated forms of STAT1, namely STAT1 Tyr 701 and STAT1 Ser 727. STAT1 was phosphorylated at both the residues following IFN-γ treatment, and this was enhanced and sustained at 8 hrs with the combined treatment of IFN-γ and TNF-α. These results indicate that the cytokines IFN-γ and TNF-α co-regulate each other's pathway, and that this regulation is associated with sustained activation of STAT1 when the cytokines are combined.

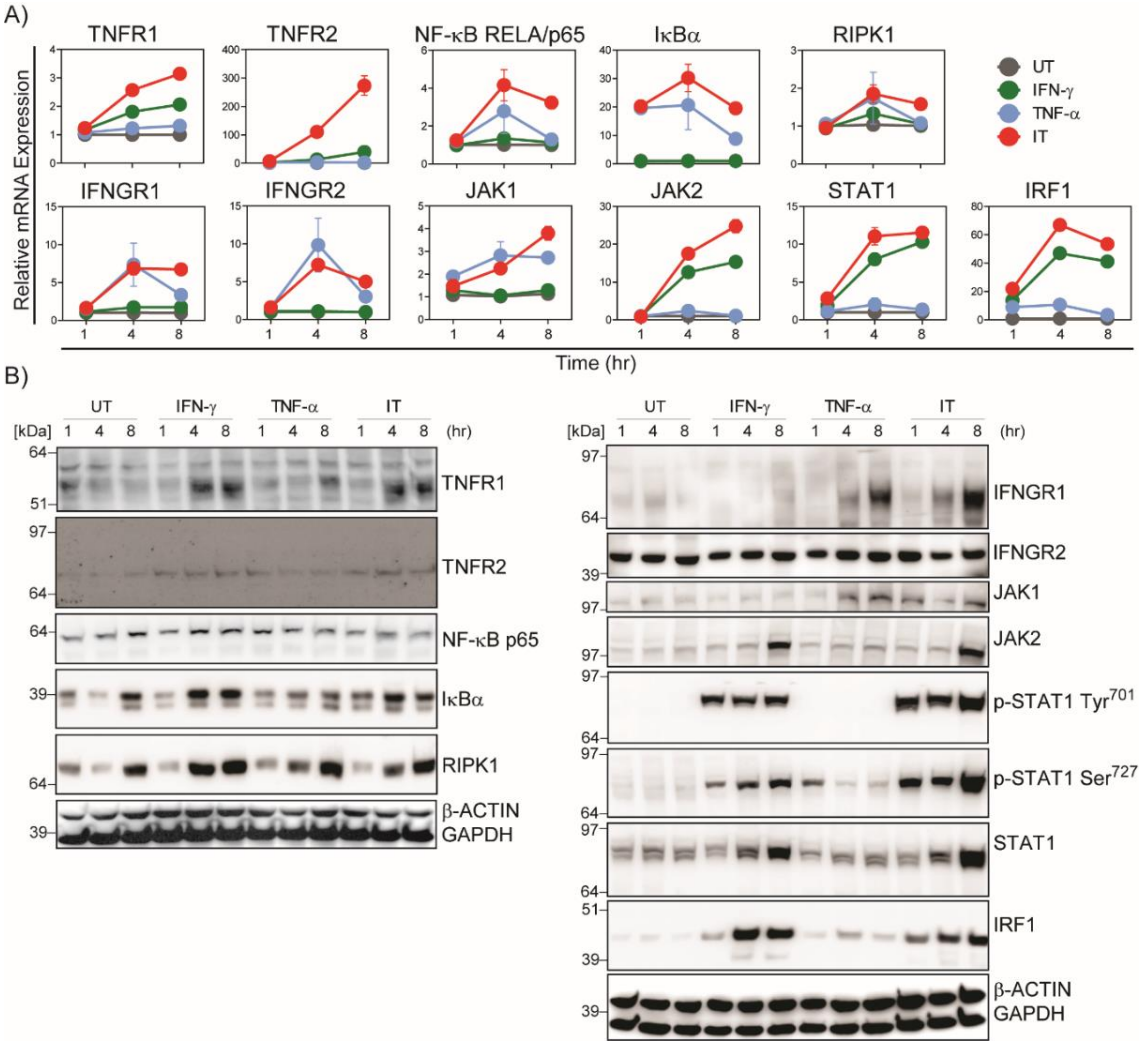


Figure 2.5: IFN-γ and TNF-α cross-regulate each other's receptor mediated signalling pathways

HT-29 cells were treated with IFN-γ, TNF-α, or IT or left untreated (UT) for 1, 4 or 8 hrs and (**A**) mRNA expression levels of selected components in IFN-γ and TNF-α signalling pathways were analysed by real-time qPCR. Relative mRNA levels were calculated with respect to the housekeeping genes *β-actin and GAPDH*. Data are represented as mean ± SEM from three independent experiments in triplicates. (**B**) Selected components of the IFN-γ and TNF-α signalling pathways were analysed at the protein level through western blotting. Blots are representative of two independent experiments.

2.4.7. IFN-γ and TNF-α induced cell death is dependent on STAT1

Next, we investigated the role of specific transcription factors associated with the two cytokine pathways (STAT1 for the IFN-γ pathway and NF-κB for the TNF-α pathway) in regulating the levels of the pathway components, mainly the receptors. RNAi-mediated knockdown of STAT1 prevented IFN-γ and IT induced expression of TNF-α receptors (*TNFR1 and TNFR2*), *JAK2, STAT1 and IRF1* at the mRNA level. Interestingly, STAT1 knockdown also reduced the expression of *IFNGR1 and IFNGR2* induced upon TNF-α and IT treatments (Fig 2.6A). Knockdown of NF-κB RELA-p65 decreased the expression of IFN-γ receptors *IFNGR1 and IFNGR2*. NF-κB RELA-p65 knockdown resulted in increased TNF-α and IT induced *TNFR1* expression while reducing the levels of *TNFR2* mRNA. Results observed at the mRNA level were similar at the protein levels except for TNFR2, where knockdown of STAT1 resulted in an increased expression of TNFR2 as opposed to the decrease seen at the mRNA level.

Knockdown of NF-κB RELA-p65 decreased the IT-enhanced expression of phosphorylated STAT1 at both Tyr 701 and Ser 727 residues, indicating that TNF-α affects the activation of STAT1. To investigate if priming of cells with individual cytokines would sensitize cells to subsequent stimulation with its co-operating cytokine, we performed a homogenous time resolved fluorescence (HTRF) assay for STAT1 and NF-κB activation on cells primed with either cytokine alone for 8 hrs and then treated with the other cytokine for 10 mins. We analysed phosphorylation of the transcription factors (p-Tyr701-STAT1 and p-Ser536-NF-κB p65) (Fig 2.7A). Priming cells with TNF-α significantly increased phosphorylation of STAT1 at Tyr701 residue in response to subsequent IFN-γ treatment compared to non-primed

cells. However, pre-treatment with IFN-γ had no significant effect on the phosphorylation of NF-κB at Ser536 site upon TNF-α treatment comparing primed to non-primed cells. Interestingly, RNAi mediated knockdown of STAT1 rescued cells from synergistic cell death by IT, while knockdown of NF-κB RELA-p65 did not **(**Fig 2.7B). In line with this, we also found that HT-29 STAT1 KO lines were resistant to IT induced cell death (Fig 2.7C). Collectively, the results suggest that TNF-α primes cells to IFN-γ and promotes IFN-γ-IFNGR1/2-JAK1/2-STAT1 dependent cell death pathway.

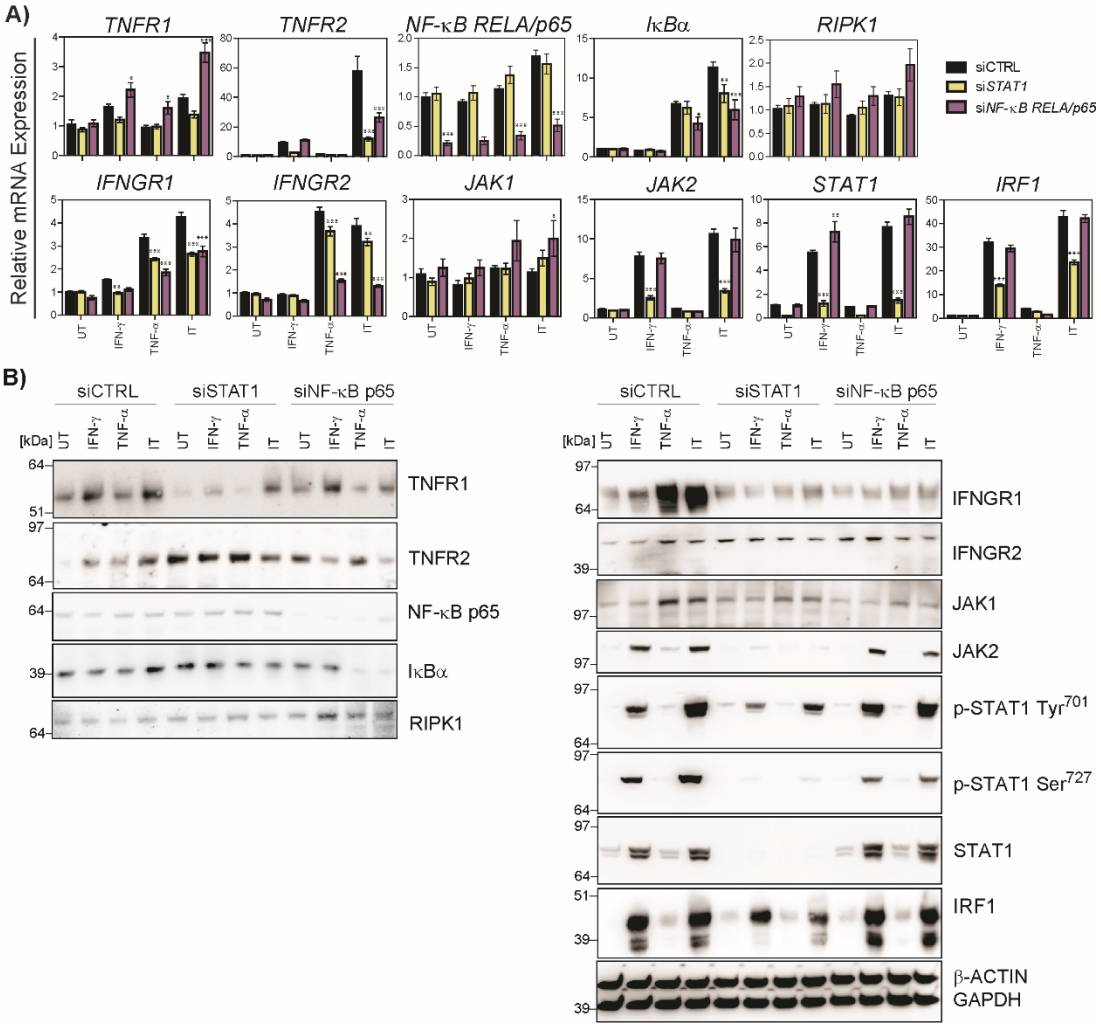


Figure 2.6: Both STAT1 and NF-κB are required for the cross-regulation of signalling components induced by IFN-γ and TNF-α

HT-29 cells were reverse transfected with si*STAT1*, si*NF-κB RELA/p65* or control siRNA (siCTRL). After 48 hrs, they were stimulated with IFN-γ, TNF-α, or IT and (**A**) mRNA expression levels of selected components of the IFN-γ and TNF-α signalling pathways as assessed by RT-qPCR after 4 hrs of treatment. Relative mRNA levels were calculated with respect to the housekeeping genes *β-actin and GAPDH*. Data are represented as means ± SEM from three independent experiments in triplicates. **p*<0.05, ***p*<0.01, ****p*<0.001, two-way ANOVA with Bonferroni posthoc test against siCTRL (**B**) Western blotting for components of the IFN-γ and TNF-α signalling pathways after 8 hrs cytokine treatment. Blots are representative of two independent experiments.

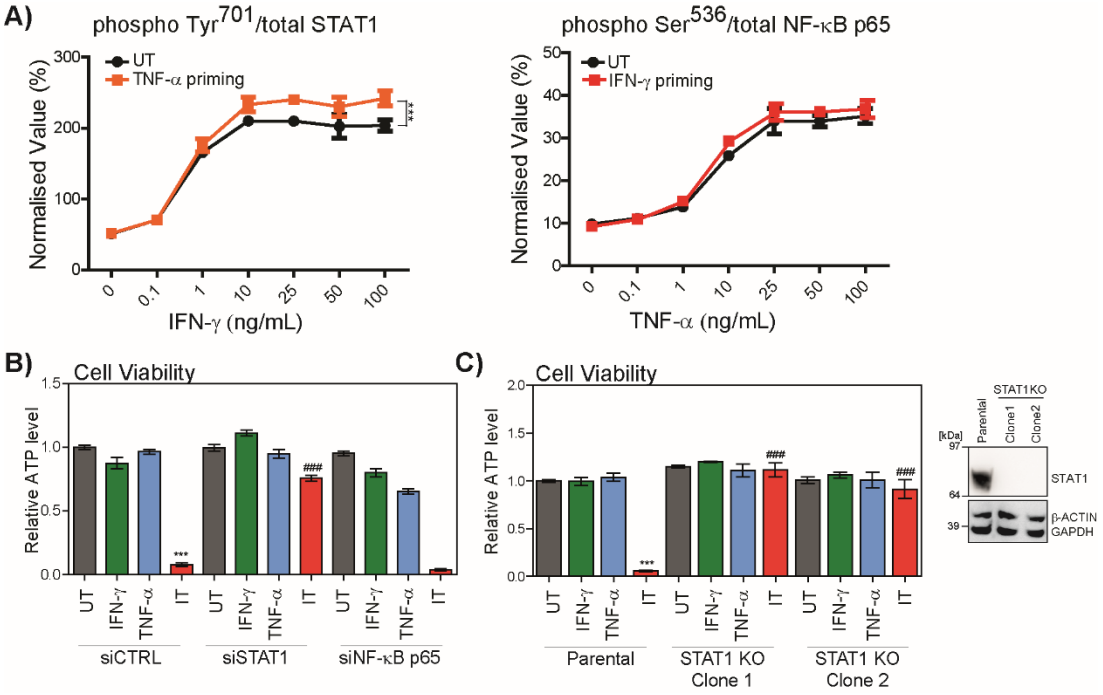


Figure 2.7: IFN-γ and TNF-α induced cell death is dependent on STAT1

(**A**) Left Panel: HT-29 cells were primed with TNF-α (50 ng/mL) for 8 hrs and treated with different concentrations of IFN-γ for 10 mins. Cells were lysed and phospho-STAT1 Tyr^{701} and total STAT1 levels were analysed by HTRF assay. Right Panel: Cells were primed with IFN-γ (25 ng/mL) for 8 hrs and treated with different concentrations of TNF-α for 10 mins. Levels of phospho-NF-κB Ser^{536} and total-NF-κB were analysed using the HTRF assay. Values are plotted as a normalized data for phospho and total proteins. (**B**) Cell viability of HT-29 cells transfected with si*STAT1*, si*NF-κB* or siCTRL for 48 hrs followed by 72 hrs cytokine treatment. Data was normalised to the untreated samples. (**C**) Cell viability of HT-29 STAT1 knockout (KO) clones treated with IFN-γ, TNF-α, and a combination of both (IT) for 72 hrs. Right Panel: Validation of HT-29 KO clones by western blots. Blots are representative of two independent experiments. Data are represented as means ± SEM from three independent experiments in triplicates. *p<0.05, **p<0.01, ***p<0.001, one-way ANOVA with Tukey's multiple comparison test.

2.4.8. IFN-γ and TNF-α cross-regulate each other signalling pathways and synergise to induce cell death through the JAK1/2-STAT1 pathway in colonic organoids

We investigated if cross-regulation of cytokine signalling pathways by IFN-γ and TNF-α occurred in a human primary 3D cell culture system. Human colonic organoids generated from colonic mucosal pinch biopsies obtained from healthy non-IBD patients were stimulated with either IFN-γ, TNF-α or IT. As observed in the HT-29 cell line model, IFN-γ induced the expression of TNF-α receptor genes (*TNFRSF1A and TNFRSF1B*), and TNF-α induced the expression of IFN-γ receptor genes (*IFNGR1 and IFNGR2*) (Fig 2.8A). However, IT treatment did not increase the levels of the receptor genes compared to the individual treatments, with the exception of *TNFR2*. In line with the cell line model, *NF-κBp65/RELA* and *IκBα* gene induction by TNF-α and *JAK2, STAT1 and IRF1* induction by IFN-γ was also observed.

Interestingly, in terms of cytotoxic effects of the cytokines, the organoids were sensitive to single cytokine treatment, and especially they were more sensitive to treatment with TNF-α compared to IFN-γ. However, the combination of these cytokines reduced organoid viability by 80% compared to the individual treatments (Fig 2.9A). A representative image of a dying organoid with IT treatment is shown in Fig 2.9B, with loss of the central lumen and breakdown of epithelial integrity observed. Because perturbation of STAT1 expression rescued HT-29 cells from IT-induced cell death, we investigated if this JAK1/2-STAT1 pathway was also required for cytotoxic effects of the cytokines in the colonic organoid cultures. We used commercially available JAK inhibitors to block the JAK1/1-STAT1 pathway. While inhibitors of individual JAK, filgotinib for JAK1 and fedratinib for JAK2, could not

block organoids from IT-induced cell death, pan-JAK inhibitors ruxolitinib and baricitinib rescued 95% of organoids from IT-mediated cell death (Fig 2.9C)

Overall, these results suggest that IFN-γ and TNF-α can cross-regulate each other's signalling pathways, thereby leading to greater and prolonged activation of the IFNGR1/IFNGR2-JAK1/2-STAT1 pathway and STAT1-dependent cell death of intestinal epithelial cells when used in combination rather than individually (Fig 2.10).

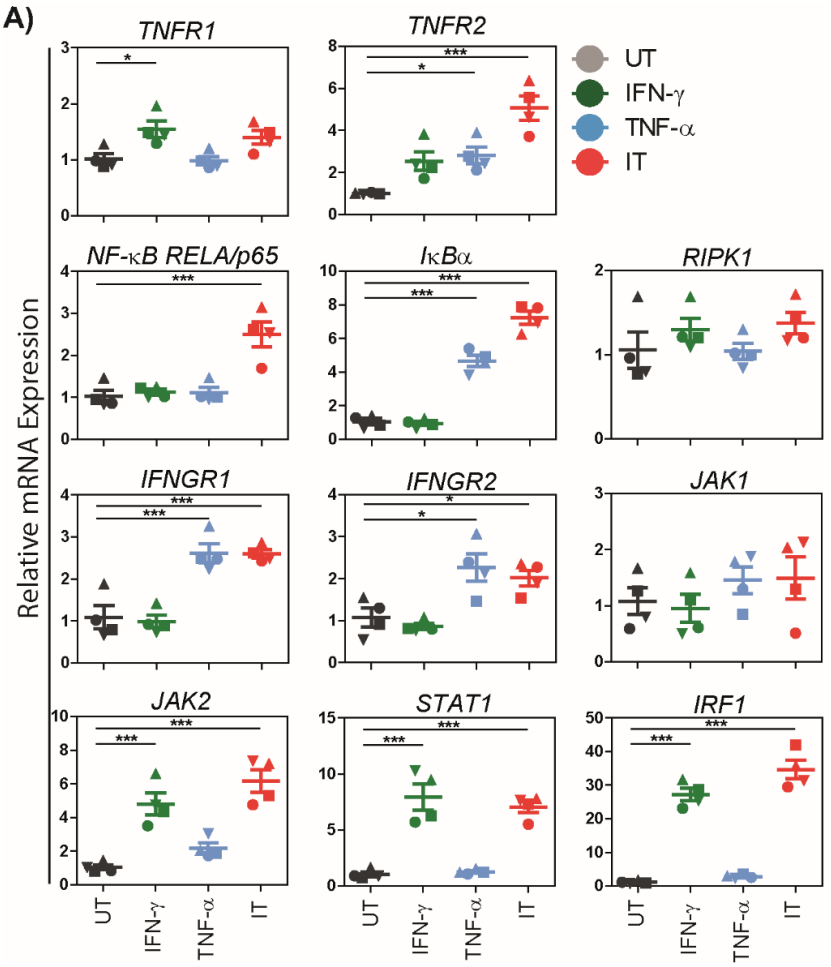


Figure 2.8: Cross regulation of the receptors and downstream signalling components by IFN-γ and TNF-α is observed in primary human healthy colonic organoids

Human primary colonic organoids were treated with 10 ng/mL IFN-γ, 10 ng/mL TNF-α, and a combination of both (IT) for 4 hrs. mRNA expression levels of selected components in the IFN-γ and TNF-α signalling pathways were analysed by RT-qPCR. Relative mRNA levels were calculated with respect to housekeeping genes- *HPRT1 and RPL13A*. Data are represented as means ± SEM from four organoid lines. **p*<0.05, ***p*<0.01, ****p*<0.001, one-way ANOVA with Tukey's multiple comparison test with UT.

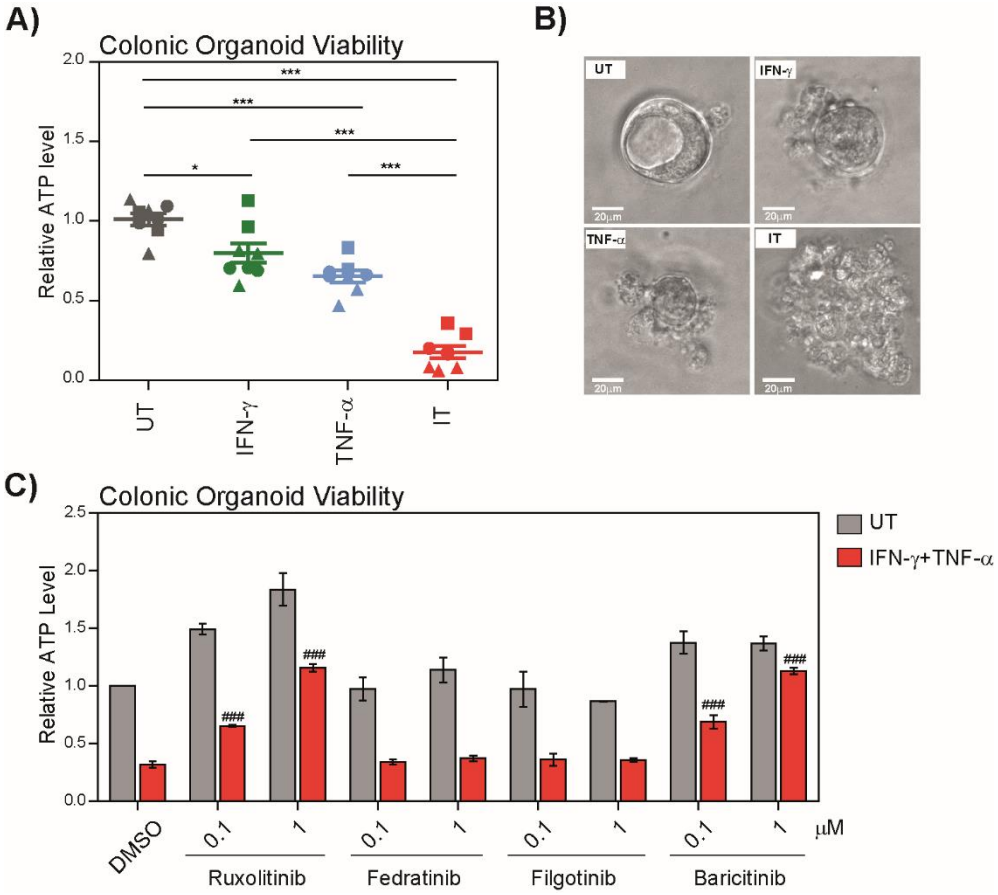


Figure 2.9: Synergistic cell death induced by the combination of IFN-γ and TNF-α in organoids is dependent on the JAK1/2-STAT1 pathway

(**A**) Cell viability of human colonic organoids treated with 10 ng/mL of cytokines either alone or in combination for 24 hrs (**B**) Representative images of one of three organoid lines treated with cytokines for 24 hrs. (**C**) Cell viability of one of the organoid line treated with IT ± four JAK inhibitors for 24 hrs. Data are represented as means ± SEM from one/three organoid lines done in triplicates. *p<0.05, **p<0.01, ***p<0.001, one-way ANOVA with Tukey's multiple comparison test: ***- with UT and ###- with IT.

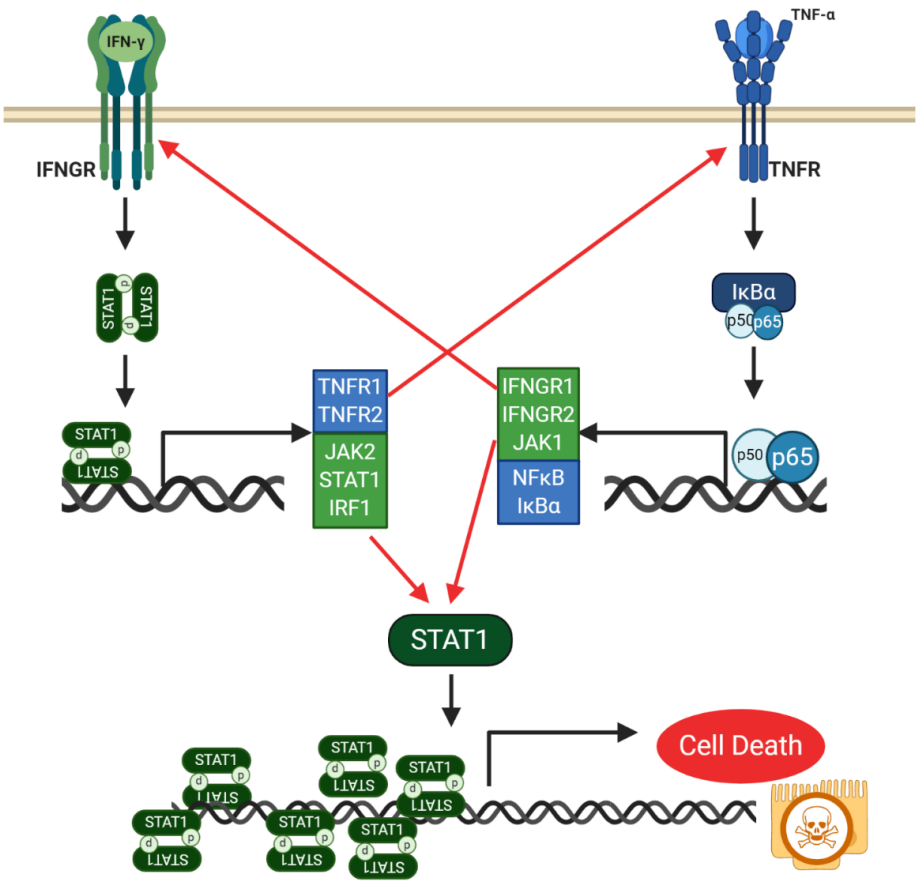


Figure 2.10: Model for cross-regulation of signalling pathways by IFN-γ and TNF-α leading to JAK1/2-STAT1 mediated synergistic cell death

The binding of IFN-γ to its receptor IFNGR1 activates downstream signalling resulting in phosphorylation of STAT1. Phosphorylated STAT1 translocates to the nucleus and induces transcription of the receptors for the TNF-α pathway (TNFR1 and TNFR2) as well as JAK2, STAT1 and IRF1. In parallel, the binding of TNF-α to its receptor TNFR1 results in NF-κB-p65 activation that further induces the expression of IFN-γ receptors (IFNGR1 and IFNGR2) the kinase JAK1 increasing sensitivity of the cells to IFN-γ. The receptors that are cross regulated form a positive feedback loop (denoted by red arrow) that results in increased and sustained activation of the two cytokine pathways, ultimately leading to STAT-1 dependent cell death.

2.5. Discussion

For the last four decades, researchers have reported on the synergistic induction of cell death across a wide variety of cell types by the cytokines IFN-γ and TNF-α[43,58–60]. Yet, the mechanisms underpinning cytokine synergism and synergistic killing of cells is still not fully understood. Our lab recently reported that TNF-α synergises with IFN-γ to induce caspase-8-JAK1/2-STAT1-dependent death of intestinal epithelial cells, with STAT1 being a critical factor[43]. In this chapter, we propose a presence of a positive feedback loop between the two cytokines signalling pathways resulting in sustained activation of STAT1, leading to STAT1-dependent cell death. Through WGCNA on microarray data from HT-29 cells treated either with IFN-γ, TNF-α or a combination of both, we identified fourteen highly correlated modules. Two modules-magenta, regulated by IFN-γ, and yellow, regulated by TNF-α, consisted of receptor genes of the opposing cytokine signalling pathway. Wet lab experiments confirmed the findings, with IFN-γ increasing the expression of TNFR1 and TNFR2 and TNF-α increasing the expression of IFNGR1 and IFNGR2. With IT-treatment, the expression of all the receptors were significantly increased compared to either cytokine treatment alone. The receptor cross-regulation phenomenon was also observed in human primary colonic organoids treated with IFN-γ and TNF-α. Gene enrichment and gene ontology of HT-29 microarray data showed enrichment of cell death pathways, such as apoptosis and necroptosis, but blocking these cell death pathways with commercially available inhibitors did not block IT-induced cell death. We observed TNF-α were priming cells to enhance activation of STAT1 with subsequent IFN-γ treatment and that STAT1 KO lines were completely resistant to IT-induced cell death. These results reiterate that STAT1 acts as a critical factor in IFN-γ+TNF-α induced cell death.

Multiple mechanisms of synergistic cell death induced by IFN-γ and TNF-α have been reported in the literature based on the types of cell lines tested. In recent studies on colon cancer lines and murine bone marrow-derived macrophages (BMDMs), IT-induced cell death was identified to be a type of inflammatory cell death called PANoptosis, with Pyroptosis, Apoptosis and Necroptosis pathways being simultaneously activated[60,61]. Studies on pancreatic β-cells report that IT can also induce ROS-dependent cell death[62]. A previous study from our lab reported that the scaffold function of caspase-8 and not its enzymatic activity was required for IT-induced cell death of intestinal epithelial cells[43]. Interestingly in our study, enrichment analysis of microarray dataset from HT-29 cells treated with cytokines also showed upregulation of genes for the enrichment of apoptotic process and necroptotic process by IT treatment. However, as previously reported by Jerzy *et al.* (2021) and this study, IT-induced HT-29 cell death could not be blocked by co-inhibition of the cell death pathways, namely apoptotic, necroptotic, ferroptotic and ROS quenching.

Enrichment analysis also found that TNF-α treatment and IT-treatment upregulated genes enriched for ER unfolded protein response (UPR). ER stress and the unfolded protein response (UPR) play a critical role in maintaining intestinal tissue homeostasis[63]. If UPR cannot alleviate ER stress, sustained ER stress leads to severe inflammation and cell death through either the apoptotic pathway or autophagic pathway[64–66]. UPR leads to PKR like ER-kinase (PERK)- mediated upregulation of C/EBP-homologous protein (CHOP), which in turn promotes transcription of pro-apoptotic BCL-2-interacting mediator of cell death (BIM) and down-regulate anti-apoptotic Bcl-2 expression and induce apoptosis[67]. However, inhibiting apoptosis through pan-caspase inhibitor z-VAD did not block cells from IT-induced cell death. UPR-ER-stress can also lead to induction of autophagy through a (PERK)- Inositol

requiring enzyme 1 (IRE1)-JNK pathway or through a calcium channel modulation by inositol-1,4,5-triphosphate (IP3) receptor (IP3-R)-beclin1-mTOR pathway[68]. This pathway was not inhibited in this study, and it would be interesting to see if autophagic inhibitors such as bafilomycin or inhibitors targeting JNK, beclin or mTOR would have any effect on IT-induced cell death, either alone or in combination with other inhibitors. TNF-α has been reported to induce UPR and ER-stress in epithelial cells[69,70] and macrophages[71]. Consistent with this, TNF-α and IT- upregulated-salmon and yellow modules from WGCNA analysis consisted of a higher ratio of genes enriched for ER-UPR response. TNF-α-induced UPR and ER-stress are ROS-dependent, and it is also reported that uncontrolled ROS- production due to TNF-α causes cell injury through DNA damage in various cell types[72,73]. It has further been shown that TNF-α/TNFR1 signalling is responsible for DNA damage in intestinal inflammation mouse[74]. In line with these findings, we identified that TNF-α and IT treatments decreased the expression of genes associated with DNA replication mechanisms (also observed with genes in pink, turquoise and green modules from WGCNA analysis). However, in contrast, scavenging ROS through N-acetyl cysteine (NAC) did not rescue HT-29 cells from IT-induced cell death.

Previous studies and studies in this thesis (chapter 3) show that IFN-γ and TNF-α can induce inflammatory chemokines such as CXCL9, CXCL10, CXCL11, CXCL8, CXCL1, CCL20, CCL5, amongst a few[41,75,76]. Consistently, inflammatory response, chemokine-mediated signalling pathways and regulation of inflammatory response are enriched in IFN-up, TNF-up and IT-up groups. Looking at the immune response, IFN-γ plays a crucial role in the maintenance of $CD4^+$ Th1 cells and inhibition of other $CD4^+$ Th cell subsets, particularly in anti-tumour immunity[77]. Genes enriched for GO terms associated with immune response are also found

enriched in the IFN-up, TNF-α-up and IT-up groups, and IFN-γ upregulated genes of the magenta module enriched for regulation of T cell activation, thereby correlating our study with previously known literature.

WGCNA is a bioinformatics tool that is now widely used to identify correlated gene networks associated with a specific phenotype[49]. It is a method extensively used to analyse microarray data, RNASeq data or multi-omics data[78]. Genes in the same module are considered to be related to each other in terms of their regulation in enriched for similar biological pathways and functions. From the WGCNA analysis, we observed for two modules, in particular, the expression of groups of genes that were positively associated with individual cytokine and combination cytokine treatments. We identified that IFN-γ receptor genes *(IFNGR1 and IFNGR2)* were present in the TNF-α regulated yellow module and closely related to *TNF, NFκB1, NFκB2, and RELB*. The IFNGR upregulation by TNF-α is in line with previous reports that show the expression of IFN-γ inducible genes IDO and B7-H1 synergistically by IT was through enhanced IFN-γ signalling and increased expression of IFNGR by TNF-α[79–81]. Parallelly, genes in the magenta module, which were upregulated by IFN-γ treatment, contained TNF-α receptors (*TNFR1 and TNFR2*) closely related to IFN-γ signalling pathway components *JAK2, IRF1 and STATs*. IFN-γ can upregulate TNF receptors, but it has been shown that this induction of TNF receptor is dispensable for the synergistic response of IT[82]. Cross-talk between TNF receptors and STAT1 has been suggested previously. In epithelial lines, STAT1 blocks interactions of TRADD -RIP or TRADD-TRAF2 interaction but favours TRADD-FADD interaction[83]. This leads to inhibition of NF-κB activity and stabilisation of DISC formation for apoptotic induction[83]. While TNF-α induced STAT1 phosphorylation at both Tyr 701 and Ser 727 residue, STAT1 did not translocate to nucleus but acted as an adaptor facilitating

DISC formation[84]. The absence of STAT1 results in decreased TNF-α induced apoptosis and IT-induced cell death, as seen in our study[84–86]. In β-cells, STAT1-IRF1 was proposed to induce apoptosis through caspase 11 but not definitely during TNF-α treatment[62]. STAT1 activity and not its upregulation were critical for IT-induced β-cell death[62]. In this study, we wanted to investigate how the presence of TNF-α affected the activation of STAT1, and we found that TNF-α pre-treatment before IFN-γ resulted in higher phosphorylation of STAT1 than IFN-γ alone. Our results also show that though NF-κB is required for cross-talk between TNF-α and IFNGR1/2, pre-treatment with IFN-γ did not affect NF-κB activation. This contrasts with studies that report treatment with IFN-γ releases STAT1 from the TNFR1-TRADD complexes, thereby increasing NF-κB-TRADD interactions leading to NF-κB signalling[87]. Hence, rather than IFN-γ sensitising cells to death ligand TNF-α dependent cell death, we report that TNF-α sensitises cells to IFN-γ-STAT1- dependent cell death.

Though there are no human trials with STAT inhibition to date, however, small-molecule JAK inhibitors are in clinical trials for IBD. Tofacitinib was approved by FDA (food and drug administration) and EMA(European medicines agency) in 2018 for the treatment of ulcerative colitis[15,88,89]. Tofacitinib was shown to rescue IECs from IFN-γ induced barrier dysfunction[90]. We tested other JAK-inibs and found pan-JAK inhibitors ruxolitinib and baricitinib rescue colonic organoids from IT-induced cell death. Though in our study, targeting JAK1 alone with filgotinib, did not protect colonic organoids from IT-induced cell death, work by Jerzy *et al.*(2021), found protection when filgotinib was used at a concentration of 10 μM[43]. Another JAK1 selective inhibitor, upadacitinb, completely blocked colonic organoids from IT-induced cell death[43]. Very recently, upadacitinib was approved by FDA for the

treatment of moderate-severely active UC[91]. One limitation of our study is that only one colonic organoid line was tested for the protective effects of JAK inhibitors.

Overall, these results suggest that IT-induced cell death is dependent on STAT-1. In HT-29 cells, TNF-α acts as an enhancer for IFN-γ-STAT1 mediated synergistic cell death in epithelial cells. Our results showing that inhibition of the JAK/STAT pathway could rescue the cells both in the cell line and organoid model supports the fact of JAK-STATs being good targets for IBD.

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Chapter 3：Screening of TNF superfamily ligands identifies TNF-α like ligands that synergise with IFN-γ

3.1. Abstract

Anti-tumor necrosis factor-alpha (TNF-α) antibodies known as anti-TNFs have been very effective drugs for inflammatory bowel diseases (IBD) over the past 25 years. However, about 60% of patients with IBD do not respond to these treatments or lose their response over time. A potential explanation for this inadequate response to anti-TNFs is that additional TNF-like cytokine pathways can compensate for TNF-α when it is blocked therapeutically. With inflammatory cytokines such as IFN-γ, TNF-α, IL-12 and IL-23 playing a key role in IBD pathogenesis, targeting other cytokines which are functionally redundant with TNF-α might represent an alternative approach for therapeutic intervention. Members of the TNF superfamily (TNFSF) of ligands and their signalling pathways have emerged as targets in many inflammatory and autoimmune diseases. Here, we screened nineteen TNFSF ligands in human colonic epithelial cell line-based phenotypic screens and in primary colonic organoids from healthy individuals and patients with Crohn's disease to identify ligands exhibiting similar function as TNF-α. We used the phenotypes of cell death and inflammatory chemokine production either alone or in synergy with IFN-γ as the functional readouts for TNF-α-like activity in cell-based screens. We identified 3 TNFSF ligands - TRAIL, TWEAK and LIGHT which exhibited properties similar to TNF-α. In colonic epithelial cell lines, the three ligands induced inflammatory chemokines in combination with IFN-γ, similar to TNF-α. However, only TRAIL could induce cell death either alone or in synergy with IFN-γ in the cell lines. In primary human colonic organoids, TWEAK and LIGHT were the major TNF-α-like ligands. Collectively, our data indicate that these TNFSF ligands could be potential targets for IBD therapy either alone or in combination with anti-TNFs.

3.2. Introduction

Inflammatory Bowel Diseases (IBD) are chronic relapsing diseases of the gastrointestinal (GI) tract, of which the two major forms are Crohn's disease (CD) and ulcerative colitis (UC). IBD is a global disease with a relatively low mortality rate but high morbidity rates[1]. Over 2.5 million people in Europe are estimated to have IBD, with a prevalence of 0.5% in the western world[2,3]. IBD is a multifactorial disease with environmental, genetic, microbial and innate-adaptive immune system imbalance modifiers contributing to pathogenesis[4–6].

Cytokines play a key role in the pathogenesis of IBD. In particular, the imbalance between pro-inflammatory and anti-inflammatory cytokines leads to the breakdown of intestinal homeostasis that characterises IBD[7–10]. Tumor necrosis factor (TNF)-α is a member of the TNF superfamily (TNFSF), which consists of 19 ligands and 29 receptors and regulates several signalling pathways leading to cell proliferation, differentiation, survival, and apoptosis in multiple cell types[11–14]. TNF-α is a critical pro-inflammatory cytokine driving pathogenesis of IBD[7,9]. Targeting TNF-α revolutionised the treatment of IBD. Infliximab, a chimeric IgG1 monoclonal antibody consisting of both human and murine variable regions, was the first anti-TNF treatment approved by the Food and Drug Administration (FDA) to treat CD in 1998. It was then approved for the treatment of UC in 2005[15]. Next, a fully humanised antibody, Adalimumab, was approved in 2007 to treat CD[15]. Certolizumab and golimumab were additional anti-TNFs subsequently approved for the treatment of CD[16]. Various mechanisms of action have been investigated and proposed to explain how anti-TNFs exert their clinical efficacy[17], including neutralisation of TNF-α and blockade of its pro-inflammatory effects on most cells[18], induction of apoptosis of

activated T-lymphocytes by blockade of TNF-α-mediated pro-survival signalling from $CD14^+$ monocytes[19,20], induction of reverse signalling effect via cell surface TNF-α itself[21], induction of antibody-dependent cell-mediated cytotoxicity[22], and modulating the immune system by inducing IL-22 in T cells[23], inducing IL-10 production[24] and inhibiting IL-12/IL-23 expression in macrophages[25]. Though anti-TNF is efficacious in IBD, 30% of patients have no initial response to anti-TNFs (primary non-responders), while 30-50% of patients lose their response over the course of anti-TNF treatment (secondary non-responders)[17,26,27]. Various studies have investigated potential mechanisms underpinning resistance to anti-TNF drugs in IBD and other inflammatory diseases. Recently, it was reported that elevated levels of the cytokine oncostatin M was associated with resistance to anti-TNF therapy[28]. Another study identified IL-23/IL-23R pathway activation in a subset of T cells expressing high TNFR2 receptor as conferring resistance to anti-TNFs[29]. Thus, targeting cytokines redundant with TNF-α or targeting other cytokine pathways might be an alternative approach to overcome the resistance to anti-TNF-α therapies[30–32].

TNFSF ligands like TNF-α can induce receptor activation both in their membrane-bound form and soluble form (released after proteolytic cleavage). The TNFSF receptors are transmembrane receptors that can be classified into three types - (1) death receptors, (2) TNFR-associated factors (TRAF) interacting receptors and (3) decoy receptors. The death receptors, TNFR1, CD95/Fas, TNF-related apoptosis-inducing ligand (TRAIL)-R1 and TRAIL-R2 contain a death domain (DD) in their cytoplasmic region that interacts with the Fas-associated death domain (FADD) and TNFR- associated death domain (TRADD). Upon activation, these receptors can induce activation of the initiator caspases- caspase-8 and caspase-10, leading to the activation of downstream effector caspases and apoptosis of target cells. The TRAF

interacting receptors have motifs that facilitate interaction with the TRAF adaptor protein family, which leads to the activation of transcription factors such as Activator protein 1 (AP-1) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), resulting in the transcription of pro-survival genes and inflammatory genes. The decoy receptors (DcR) – including osteoprotegerin (OPG), TRAIL-R3, TRAIL-R4 and DcR3, compete for ligand binding and lead to inhibition of ligand signalling[14,33–35]. Most of the TNFSF ligands and receptors are expressed on immune cells, and hence they play an important role in maintaining the equilibrium of the intestinal and extra-intestinal immune system. Recent studies also show that these ligand-receptor pathways are emerging targets in many autoimmune and inflammatory diseases[12,36–38].

IFN-γ is another pro-inflammatory cytokine that is implicated in the pathogenesis of IBD. IFN-γ is the sole type II interferon and is produced by NK cells, Th1 $CD4^+$ T cells, antigen-presenting cells, $CD8^+$ cytotoxic T lymphocytes (CTLs), and innate lymphoid cells (ILCs)[39]. The pathophysiological role of IFN-γ has been attributed to its inflammatory and cytotoxic effects on monocytes and macrophages, host barrier epithelial cells, endothelial cells, either alone or in synergy with TNF-α. Through the downstream Janus kinase (JAK)1/2- signal transducer and activator of transcription 1 (STAT1) pathway, IFN-γ can induce apoptosis in epithelial cell lines and Paneth cells[40–44]. It can also induce the expression of death-inducing TNFSF ligands such as FasL or TRAIL and initiate cell death indirectly via death receptor pathways[45–48]. IFN-γ also affects barrier integrity by targeting tight junction proteins, reducing protein expression, rapid internalisation, and endocytosis[49–51]. Apart from inducing epithelial cell death, IFN-γ induces the expression of inflammatory chemokines in most cell types, especially the T cell and NK cell-selective CXCR3

ligands- CXCL9, CXCL10 and CXCL11[52]. These chemokines direct the spatial migration of activated T cells and NK cells to inflamed mucosa via the CXCR3 receptor on these cells. IFN-γ also increases levels of CCL2 along with CXCL10 and CXCL11 in an experimental animal model of colitis[53]. These effects of IFN-γ on target tissues, including the gut, are often exacerbated in the presence of TNF-α.

Synergism between the two Th1 cytokines, IFN-γ and TNF-α, has been observed in various cell types and investigated in various autoimmune and inflammatory diseases, including IBD, for the past 40 years[54]. These two cytokines are associated with increased IEC death, disruption of gut barrier function and increased levels of other inflammatory chemokines and cytokines in the inflamed gut. IFN-γ and TNF-α can co-operate to promote intestinal damage by inducing synergistic cell death of IECs[55–58]. The STAT1/IRF1 pathway plays a key role in IFN-γ and TNF-α synergism by either inhibiting cytoprotective NF-κB[59], upregulation of caspase-8[47] or by the activation of caspase-3 through caspase-1[60]. Studies on pancreatic β-cells reported that the two cytokines induced ROS-dependent cell death and loss of mitochondrial integrity[61,62]. We recently reported that IFN-γ and TNF-α synergistically kill intestinal epithelial cells (IECs) through a caspase-8-JAK1/2-STAT1 pathway, with STAT1 as one of the key effectors[63]. In chapter 2 of this thesis, we report that the cell death was atypical and was neither apoptotic, necroptotic, ferroptotic or a combination of all. Another study from our lab reported that IFN-γ and TNF-α can synergise to induce the inflammatory CXCR3 chemokines, CCL5 and CCL20[57].

Based on the collected evidence, the aim of this study was to screen the known and commercially available TNFSF ligands to identify ligands displaying similar properties to TNF-α and its ability to synergise with IFN-γ. We use colorectal

adenocarcinoma cell lines as cell line models for colonic epithelial cells and two phenotypic readouts – cell death and inflammatory chemokine/cytokine production, to screen all 19 TNFSF ligands and then validate the identified target ligands in primary human colonic organoids.

3.3. Materials and Methods

3.3.1. Cell Culture

The human colorectal adenocarcinoma cell lines HT-29 (# HTB-38) and DLD-1 (#CCL-221) were obtained from American Type Culture Collection. HT-29 was cultured in McCoy's 5A medium (#M9309, Sigma- Aldrich) and DLD-1 in RPMI-1640 (#R8758, Sigma-Aldrich). Both media were supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin and 0.1 mg/mL streptomycin (#P4333, Sigma-Aldrich). The cell lines were incubated at 37 ºC supplemented with 5% CO_2. The lines were passaged 1-2 times per week and routinely checked for mycoplasma contamination using MycoAlert Mycoplasma Detection Kit (#LT07-118, Lonza).

3.3.2. Human Studies

The collection and use of colonic pinch biopsies was ethically approved by the Clinical Research Ethics Committee of the Cork Teaching Hospitals (CREC). All patients provided informed consent in agreement with the Declaration of Helsinki. The biopsies were collected from non-IBD (healthy control) tissues from consented adult patients or from patients with Crohn's disease at Cork University Hospital and Mercy University Hospital. All non-IBD patients were comprised of otherwise healthy individuals undergoing investigation of symptoms such as weight loss, anaemia or altered bowel habit. All patients with CD had a confirmed diagnosis, as evidenced by previous histopathological findings.

3.3.3. Primary human colonic organoid culture

Protocols for crypt isolation and organoid culture was followed as described in Section 2.3.4. Undifferentiated organoids were seeded in 96 well plates, overlaid with proliferation medium and left to recover for three days. They were treated with 100 ng/mL of the selected TNF superfamily members either alone or in combination with 10 pg/mL of IFN-γ. Supernatants were collected after 24 hrs for MSD immunoassay, and the cell viability assay was performed.

3.3.4. Recombinant proteins, reagents and antibodies

Recombinant human IFN-γ (#300-02) and TNF-α (#300-01A) were purchased from Peprotech and used at a concentration of 10 ng/mL each. Recombinant human TNF superfamily ligands were purchased from R&D Systems and diluted in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA). The TNFSF ligands were used at 100 ng/mL concentration either alone or in combination with 10 ng/mL of IFN-γ: TNFSF ligands included CD30L (#1028-CL), CD40L (#6420-CL), EDA-A1 (#3944-ED), EDA-A2 (#922-ED), FasL (#126-FL), LTα1/β2 (#678-LY), LTα2/β1 (#679-TX), LTα1/β2 (#8884-LY), TNF-α (#210-TA), OX40L (#1054-OX), 4-1BBL (#2295-4L), TRAIL (#375-TL), RANKL (#390-TN), TWEAK (#1090-TW), APRIL (#5860-AP), BAFF (#2419-BF), LIGHT (#664-LI), TL1A (#1319-TL), GITRL (#694-GL) and β-NGF (#256-GF). The ligand for CD27 (CD27L/CD70) was purchased from Peprotech (#310-30).

3.3.5. Endpoint cell viability assay

HT-29 cells ($2*10^4$ cells/well) and DLD-1 cells ($1*10^4$ cells/well) were seeded in 96-well plates and incubated overnight in media containing antibiotics and 10% FBS until they reached 80% confluency. Cells were treated with cytokines for 72 hrs. Cell viability was measured using CellTiter-Glo® (#G7573, Promega) following the manufacturer's instructions and as previously described in Woznicki *et al.*,2020[47]. The luminescence reading was captured using a Synergy2 microplate reader (BioTek).

3.3.6. Crystal violet staining

HT-29 ($2*10^4$ cells/well) and DLD-1 ($1*10^4$ cells/well) cells were seeded in 96-well plates and incubated overnight in media containing antibiotics and 10% FBS until they reached 80% confluency. Cells were then treated with either IFN-γ alone, TNF-α alone or the combination of both cytokines for 72 hrs. Following the treatment, cells were washed twice with PBS (#D8537, Sigma-Aldrich) and fixed in 100% methanol (#10675112, Fisher Chemical) for 10 mins at -20ºC. The cells were stained using 0.5% crystal violet solution (#V5265, Sigma- Aldrich) along with 20% methanol for 15 mins. The cells were washed with PBS and left to dry overnight. The plates were scanned using the Odyssey 9120 imaging system (LI-COR), and digital images were processed in ImageJ software.

3.3.7.Screening Protocol Optimisation

IFN-γ has been shown to prime multiple cell types to inflammatory stimuli. Hence, we tested two different protocols to optimise functional readouts for the screens - Protocol A with a specific time tested for IFN-γ priming before subsequent treatment

of washed cells with TNF-α and Protocol B- with IFN-γ priming followed immediately by continuous treatment with TNF-α without removal of IFN-γ from the cells. The detailed protocol is described below and in Figure 3.1.

Protocol A- Time-specified IFN-γ priming

HT-29 ($2*10^4$ cells/well) and DLD1 ($1*10^4$ cells/well) cells were seeded in 96-well plates and incubated. After 24 hrs, they were either treated with 200 µL of 0.1, 1 and 10 ng/mL of IFN-γ in media or left untreated for 16 hours. After priming, the cells were washed twice with media. Cells were further treated with media containing 200 µL of 10 ng/mL TNF-α or not. For chemokine analysis, supernatants were collected at 12 and 24 hrs after TNF-α treatment. Cell viability was measured after 72 hrs of TNF-α treatment.

Protocol B- Continuous IFN-γ treatment

HT-29 (2*104 cells/well) and DLD1 (1*104 cells/well) cells were seeded in 96-well plates and incubated. After 24 hrs, they were either treated with 180 µL of 0.1, 1 and 10 ng/mL of IFN-γ or left untreated for 16 hours. After 16 hrs, 20 µL of media containing 100 ng/mL TNF-α or not was added to the wells, bringing the final concentration of TNF-α in the wells to 10 ng/mL. For chemokine analysis, supernatants were collected at 12 and 24 hrs after TNF-α treatment. Cell viability was measured after 72 hrs of TNF-α treatment.

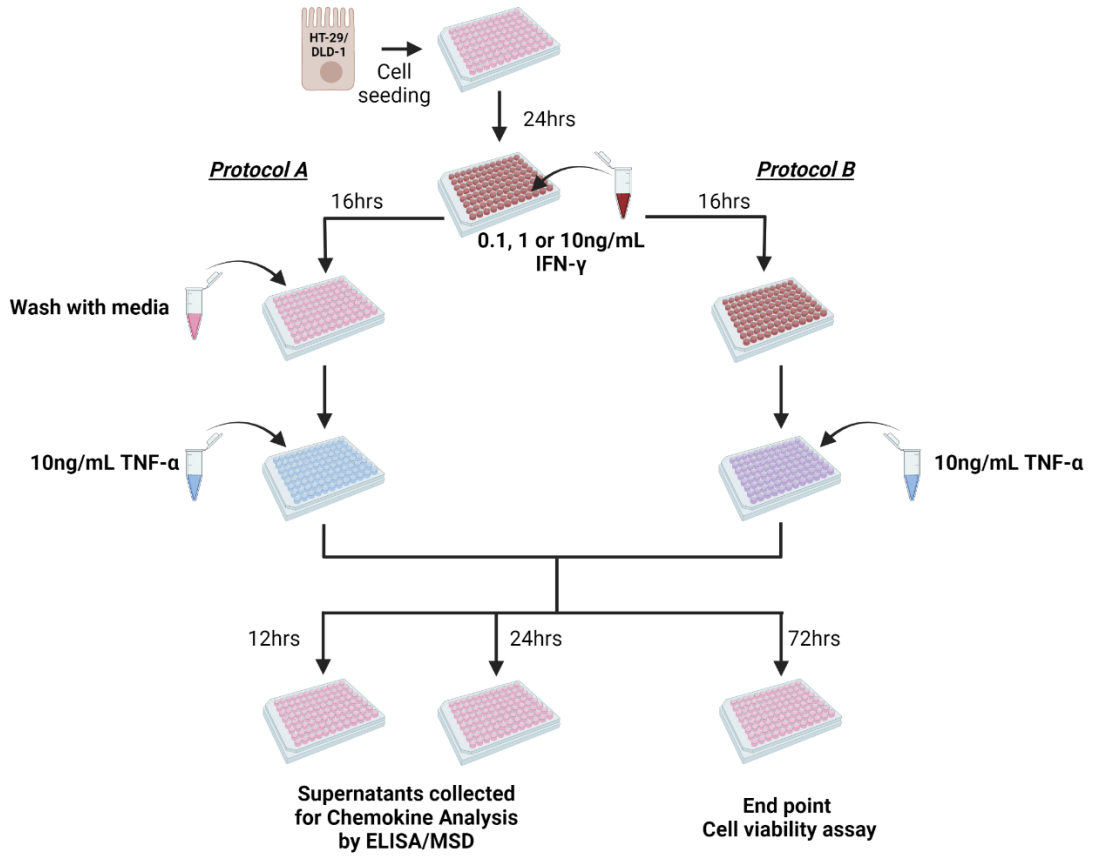


Figure 3.1: TNFSF screen optimisation protocols

Protocol A follows a defined priming time with different concentrations of IFN-γ for 16 hrs. Following this, the cells are gently washed to remove IFN-γ and then treated with 10 ng/mL of TNF-α. In Protocol B, cells are primed with different concentrations of IFN-γ for 16 hrs. The TNF-α is then added to the wells that contain IFN-γ. Here the cells are exposed to IFN-γ for the entire time. In both protocols, supernatants are collected 12 and 24 hrs after TNF-α treatment for chemokine analysis by ELISA/MSD assay. Cells were assessed for cell viability after 72 hrs of TNF-α treatment using CellTiter-Glo® assay.

3.3.8. ELISA for chemokine analysis

Supernatants collected after treatments were stored at -20 ℃ until analysed by ELISA. Human DuoSet ELISA kits for CXCL10 (#DY266) and CXCL11 (#DY672) were purchased from R&D Systems, and the assay was performed according to the manufacturer's instructions. The plates were read using 1 volume of 1-Step™ Ultra TMB-ELISA Substrate Solution (#34028, ThermoFisher) and ½ volume of 1 M sulphuric acid (#10325960, Fisher Chemical). The optical density at 450 nm and 570 nm was obtained using a Synergy plate reader (BioTek). The final concentration of the chemokine was obtained by subtracting $OD_{570\text{-blank}}$ from $OD_{450\text{-blank}}$ and extrapolated from the standard curve using the Gen 5 software (BioTek).

3.3.9. Meso Scale Discovery (MSD) immunoassay for chemokine analysis

Supernatants collected after treatments were stored at -20 ℃ until analysed by MSD. The MSD immunoassay was performed following the manufacturer's instructions. The plates were read in the MESO QuickPlex instrument, and analyte concentrations were extrapolated using the four-parameter logistic fit using the MSD workbench software (Meso scale discovery). The following assay kits were used for the experiments: U-PLEX 10-plex assay- CCL2, CCL22, CX3CL1, CXCL5, CXCL10, CXCL11, IL1α, IL13, IL29 and IL33; human U-PLEX chemokine combo 1 and 2 (#K15046K, #K15047K); U-PLEX 8-plex assay- CXCL10, CXCL11, CXCL8. CXCL1, CX3CL1, CCL22, CCL2 and MIF; U-PLEX 5-plex assay- CXCL10, CXCL11, CXCL8, CX3CL1.

3.3.10. Heat map generation

The chemokine concentration data acquired from MSD U-Plex 10-plex assay (Fig 3.3 and 3.4) and the U-plex chemokine combo assay (Fig 3.5) were log10 transformed, and hierarchical clustering was performed using a heat-plot function from made4 package using Euclidean distance and ward.D2 method in R.

3.3.11. Statistics

Statistical analysis was carried out using GraphPad Prism version 5 as described in the figure legends. P-values of $P<0.05$, $P<0.01$ and $P<0.001$ were considered to be statistically significant.

3.4. Results

3.4.1. IFN-γ and TNF-α synergise to induce Th1 chemokine production and cell death in colonic epithelial cancer cell lines

The Th1 cytokines IFN-γ and TNF-α have been shown to synergise to induce cell death and produce Th1 chemokines in many different cell types[63–66]. To optimize and validate cell-based assays for screening ligands of the TNFSF for TNF-α-like phenotypes in the context of co-treatment/priming with IFN-γ, we chose HT-29 (a hyper triploid colorectal adenocarcinoma) and DLD-1 (a pseudodiploid colorectal adenocarcinoma) cell lines. In both HT-29 and DLD-1 lines, treatment with both IFN-γ and TNF-α (IT) resulted in the secretion of the Th1 chemokines CXCL10 and CXCL11 (Fig 3.2 A). In HT-29 cells, IFN-γ treatment alone resulted in the release of both chemokines with maximum production after 24 hrs of treatment. With the addition of TNF-α, the concentration increased further to about seven-fold for CXCL10 and nine-fold for CXCL11 after 24 hrs of treatment compared to that produced by IFN-γ treatment alone (Fig 3.2A). However, in DLD-1 cells, IFN-γ treatment alone did not have any effect on chemokine secretion by 24 hrs, but when combined with TNF-α, there was an increase in the production of chemokines that was time-dependent with maximum chemokine release observed after 24 hrs of cytokine treatment (Fig 3.2B). After 72 hrs of cytokine treatment, a significant reduction in cell viability was observed with the combination of IFN-γ and TNF-α (IT) compared to treatment with either cytokine alone (Fig 3.2C-D). While the reduction in cell viabilitywas dramatic in HT-29 cells (about 80%), it was only marginally significant in DLD-1 cells (approx. 15%).

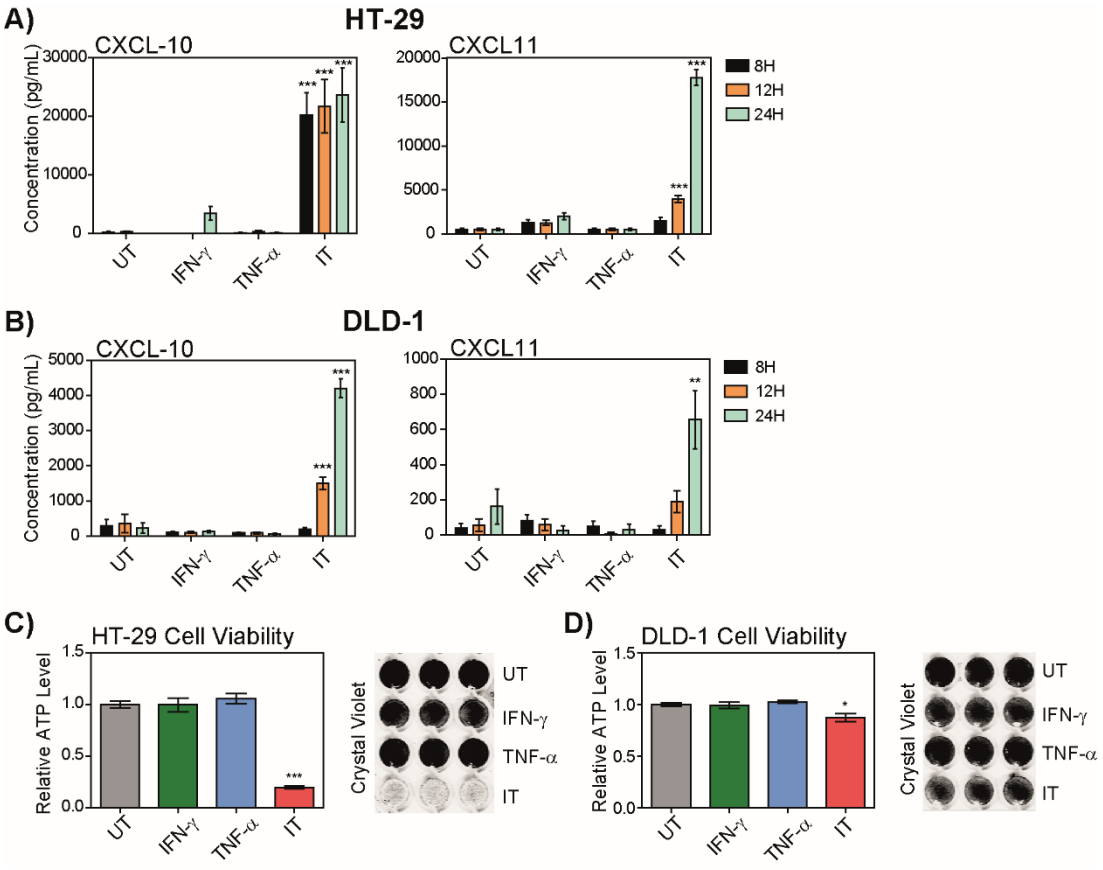


Figure 3.2: IFN-γ and TNF-α synergise to induce CXCR3 chemokine production and cell death

HT-29 and DLD-1 cells were stimulated with 10 ng/mL of IFN-γ, TNF-α or a combination of both (IT) or left untreated (UT). CXCL10 and CXCL11 were measured by ELISA from **(A)** HT-29 and **(B)** DLD-1 supernatants. (**C**) HT-29 and (**D**) DLD-1 cell viability were analysed using CellTitre-Glo® (left panel) and crystal violet staining (right panel). Data are represented as mean ± SEM from three independent experiments in triplicates. *p<0.05, **p<0.01, ***p<0.001, one-way ANOVA with Tukey's multiple comparison test with UT.

3.4.2. Optimisation of cell-based assays for screening TNFSF ligands for TNF-α-like biology

IFN-γ is known to prime cells, especially macrophages, to subsequent treatment with inflammatory stimuli leading to the increased secretion of pro-inflammatory cytokines and chemokines[67,68]. It has also been reported that the concentration or dose of IFN-γ also plays a vital role in priming and activation of negative feedback mechanisms[69–71]. Hence, to maximise chemokine levels as a readout for cell-based screens, we investigated various protocols used previously by our lab and other groups involving priming with different doses of IFN-γ before subsequent TNF-α treatment. As described in the Methods section (3.3.7), we tested two protocols, A and B, to identify a set of experimental conditions leading to maximum phenotypic readouts relevant to TNF-α biology – cytokine/chemokine secretion and cell death response. From previous work in our lab, we selected ten cytokines/chemokines whose production was greatly enhanced by the combination treatment of IFN-γ and TNF-α. We observed that in both cell lines HT-29 (Fig 3.3A-B) and DLD-1 (Fig 3.4A-B), the production and secretion of the ten cytokines/chemokines selected were greatly enhanced with the combination of IFN-γ and TNF-α (IT) compared to treatment of cells with either of the cytokines alone. The maximum levels of chemokines secreted were observed after 24 hrs of treatment with 10 ng/mL of IFN-γ in combination with 10 ng/mL of TNF-α. Protocol B, with continuous IFN-γ treatment, gave the maximum release of chemokines assayed compared to Protocol A, with time-specified IFN-γ priming alone.

To evaluate cell death phenotype, we selected the highest concentration of IFN-γ used (10 ng/mL) and tested the two protocols A and B. In HT-29 cells, we

observed that IT treatment resulted in higher cell death in the two protocols (Fig 3.3C-D). However, interestingly we observed that time-specified IFN-γ priming alone (Protocol A) had a potential proliferative effect, identified by the increase in ATP levels (Fig 3.3C), while continuous IFN-γ treatment (Protocol B) did not (Fig 3.3D). Also, the effect on cell death was greater following protocol B (about 80% - Fig 3.3D) compared to protocol A (about 40% - Fig 3.3C). In DLD-1 cells, we found that protocol A, with time-specified IFN-γ priming, IFN-γ treatment induced very small but significant cell death (about 10%) compared to untreated cells (Fig 3.4C). The cell death was enhanced with TNF-α treatment (in IT) but only marginally (about 20%). However, IT induced cell death was about 50% with protocol B, using continuous IFN-γ treatment along with TNF-α (Fig 3.4D). IFN-γ alone had a similar effect on cell death (about 10%). In both protocols, TNF-α treatment alone did not have any effect on DLD-1 cell viability. Considering all these results, we used protocol B, with continuous IFN-γ treatment along with TNF-α, to screen TNFSF ligands and identify potential TNF-α-like targets.

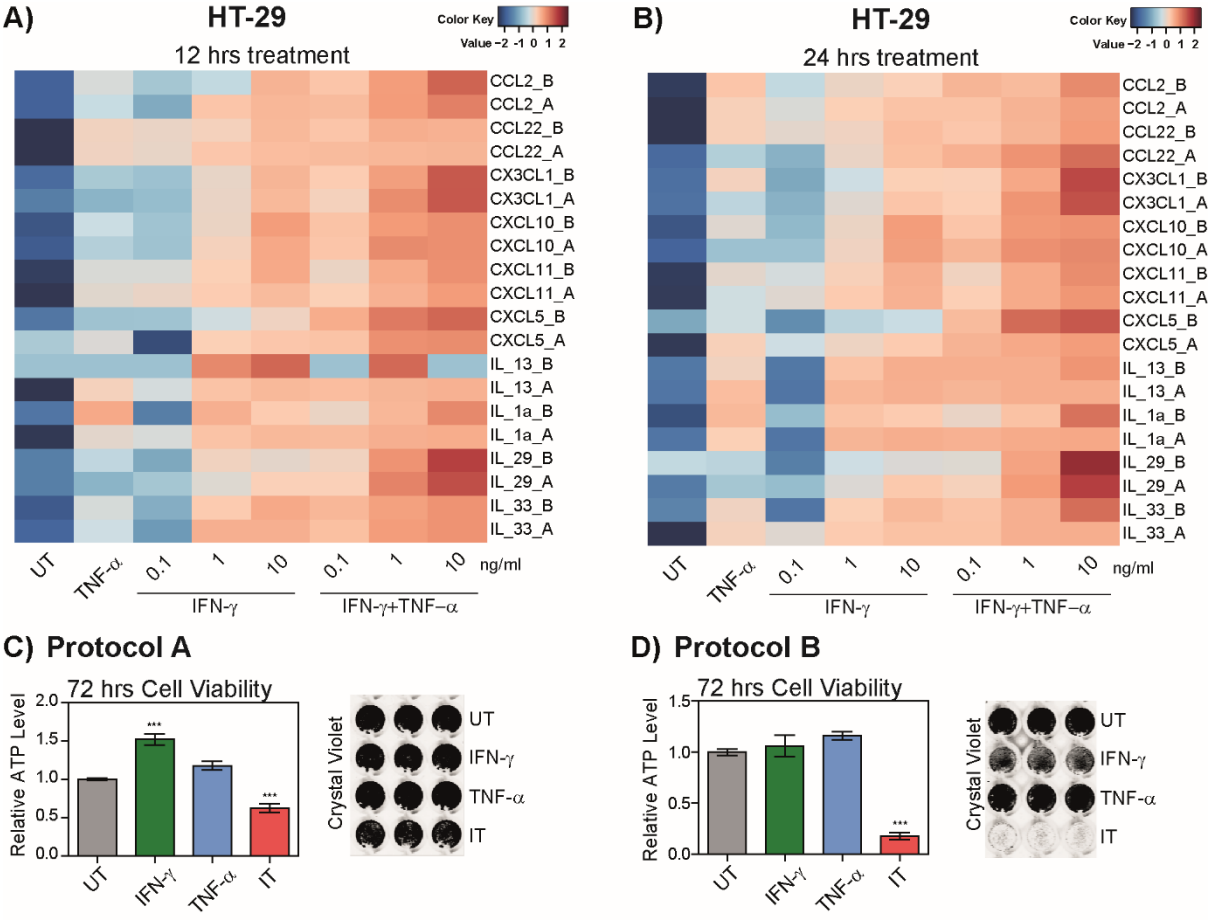


Figure 3.3: IFN-γ and TNF-α synergise to enhance inflammatory chemokine/cytokine production and induce cell death in HT-29 cells.

HT-29 cells were either untreated (UT) or treated with different concentrations of IFN-γ, 10 ng/mL of TNF-α or both cytokines (IT) following two protocols- protocol A (time-specified IFN-γ priming) and protocol B (continuous IFN-γ treatment). Production of ten selected cytokines/chemokines were analysed by MSD on supernatants collected after (**A**) 12 hrs and (**B**) 24 hrs of cytokine treatments. Data are represented as log10 transformed of mean ± SEM from three-five independent experiments done in triplicate. Cell viability was measured by CellTiter-Glo® after 72 hrs of cytokine treatments using (**C**) time-specified 10 ng/mL of IFN-γ priming of Protocol A or (**D**) continuous 10 ng/mL of IFN-γ priming of Protocol B (as outlined in Figure 3.1), and followed by treatment with 10 ng/mL of TNF-α. Data are represented as mean ± SEM from three independent experiments done in triplicates. *p<0.05, **p<0.01, ***p<0.001, one-way ANOVA with Tukey's multiple comparison test with UT.

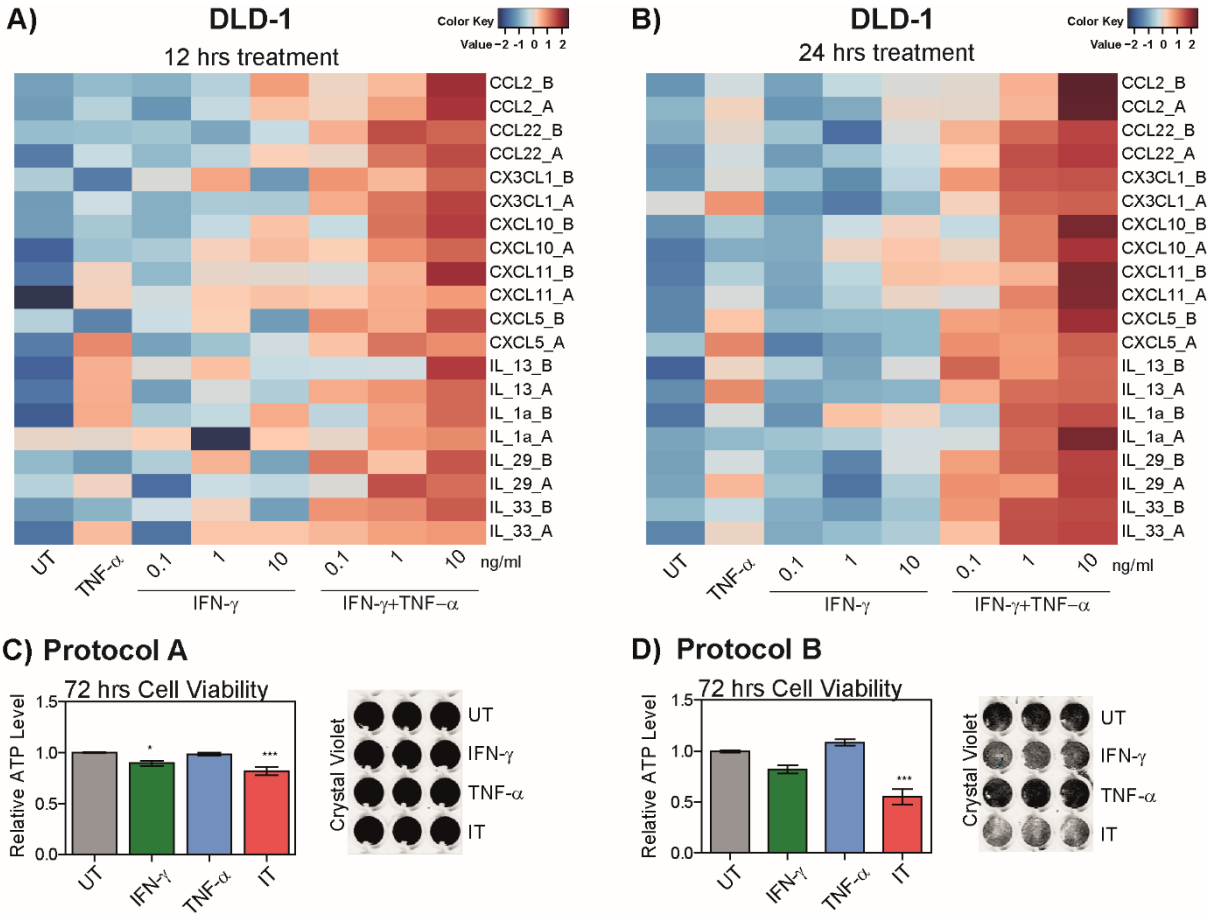


Figure 3.4: IFN-γ and TNF-α synergise to enhance inflammatory chemokine/cytokine production and induce cell death in DLD-1 cells

DLD-1 cells were either untreated (UT) or treated with different concentrations of IFN-γ, 10 ng/mL of TNF-α or both cytokines (IT) following two protocols- protocol A (time-specified IFN-γ priming) and protocol B (continuous IFN-γ treatment). Production of ten selected cytokines/chemokines were analysed by MSD on supernatants collected after (**A**) 12 hrs and (**B**) 24 hrs of cytokine treatments. Data are represented as log10 transformed of mean ± SEM from three-five independent experiments done in triplicates. Cell viability was measured by CellTiter-Glo® after 72 hrs of cytokine treatments using (**C**) time-specified 10 ng/mL of IFN-γ priming of Protocol A or (**D**) continuous 10 ng/mL of IFN-γ priming of Protocol B (as outlined in Figure 3.1), and followed by treatment with 10 ng/mL of TNF-α. Data are represented as mean ± SEM from three independent experiments done in triplicates. *p<0.05, **p<0.01, ***p<0.001, one-way ANOVA with Tukey's multiple comparison test with UT.

3.4.3. FasL, LT-α/β, TRAIL, TWEAK, BAFF and LIGHT synergise with IFN-γ to induce inflammatory cytokine/chemokine production and cell death

We screened 19 TNF superfamily ligands - including TNF-α as positive control - to identify ligand(s) that synergise with IFN-γ to induce production and secretion of inflammatory cytokines/chemokines and cell death similar or comparable to that induced by TNF-α. Firstly, we evaluated the effects of treating the cells with the TNFSF ligands alone. Of the 24 inflammatory cytokines/chemokines analysed, we found that TNF-α alone increased the secretion of almost all of the cytokines/chemokines in the panel. In HT-29 cells, TNF-α treatment alone increased secretion of 16 out of 24 cytokines/chemokines analysed except for CCL2, CCL3, CCL4, CCL17, CXCL11, MIF, CCL15, CXCL12a. Based on the expression pattern of the chemokines, we identified 5 clusters of TNFSF ligands. BAFF, lymphotoxin (LTα1/β2), and TRAIL clustered close to TNF-α (Fig 3.5A). Of the 24 cytokines/chemokines analysed, these ligands induced secretion of CXCL8, CCL8, CCL13, CCL1, and CXCL1 similar to that of TNF-α. However, the concentrations of the secreted proteins were at least 3-4 fold lower compared with the TNF-α treatment. In DLD-1 cells, TNF-α treatment alone increased the expression of only 6 of 24 chemokines - CXCL8, CCL27, CX3CL1, CCL1, CXCL1, and CCL20 while for other TNFSF ligands, the concentration of chemokines measured was negligible. Focussing on the clustering for DLD-1 cells, lymphotoxin (LTα2/β1), EDA-A2, TWEAK, LIGHT and TL1A clustered closely with TNF-α (Fig 3.5B). We identified that treatment of cells with the TNFSF ligands alone did not have any effect on cell viability of either HT29 (Fig 3.6A) or DLD-1 (Fig 3.6B) cell lines.

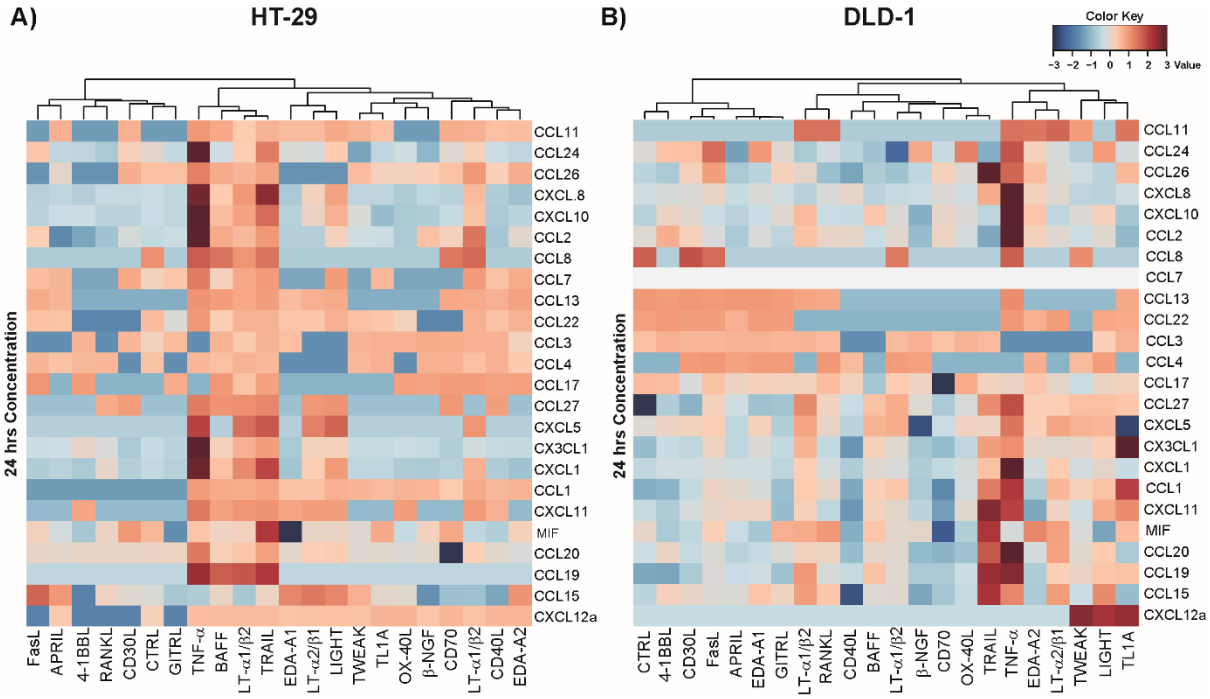


Figure 3.5: Inflammatory chemokine/cytokine production after 24 hrs of treatment with TNFSF ligands

(A) HT-29 and **(B)** DLD-1 cells were treated with BSA (CTRL - control) or 100 ng/mL of the indicated TNFSF ligands alone. Following 24 hrs of treatment, supernatants were collected and used to measure 24 inflammatory chemokines/cytokines by MSD assay. Data are represented as log10 transformed of mean ± SEM from three independent experiments in a single replicate.

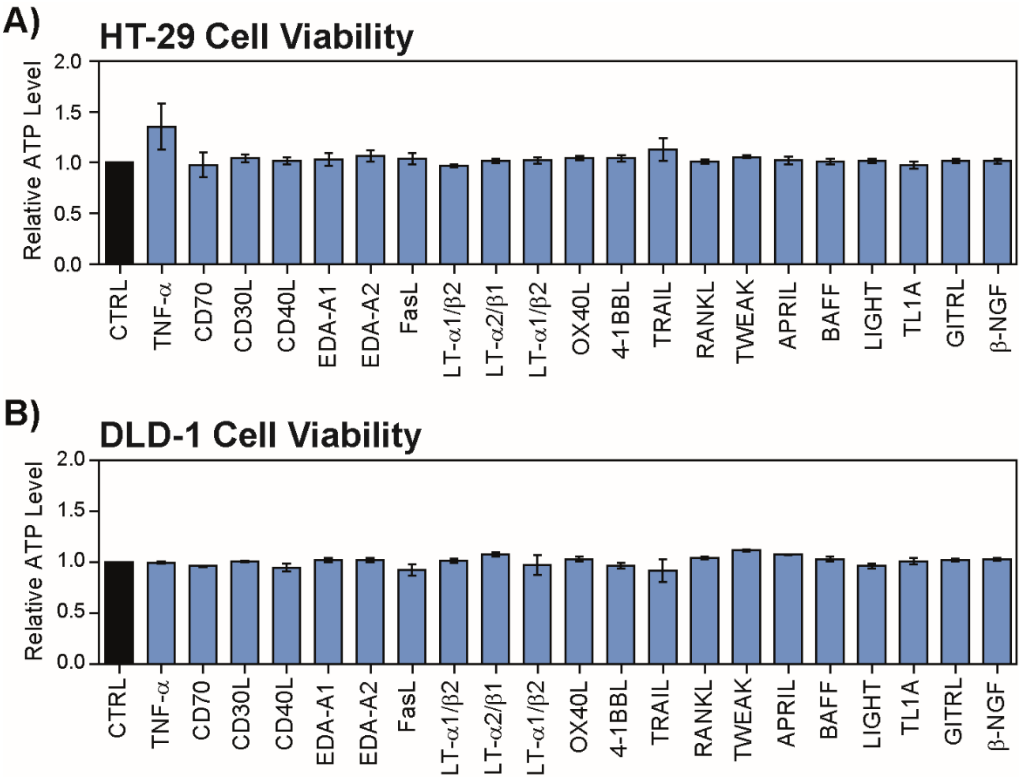


***Figure 3.6: TNFSF ligands did not affect cell viability of* colonic epithelial cancer cell lines**

(A) HT-29 cells and **(B)** DLD-1 cells were treated with BSA (CTRL - control) or 100 ng/mL of the indicated TNFSF ligands alone. Cell viability was measured by CellTitre-Glo® assay after 72 hrs of treatment. Data are represented as mean ± SEM from three independent experiments in a single replicate.

Next, we investigated the ability of TNFSF ligands to synergise with IFN-γ. As readouts of potential synergism, we selected the CXCR3 chemokines, CXCL10 and CXCL11, which are synergistically regulated by the combination of IFN-γ and TNF-α. As expected, TNF-α synergised with IFN-γ to increase the expression of CXCL10 and CXCL11 in HT-29 cells. Lymphotoxin, BAFF and LIGHT synergised with IFN-γ to enhance the expression of CXCL10, similar to TNF-α. These ligands, along with TRAIL, APRIL, and TL1A, also enhanced the expression of CXCL11 in combination with IFN-γ in HT-29 cells (Fig 3.7A). In the DLD-1 cell line, out of the 19 ligands tested, only TRAIL synergised with IFN-γ to enhance CXCL10 and CXCL11 production, similar to TNF-α (Fig 3.7B). Lymphotoxin and APRIL affected CXCL10 expression, but this was not statistically significant.

With regards effects of cytokine combinations on cell viability, we confirmed that TNF-α synergised with IFN-γ to induce cell death in both HT-29 and DLD-1 cells (Fig 3.8). In HT-29 cells, the death ligands FasL and TRAIL, as well as lymphotoxin, synergised with IFN-γ to induce cell death in HT-29 cells (Fig 3.8A). In DLD-1 cells, TRAIL synergised with IFN-γ and reduced cell viability by 90% in combination with IFN-γ, which was more than that observed by the combination of IFN-γ+TNF-α (Fig 3.8B). Based on the overall findings using both cell lines, we selected 7 TNFSF ligands - FasL, lymphotoxin, TRAIL, TWEAK, BAFF, and LIGHT for further validation screening. We also included TL1A as it is an identified risk factor gene for IBD[36].

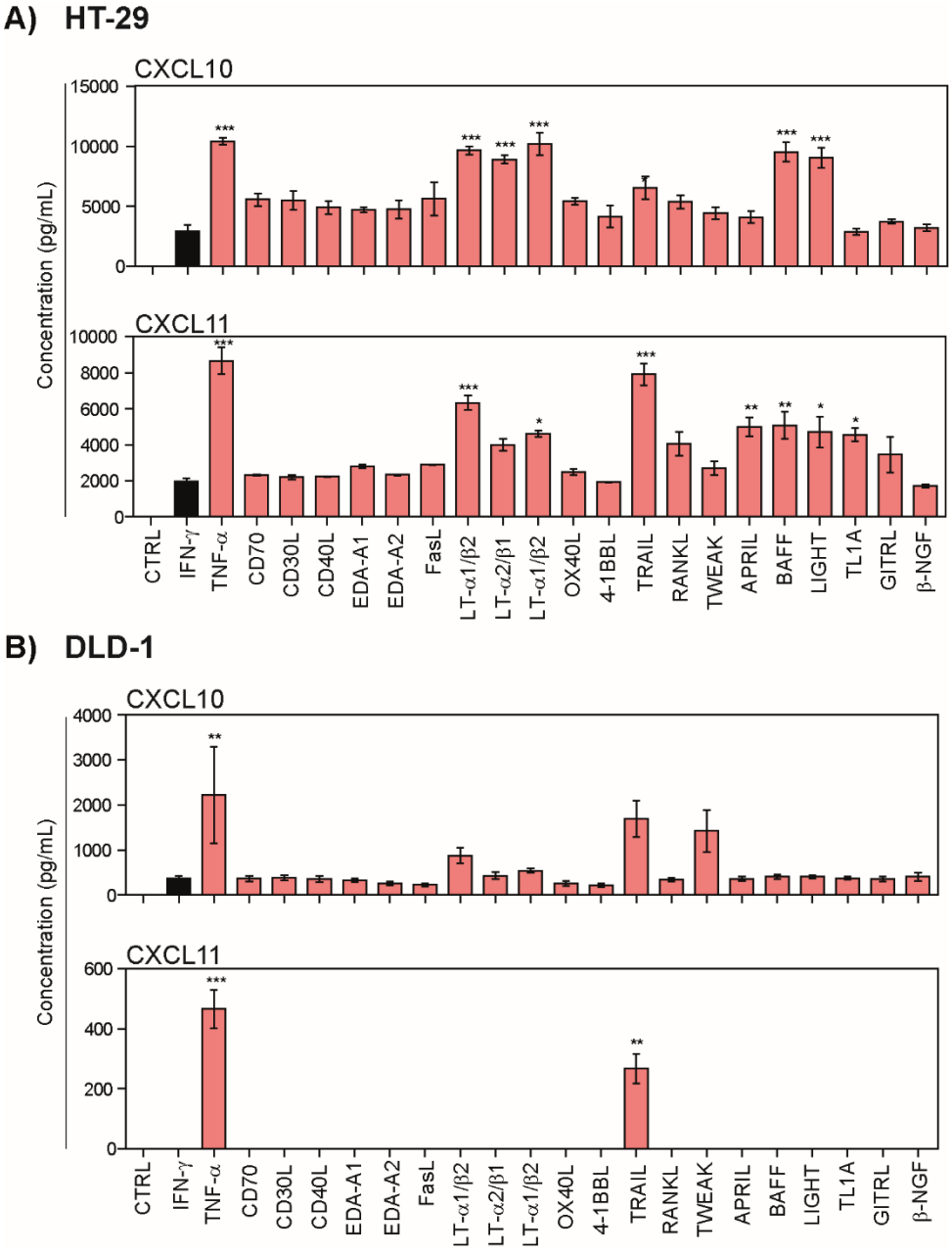


Figure 3.7: Identification of TNFSF ligands with TNF-α-like properties based on synergistic regulation of CXCR3 chemokines in combination with IFN-γ

(A) HT-29 cells and **(B)** DLD-1 cells were treated with BSA (CTRL) or primed with 10 ng/mL of IFN-γ for 16 hrs and treated with 100 ng/mL of the indicated TNFSF ligands. CXCL10 and CXCL11 levels were measured by MSD on supernatants collected after 24 hrs of treatment. Data are represented as mean ± SEM from three independent experiments with a single replicate. *p<0.05, **p<0.01, ***p<0.001, one-way ANOVA with Tukey's multiple comparison test.

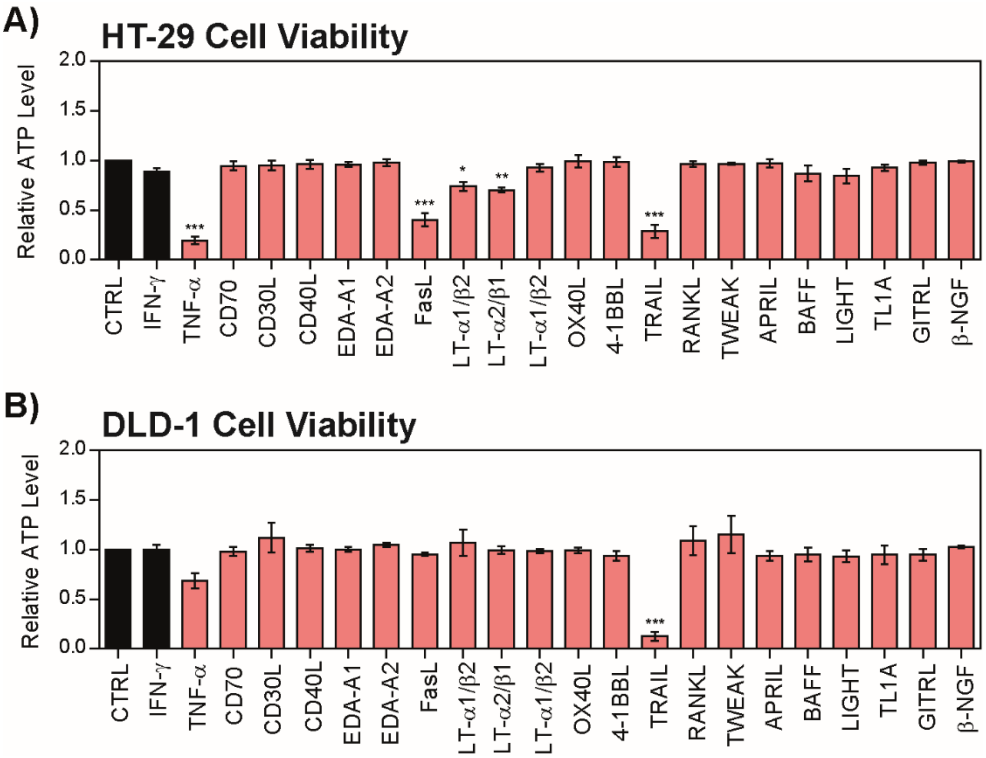


Figure 3.8: Identification of TNFSF ligands with TNF-α like property based on synergistic induction of cell death along with IFN-γ

(A) HT-29 cells and **(B)** DLD-1 cells were treated with BSA (CTRL) or primed with 10 ng/mL of IFN-γ for 16 hrs and treated with 100 ng/mL of the indicated TNFSF ligands. Cell viability was assessed by CellTitre-Glo® assay after 72 hrs of treatment. Data are represented as mean ± SEM from three independent experiments with a single replicate. *p<0.05, **p<0.01, ***p<0.001, one-way ANOVA with Tukey's multiple comparison test.

3.4.4. Validation screen confirms TRAIL, TWEAK and LIGHT as TNF-α-like ligands

Next, we performed a further validation screen in both cell lines for the seven selected TNFSF ligands (from section 3.4.3) along with TNF-α. We selected a panel of 8 inflammatory chemokines from the 24-chemokine panel used in the initial screen of cells treated with TNFSF ligands alone (Fig 3.5) that had a significant difference in their production following treatment with IFN-γ and TNF-α. In HT-29 cells, TNF-α synergised with IFN-γ to induce 7 out of 8 chemokines compared to individual TNF-α and IFN-γ treatment (Fig 3.9A). TNF-α treatment alone induced the expression of CXCL8, CXCL1, CX3CL1, CCL22, and CCL2. Combined treatment of TNF-α with IFN-γ had no effect on CXCL8 levels, a marginal increase in CXCL1 levels, and a significant increase in the rest of the chemokines compared to TNF-α alone treatment. The seven TNFSF ligands on their own did not have any effect on the expression of the selected chemokines except for TRAIL, which increased the expression of CXCL8, CXCL1, and MIF (Fig 3.9A). Five out of the seven TNFSF ligands synergised with IFN-γ to increase the expression of CXCL10 and CXCL11, as observed in the previous larger screen (Fig 3.7). Only BAFF, which increased the production and secretion of both CXCL10 and CXCL11 in the primary screen, did not have any effect on chemokine production in this secondary screen. IFN-γ synergised with FasL, lymphotoxin (LT-α1/β2), and LIGHT to enhance levels of CX3CL1 compared to TNFSF-alone treatments. TNF-α treatment of DLD1 cells induced most of the chemokines analysed in combination with IFN-γ (Fig 3.10A). Only TWEAK, in combination with IFN-γ, increased the expression of 4 out of 8 chemokines

analysed. Treatment with TRAIL alone enhanced the expression of CXCL8, CCL22, and MIF. TRAIL synergised with IFN-γ to increase the expression of only CXCL-11.

In terms of effects on relative cell viability, both FasL and TRAIL, in combination with IFN-γ, induced cell death in HT-29 cells, similar to TNF-α, with TRAIL being the most potent inducer (85% reduction in cell viability) followed by TNF-α (70%) and FasL (35%). However, lymphotoxin did affect the cell viability in this secondary validation screen (Fig 3.9B). In contrast, DLD-1 cells were more sensitive to treatment with TRAIL alone, and cell viability was further decreased by IFN-γ priming followed by TRAIL treatment (Fig 3.10B). Based on these findings, we identified TRAIL as being the most similar ligand to TNF-α in inducing cell death and inflammatory chemokines in combination with IFN-γ. TWEAK and LIGHT are the next TNFSF ligands most similar to TNF-α in terms of their ability to induce inflammatory chemokines/cytokines in combination with IFN-γ.

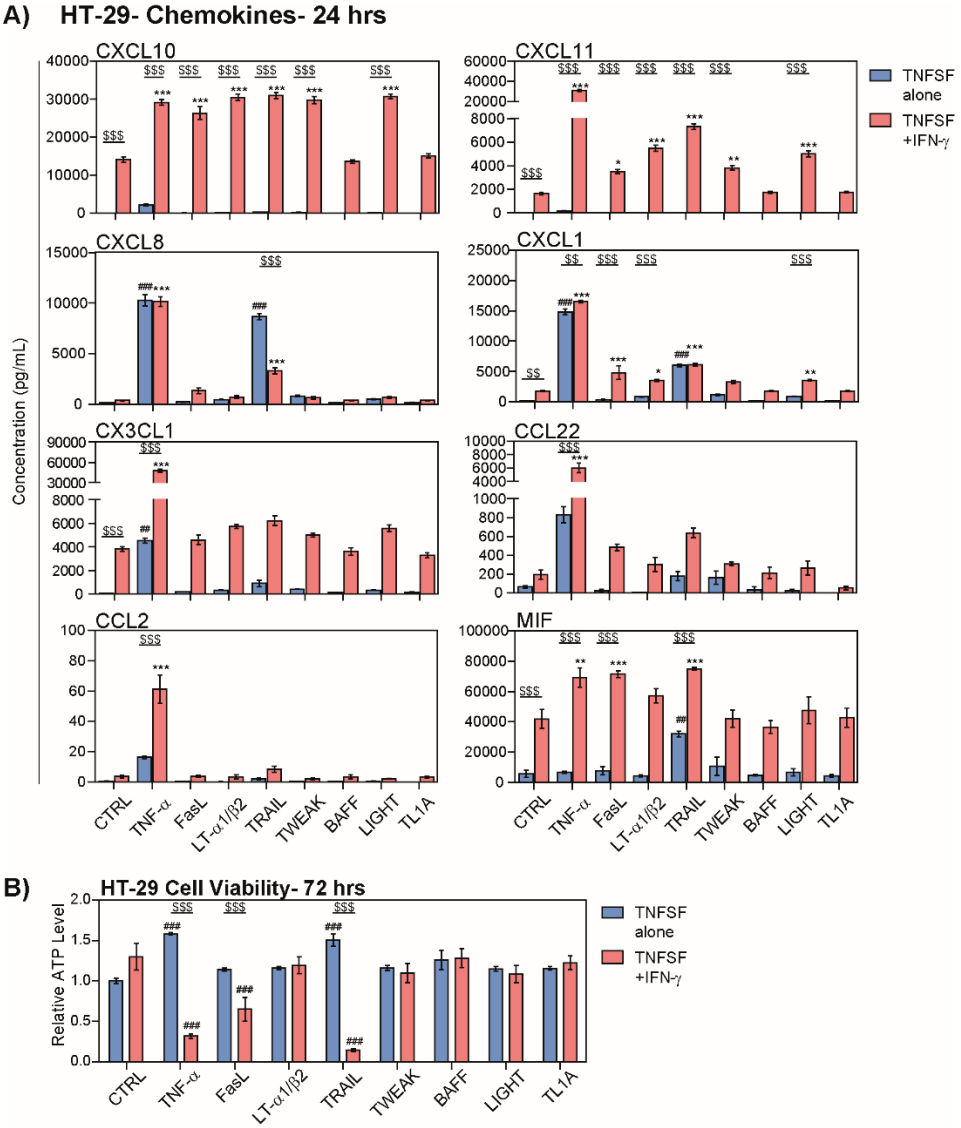


Figure 3.9: Validation screen of seven selected TNFSF ligands alone and in combination with IFN-γ in HT-29 cells

HT-29 cells were treated with 100 ng/mL of the seven selected TNFSF ligands alone or in combination with 10 ng/mL of IFN-γ. Cells treated with BSA ± IFN-γ are used as CTRL. (**A**) Chemokine production was measured by MSD 8-spot U-plex assay after 24 hrs of cytokine stimulation. (**B**) Cell viability was measured by CellTitre-Glo® assay after 72 hrs treatment. Data are represented as mean ± SEM from three independent experiments with a single replicate. *p<0.05, **p<0.01, ***p<0.001. Two-way ANOVA with Bonferroni posthoc test - $$$ and one-way ANOVA with Tukey's multiple comparison test- * with IFN-γ and # with CTRL.

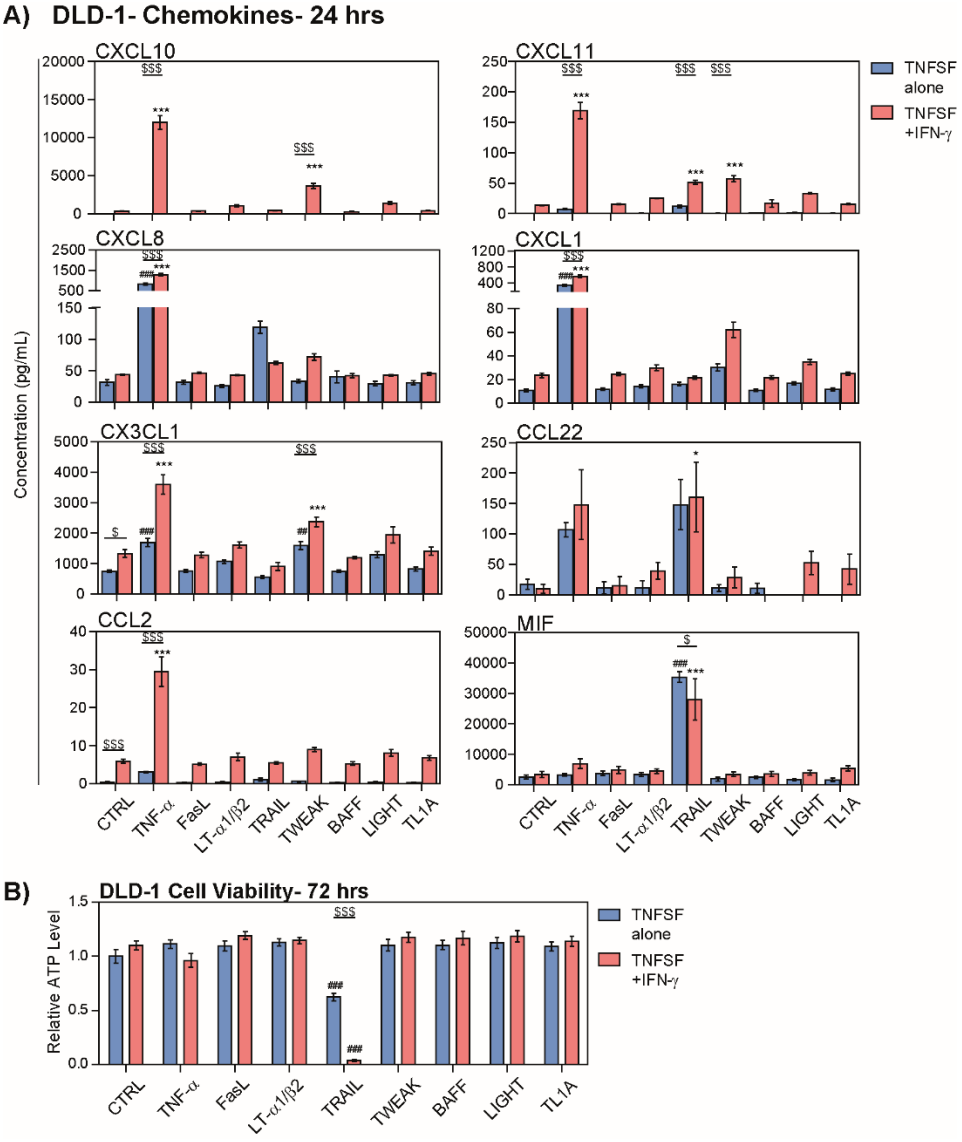


Figure 3.10: Validation screening of seven selected TNFSF ligands alone and in combination with IFN-γ in DLD-1 cells

DLD-1 cells were treated with 100 ng/mL of the seven selected TNFSF ligands alone or in combination with 10 ng/mL of IFN-γ. Cells treated with BSA ± IFN-γ are used as CTRL. (**A**) Chemokine production was measured by MSD 8-spot U-plex assay after 24 hrs of cytokine stimulation. (**B**) Cell viability was measured by CellTitre-Glo® assay after 72 hrs treatment. Data are represented as mean ± SEM from three independent experiments with a single replicate. *p<0.05, **p<0.01, ***p<0.001. Two-way ANOVA with Bonferroni posthoc test - $$$ and one-way ANOVA with Tukey's multiple comparison test- * with IFN-γ and # with CTRL.

3.4.5. TRAIL, TWEAK and LIGHT are confirmed as potential TNFSF ligand targets in human colonic organoids

We investigated if the effect of selected TNFSF ligands alone or in combination with IFN-γ elicited similar effects in a non-transformed primary human epithelial cell model, i.e. human colonic organoids. We selected four out of seven TNFSF ligands (lymphotoxin, TRAIL, TWEAK and LIGHT) from the validation screens that gave the strongest response in terms of production of cytokines/chemokines and cell death. We used colonic organoid lines derived from non-IBD/healthy controls as well as patients with Crohn's disease for these experiments. In healthy organoids, TNF-α induced CXCL8 and IFN-γ+TNF-α reduced CXCL8 levels but not significantly compared to TNF-α alone (Fig 3.11A). Co-treating healthy organoids with TNF-α and IFN-γ resulted in synergistic production of CXCL10, CXCL11 and CX3CL1. Of the four ligands tested, TWEAK, alone or in combination with IFN-γ, had the most similar response to TNF-α in the induction of the four chemokines tested. LIGHT synergised with IFN-γ to induce CXCL10 and CXCL11 but had no effect on CXCL8 or CX3CL1 production. Lymphotoxin synergised with IFN-γ to induce CXCL10 but not the three other cytokines, while TRAIL, either alone or in combination with IFN-γ had no major effect on any chemokine production (Fig 3.11A). Similar to healthy organoids, in organoid lines derived from colonic mucosal biopsies of patients with CD, TNF-α treatment increased CXCL-8 production but was not significant, and the combination of TNF-α+IFN-γ increased the expression of the other three chemokines (Fig 3.12A). TWEAK displayed more TNF-α like effect in Crohn's lines as on its own induced CXCL8 and in synergism with IFN-γ induced CXCL10, CXCL11 and CX3CL1. While both lymphotoxin and LIGHT in combination with IFN-γ induced CX3CL1,

only lymphotoxin in combination with IFN-γ induced CXCL10 production (Fig 3.12A). TRAIL had no effect on chemokine production in CD organoid lines.

Treatment with TRAIL and TWEAK alone induced a considerable reduction in the cell viability of healthy organoid lines. Co-treatment of TNFSF with IFN-γ had negligible effects on healthy organoid line viability (Fig 3.11B). Organoid lines derived from CD tissue were very sensitive to TRAIL treatment alone (70% reduction in cell viability) and less sensitive to TWEAK treatment (20% reduction). Treatment with the other TNFSF ligands, either alone or in combination with IFN-γ, had negligible effects on cell viability (Fig 3.12B).

Overall, of the 19 TNFSF ligands screened in this study, we identified the following ligands displaying TNF-α like properties: i) TRAIL, TWEAK, and LIGHT induce inflammatory chemokines in both the cell lines and colon organoid lines; ii) FasL, Lymphotoxin, and TRAIL induce cell death in the cell line models; iii) TRAIL induce cell death in both healthy and Crohn's disease organoid lines.

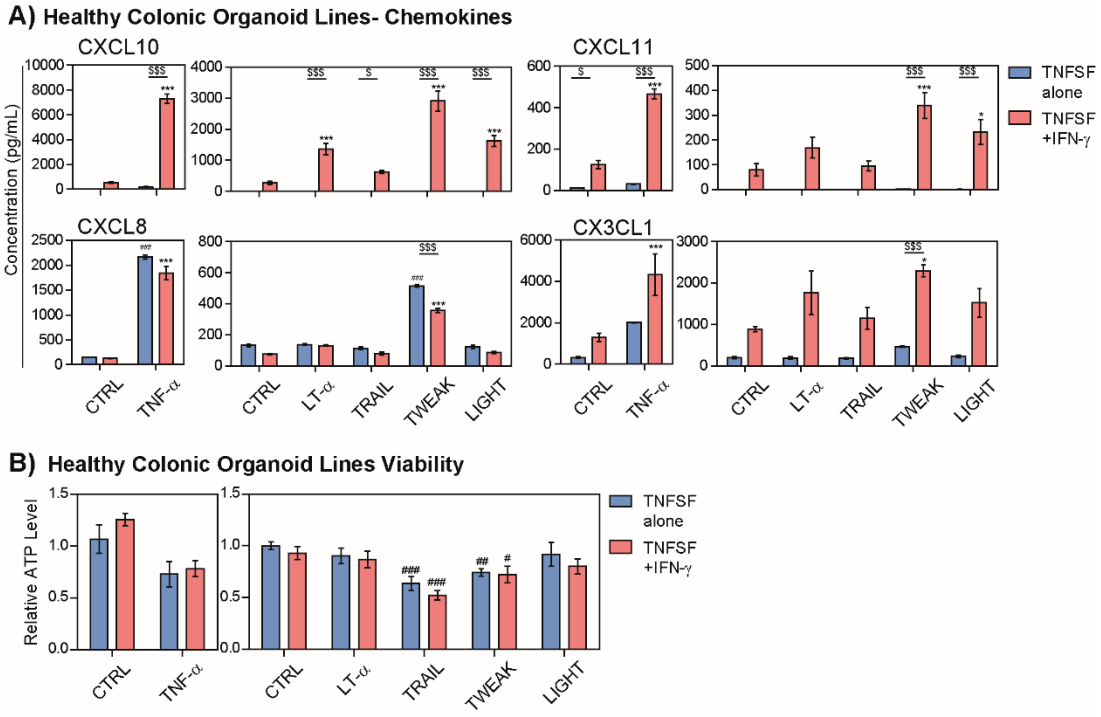


Figure 3.11: TNFSF ligands synergise with IFN-γ to induce Th1 chemokines in human healthy colonic organoids

Human colonic organoids were generated from colonic mucosal biopsies collected from healthy controls and treated with 100 ng/mL of the indicated TNFSF ligands alone or in combination with 10 pg/mL of IFN-γ for 24 hrs. Treatment with BSA ± IFN-γ was considered as CTRL (**A**) Chemokine production assessed by MSD 5-spot U-plex assay after 24 hrs of cytokine stimulation and (**B**) Cell viability was measured by CellTitre-Glo® assay after 24 hrs of cytokine treatments. Data are represented as mean ± SEM from one (for TNF-α) and two organoid lines with duplicates. **p*<0.05, ***p*<0.01, ****p*<0.001, *- two-way ANOVA with Bonferroni posthoc test compared to CTRL.

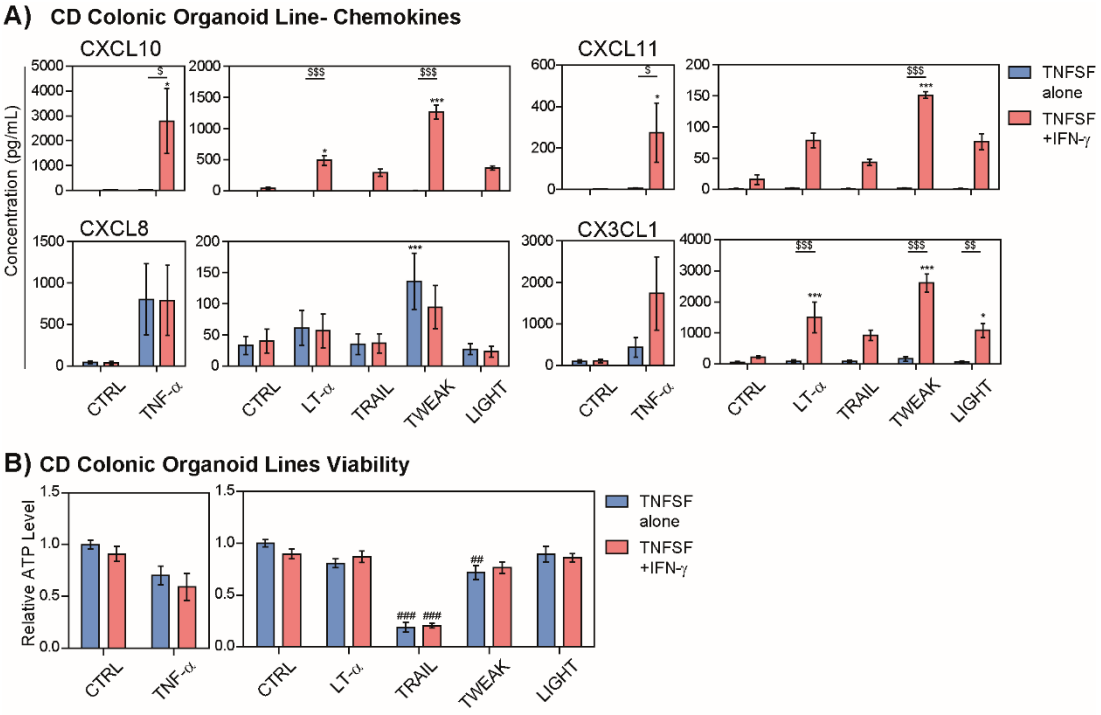


Figure 3.12: TNFSF ligands synergise with IFN-γ to induce Th1 chemokines in human Crohn's disease colonic organoids

Human colonic organoids were generated from colonic mucosal biopsies of patients with Crohn's disease and treated with 100 ng/mL of the indicated TNFSF ligands alone or in combination with 10 pg/mL of IFN-γ for 24 hrs. Treatment with BSA ± IFN-γ was considered as CTRL. (**A**) Chemokine production was assessed by MSD 5-spot U-plex assay after 24hr of cytokine stimulation, and (**B**) Cell viability was measured by CellTitre-Glo® assay after 24 hrs of cytokine treatments. Data are represented as mean ± SEM from one (for TNF-α) and two organoid lines with duplicates. *p<0.05, **p<0.01, ***p<0.001, *- two-way ANOVA with Bonferroni posthoc test compared to CTRL.

3.5. Discussion

Resistance to anti-TNF therapies constitutes a major clinical problem for patients with IBD, and the therapeutic gap in these diseases needs to be addressed through the identification of drug new targets for IBD treatment[16,17]. Recently, additional cytokines such as oncostatin M[28] and IL-23[29] have been identified as potential resistance mechanisms for anti-TNF therapy failure; hence, targeting redundant cytokine pathways might be one way to overcome resistance. In this regard, the closest family members to TNF-α belong to the TNF superfamily (TNFSF), and this family of ligands might contain members with TNF-α-like biological activity. Here, we used epithelial cell line models and primary human colonic organoid lines to identify target ligands exhibiting similar biological properties to TNF-α. Previously, TNF-α has been shown to synergise with IFN-γ to induce inflammatory chemokines and cell death in epithelial cell lines[57,63]. We used these two phenotypic readouts in epithelial cell line models and primary human colonic organoid lines treated with either TNFSF alone or in combination with IFN-γ to identify ligands with functions most similar to TNF-α. From our cell line (HT-29 and DLD-1) and primary organoid screens, TRAIL, TWEAK and LIGHT were identified to induce inflammatory chemokines both alone and in combination with IFN-γ. In our cell line models and organoid lines, we identified TRAIL to induce cell death either alone or in combination with IFN-γ in a similar manner to TNF-α. Overall, we also observed that most of our results in colonic cancer cell line models translated well across into human primary colonic epithelial organoids.

The TNF superfamily of ligands consists of 19 ligands that are expressed on various cell types of the immune system. They are type II transmembrane proteins and

signal for proliferation, differentiation, cell survival and apoptosis[72]. Various clinical trials are now focusing on targeting TNFSF members in different autoimmune and inflammatory diseases[12,73]. Brentuximab targeting CD30L is approved for Hodgkin's lymphoma, Belumimab targeting BAFF is approved for systemic lupus erythematosus (SLE), and Denosuma- antibody targeting RANKL for osteoporosis has been approved[13]. TNFSF ligands may act as redundant pathways during TNF-α blockade with anti-TNFs in human chronic inflammatory disease and therefore are potential targets for the treatment of IBD.

TWEAK is a multifunctional cytokine that signals through Fn14, a TRAF2 interacting receptor[74–76]. It is well documented that TWEAK/Fn14 is highly inducible and is elevated in various autoimmune and inflammatory diseases[73,77–82]. The activation of TWEAK/Fn14 results in the induction of pro-inflammatory cytokines and chemokines such as CCL2, CCL3, CCL5, CCL21, CXCL10, CXCL1, IL-13, IL-17 in many cell types through canonical and non-canonical NF-κB pathways[81,83–86]. In this study, we did not find any significant induction of 10 key chemokines with TWEAK treatment alone either in our cell line models or in primary colonic organoids. In contrast, CXCL10, CXCL11 and CX3CL1 were induced by TWEAK following IFN-γ priming. Previous reports have shown that TWEAK induces HT-29 cell death along with IFN-γ through a JAK-STAT dependent pathway[87–89]. However, the concentration of IFN-γ used in those studies was at least three-fold higher up to 30 ng/mL than the concentration used in this study which might explain the observation of no induction of cell death with IFN-γ and TWEAK in our study. Studies in animal models of colitis show that targeting TWEAK through antibodies or knockout of TWEAK offered protection to chemically-induced colitis[90–92]. Dampening of TWEAK signalling reduced levels of inflammatory cell infiltrates, crypt loss and

collagen accumulation[93]. In patients with UC, mucosal samples had elevated expression of TWEAK and its receptor Fn14[94]. In the ileal samples of patients with CD, TWEAK was elevated both at the mRNA and protein levels[95]. However, in DSS (dextran sodium sulfate)-induced animal model of colitis, the receptor FN14 knockout increased susceptibility to colitis[96]. A study from Norway on serum TWEAK levels in newly identified IBD cases between 2012-2015 found no significant difference between IBD and healthy cohorts[97]. These observations may stem from the fact that TWEAK might play a dual role in intestinal injury and mucosal repair.

LIGHT (TNFSF14) can bind to three receptors, herpes virus entry mediator receptor (HVEM), lymphotoxin β receptor (LTβR) and the decoy receptor DcR3. LIGHT signalling through HVEM is an important co-stimulator for the activation and proliferation of T-cells [98–100]. LIGHT can induce intestinal inflammation by promoting the expansion and activation of Th1 cells [100,101]. Overexpression of LIGHT resulted in induction of colitis, and LIGHT/HVEM deficiency exacerbated DSS-induced colitis[101–106]. In fibroblasts, LIGHT, either alone or in combination with IFN-γ, induced the production of inflammatory chemokines such as CXCL10, CXCL11, CCL5, and CXCL5, and downregulated homeostatic factors[107,108]. Here, we show that in intestinal epithelial cells, LIGHT can synergise with IFN-γ to induce CXCL10 and CXCL11. LIGHT has been shown to sensitise cells to IFN-γ induced cell death, and the synergism results in a mixed-type of cell death, with mitochondrial dysfunction - due to downregulation of Bcl2 and Bcl-xL, activation of as well as caspase activation[109–113]. However, this cell death appears to be dose-dependent for both the cytokines [109–111]. In all of these studies, the concentration of LIGHT used ranged from 50 ng/mL – 5 µg/mL, whereas in our work, we used 100 ng/mL concentration, which might not have been enough to induce cell death. The specific role of LIGHT in IBD

is yet to be identified. However, animal models with a deficiency in LIGHT have reduced inflammation compared to wild type[104]. Also, overexpression of LIGHT on T-cells results in the spontaneous development of intestinal inflammation[102,114]. High levels of LIGHT were detected in the activated T-cells of the gut, and LIGHT mRNA expression was significantly increased in the intestines of patients with active CD[115,116]. LIGHT was found to be elevated in a set of stromal cells that were expanding in the UC subset compared to healthy individuals[117]. CERC-002, an anti-LIGHT antibody, has shown positive phase-I results in patients with CD who are also refractory to anti-TNF therapy[118].

TRAIL is one of the most extensively studied death ligands of the TNF superfamily[119]. TRAIL induces apoptosis through the TRAIL-R1/R2, FADD and caspase-8 complex[120]. In our study, we found that DLD-1 cells and the primary organoid lines were sensitive to TRAIL-induced cell death compared to the HT-29 line. However, priming with IFN-γ enhanced the sensitivity of HT-29 cells to TRAIL. IFN-γ can sensitise epithelial cells by upregulating caspase-8 expression or by inhibiting anti-apoptotic proteins like IAP-2[45,47,121,122]. In this study, TRAIL-induced the production of most of the chemokines tested in a similar way to TNF-α, and this response was enhanced with IFN-γ pre-treatment. This is in line with previous reports showing that TRAIL, in various cancer cell lines, including HT-29 cells, can induce inflammatory chemokines through a FADD-caspase 8 'FADDosome' complex [119,123–125]. Since we have HT-29 as one of our cell line models, the increase in inflammatory chemokines such as CXCL8 and CXCL1 might be through the FADDosome pathway. Begue *et al.* (2006) found that TRAIL was upregulated in intestinal epithelial cells in inflamed colonic and ileal tissues[126]. TRAIL could potentially augment IEC apoptosis through auto/paracrine signalling. Brost *et al.* found in patients with IBD that TRAIL

was upregulated in mononuclear cells in active inflammatory sites[127]. However, as seen with TWEAK, TRAIL-R deficiency increased susceptibility to DSS-induced colitis, with increased epithelial damage, loss of colonic crypts and inflammatory cell infiltration[128]. The reason might be that chemically-induced colitis may be much different from the IBD signature observed in humans or that the TRAIL pathway can also have a role in the homeostasis of epithelial cells.

Other TNFSF family ligands, including TL1A and RANKL, are identified as susceptible genes for IBD, particularly for Crohn's disease[129]. However, in our screens, these did not validate as potential TNF-α-like targets in terms of synergism with IFN-γ. Our study has a few limitations. One is the lack of information regarding TNFSF receptor expression in non-treated and IFN-γ primed cell lines and colonic organoid lines. Another limitation was the batch to batch variations observed with commercially bought ligands resulting in varied results between the primary and secondary screens. Another is the number of lines used for the screens, two for the cell lines and two each for the organoid lines. Overall, our cell line models and our preliminary validation using the human primary organoid lines from healthy/non-IBD and CD patients indicate that TWEAK, LIGHT and TRAIL are potential targets for IBD. Further validation in pre-clinical models, including combinatorial treatment with anti-TNFSF and anti-TNF, would provide further evidence of their potential therapeutic value for future clinical trials.

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Chapter 4 : IFN-γ and TRAIL synergise to induce Th1 chemokines and cell death in human colon cancer cells in a caspase8-JAK1/2-STAT1 dependent manner

4.1. Abstract

Screening TNF superfamily ligands for a tumor necrosis factor (TNF)-α-like molecule with respect to inflammatory chemokine secretion and cell death, we identified TNF-α related apoptosis-inducing ligand (TRAIL) as a major hit. We aimed to investigate more the effects of TRAIL because of its role in tumour immunology. TRAIL was initially discovered to be an anti-tumour molecule that can induce apoptosis in tumour cells without having lethal effects on normal cells. However, recent studies have shed light on TRAIL's tumour promoting activity rather than its anti-tumorigenic effects. IFN-γ, a pro-inflammatory T helper 1 (Th1) cytokine, is a potent tumour suppressor cytokine. In this study, we investigated whether IFN-γ can switch TRAIL's effect from a pro-tumorigenic cytokine to an inflammatory anti-tumourigenic cytokine and the molecular mechanism behind the interaction. We chose a panel of eleven colon cancer lines to test for sensitivity towards TRAIL and IFN-γ and their combination. We investigated the molecular mechanism behind IFN-γ and TRAIL synergism on HT-29 cancer cell lines using CRISPR/Cas9 KO cell models and perturbation experiments using inhibitors and RNAi. Of eleven colon cancer lines tested, seven were found to be TRAIL-sensitive. Of the remaining four TRAIL-resistant cell lines, IFN-γ+TRAIL could synergise to induce cell death. IFN-γ and TRAIL had a cell line dependent effect on the secretion of pro-inflammatory chemokines. In HT-29 cell lines, IFN-γ and TRAIL synergised to induce a high degree of cell death and inflammatory chemokines. While TRAIL induced the tumour promoting CXCL8 and CXCL1 chemokines, IFN-γ induced the tumour suppressing CXCL10 chemokine. The combination of the two cytokines increased the levels of CXCL-10 with no change in CXCL8 and CXCL1 levels. IFN-γ+TRAIL induced cell death of HT-29 cells was independent of canonical apoptotic and necroptotic pathways as inhibitors targeting key molecules of these

death pathways could not block IFN-γ+TRAIL induced cell death. Knockout of caspase-8 partially blocked IFN-γ+TRAIL induced cell death, and IFN-γ+TRAIL induced CXCL10 production. Perturbation of the JAK-STAT pathway also blocked cells from IFN-γ+TRAIL induced cell death and reduced CXCL10 levels but had no effect on levels of CXCL8 and CXCL1. Moreover, IFN-γ and TRAIL resistant colon cancer lines had reduced phosphorylation of STAT1 at the Tyr 701 residue during cytokine treatments compared to lines that were sensitive to IFN-γ and TRAIL. Overall, our data suggest that IFN-γ can modulate TRAIL responses in the colon cancer lines by inducing cell death and increasing the levels of Th1 chemokine CXCL10 through a caspase-8-JAK1/2-STAT1 dependent pathway.

4.2. Introduction

TNF-related apoptosis-inducing ligand (TRAIL) is a type II transmembrane protein belonging to the TNF superfamily (TNFSF). TRAIL is a death ligand known for its ability to induce death receptor mediated-apoptosis across a wide variety of cancer cell lines through its interaction with death domain-containing receptors- TRAIL-R1 (DR4) and TRAIL-R2 (DR5)[1]. It can also bind to three other decoy receptors- TRAIL-R3 (DcR1), TRAIL-R4 (DcR2) and osteoprotegerin (OPG)[2]. Binding of TRAIL to death receptors TRAIL-R1 and TRAIL-R2 recruits Fas-associated death domain (FADD) followed by subsequent caspase-8 and caspase-10 recruitment via their death effector domain (DED)[1,3]. This leads to the assembly of a signalling complex known as the death-inducing signalling complex (DISC). DISC promotes autoprocessing and activation of caspase-8 and caspase-10[4]. Activation of caspase-8 leads to activation of both extrinsic and intrinsic apoptosis in epithelial cell lineages[5]. In the extrinsic pathway, caspase-8 stimulates effector caspases 3, 6 and 7 and induces apoptosis[6]. In the intrinsic/mitochondrial pathway, caspase-8 processes Bid to truncated Bid (tBid), which translocates to the mitochondrial membrane and, along with Bak and Bax, induces mitochondrial outer membrane permeabilization (MOMP)[7]. Cytochrome-c is subsequently released from mitochondria, which recruits apoptotic protease activating factor-1 (Apaf-1) and caspase-9 to form the apoptosome in the cytosol, leading to the activation of caspase-9 and downstream effector caspases[8]. Smac is also released from mitochondria and can interact with the inhibitory of apoptotic proteins (IAPs) blocking their inhibitory effects on caspase activity[7].

In addition to inducing apoptosis, TRAIL can also activate the pro-survival and pro-inflammatory transcription factor nuclear factor kappa B (NF-κB) through its receptors TRAIL-R1, TRAIL-R2 and TRAIL-R4[9–12]. TRAIL-mediated activation of

NF-κB increases survival and proliferation of TRAIL-resistant cancer lines[13–15]. TNFR1-associated death domain (TRADD) and TNF receptor-associated factor 2 (TRAF2) play an important role in TRAIL-dependent activation of NF-κB[16–18]. TRAF2, recruited to the receptors via TRADD, can act as an E3 ubiquitin ligase and lead to proteasomal degradation of active caspase-8[19]. However, recent studies report that FADD, caspase-8 and receptor-interacting protein kinase 1 (RIPK1) are all involved in TRAIL-mediated NF-κB activation[12]. TRAIL-mediated NF-κB activation is strongly reduced in FADD-deficient or caspase-8 deficient Jurkat cell lines[17,20]. Through a complex called the FADDosome, consisting of Caspase-8-FADD-RIPK1, TRAIL can activate NF-κB and induce inflammatory chemokines[21]. The linear ubiquitin chain assembly complex (LUBAC), recruited to TRAIL receptor through a FADD-dependent manner, is essential for recruitment and activation of the inhibitor of NF-κB (IκB) kinase (IKK) complex, which in turn activates NF-κB leading to the increased expression of downstream target genes[22–24]. TRAIL-induced NF-κB can also induce the expression of anti-apoptotic genes such as *cFLIP, Bcl-X_L, and cIAPs* and induces resistance to cancer cells against TRAIL[25–28]. Cellular FLICE-inhibitory protein (c-FLIP) forms heterodimers with pro-caspase-8 and inhibits its activation. c-FLIP can bind to the DED of FADD and prevent the recruitment of caspase-8 and caspase-10[29]. High levels of c-FLIP contribute to TRAIL resistance in cancers such as breast cancers[30,31]. Targeting c-FLIP by RNAi sensitised TRAIL-resistant cancer lines to TRAIL-induced apoptosis by enhancing recruitment of caspase-8 to the DISC assembly and initiating apoptosis[32,33]. Elevated levels of anti-apoptotic proteins such as Bcl-2[34,35] and Bcl-xL[36,37] are also observed in TRAIL-resistant cancers. Smac mimetics targeting inhibitor of apoptosis proteins (IAP)s, especially XIAP, sensitise cells to TRAIL-induced apoptosis by modulating caspase-8 activation and TRAIL-

induced NF-κB signalling[38–40]. Higher expression levels of TRAIL-R3 and TRAIL-R4, especially in breast, lung and prostate cancers, can also confer resistance to TRAIL-induced cell death[41–43]. These decoy receptors bind TRAIL and lower TRAIL's availability for death receptors to induce apoptotic signals. TRAIL-mediated NF-κB signalling also leads to the secretion of pro-inflammatory chemokines that may modulate the tumour microenvironment leading to increased tumour growth and proliferation. For example, TRAIL-induced CXCL8 in ductal adenocarcinoma[28]. CXCL8 acts as a chemoattractant for neutrophils and, in the tumour microenvironment, helps cancer cells maintain epithelial-mesenchymal transition (EMT)[44]. In lung cancers, TRAIL increased levels of CXCL8 and CXCL1, which are neutrophil chemoattractants, and increased levels of CCL2, which polarised myeloid cells towards M2-like pro-tumorigenic cells[21,45]. Our results in the previous chapter of the thesis (Chapter 3) also reported that TRAIL induced CXCL8 and CXCL1 in colon cancer lines.

IFN-γ is a pro-inflammatory cytokine, a major effector of the immune system, and is implicated in various diseases[46]. IFN-γ is a pleiotropic cytokine with associated pro-apoptotic and anti-tumour mechanisms[47]. IFN-γ is predominantly produced by immune cells such as natural killer (NK cells), Th1 $CD4^+$ T-cells, cytotoxic T-cells and innate lymphoid cells (ILC1)[48]. IFN-γ signals through its receptors IFNGR1 and IFNGR2[49]. Ligand binding leads to phosphorylation and activation of Janus kinase (JAK) 1 and 2, which further phosphorylate signal transducer and activator of transcription 1 (STAT1)[46]. Phosphorylated STAT1 dimerises and translocates to the nucleus, where it binds to the gamma activated sequence (GAS) and activates primary response genes such as *IRF1*[50]. A main function of IFN-γ in anti-tumour immunity is the maintenance of $CD4^+$ Th1 cells and inhibition of other $CD4^+$ Th cell subsets[51]. In

$CD4^+$ T cells, IFN-γ induces expression of the transcription factor T-bet via STAT1, and T-bet inhibits Th2 cell differentiation, thereby maintaining Th1-polarizing conditions[52,53]. IFN-γ induces CXCR3 chemokines such as CXCL9, CXCL10, and CXCL11, resulting in the trafficking of CXCR3 expressing T-cells, NK cells, and NKT cells into the tumour microenvironment (TME)[54,55]. IFN-γ also polarises macrophages in the TME towards an M1-phenotype that sustains anti-tumour immunity by increasing levels of IL-1β, TNF-α, IL-12, IL-18, IL-23 and enhanced nitric oxide production[56,57]. IFN-γ up-regulates major histocompatibility complex (MHC)- class I on antigen-presenting cells (APCs) through IRF-1 and mediates activation of $CD4^+$ and $CD8^+$ T-cells[58,59]. IFN-γ can also induce apoptosis in cancer cells[59]. IFN-γ up-regulates caspase-8 via the STAT1-IRF1 pathway in tumours resistant to cytotoxic therapies[60]. In pancreatic carcinoma, IRF-1 can initiate caspase-1 activation and trigger caspase-mediated apoptosis[61]. IFN-γ can also induce necroptosis via a ZBP1-RIPK3 complex formed in RIPK1 deficient cells[62,63]. In apoptotic-resistant cancer cells, due to the blocking of caspase activation, IFN-γ synergises with Smac mimetics and induces necroptosis through a caspase-10 and RIPK1 dependent pathway[64,65].

IFN-γ has been implicated in the sensitisation of cancer cells to TRAIL-dependent apoptosis through various mechanisms. IFN-γ induces TRAIL expression in various tumours such as hepatoma[66], neuroblastoma[67], and in Ewing tumours in combination with TNF-α[68], resulting in paracrine and autocrine TRAIL-induced cell death. The upregulation of TRAIL by IFN-γ was mediated through activated STAT1, and IRF-1 and TRAIL upregulation by IFN-γ was blocked by a JAK/STAT pathway inhibitor[69,70]. IFN-γ increased levels of caspase-8 to sensitise various cancer cells[71–76], including colon carcinoma[77] to TRAIL-mediated cell death. IFN-γ, along with TNF-

α, repressed the levels of anti-apoptotic protein Bcl-X_L in resistant colon carcinoma and sensitised them to TRAIL-mediated apoptosis[78]. IFN-γ can also block TRAIL-induced upregulation of anti-apoptotic protein IAP-2 protein and sensitise cells to enhanced TRAIL-induced apoptosis[79]. Though studies have focussed on how IFN-γ can sensitise cells to TRAIL-mediated apoptosis, the synergistic interaction of the two cytokines in modulating pro-inflammatory chemokine profiles in cancer cells has not yet been reported.

In this study, we investigated the molecular mechanisms underpinning the crosstalk and synergistic effects of IFN-γ and TRAIL on colon cancer cell lines and colon cancer organoid cultures. In particular, our group recently reported a Casp8-JAK1/2-STAT1 pathway required for IFN-γ+TNF-α induced cell death of colonic epithelial cells[80], and we wanted to investigate the role of this pathway in IFN-γ+TRAIL induced chemokine secretion and cell death.

4.3. Materials and Methods

4.3.1. Cell Culture

The following human colorectal cancer cell lines were obtained from American Type Culture Collection. DLD-1 (#CCL-221), HCT-15 (#CCL-225) and COLO205 (#CCL-222) cell lines were cultured in RPMI-1640 (#R8758, Sigma-Aldrich); HCT-116 (#CCL-247) and HT-29 (#HTB-38) cell lines in McCoy's 5A medium (#M-9309, Sigma-Aldrich); LS-174T (#CL-188) and RKO (#CRL-2577) cell lines in DMEM medium (#D5796, Sigma-Aldrich), LoVo (#CCL-229) cell line in F-12K nutrient mixture (#21127022, Gibco), and SW-48 (#CCL-231) and SW-948 (#CCL-237) cell lines in Leibovitz L-15 medium (#11415049, Gibco) supplemented with 1.5 g/L sodium bicarbonate (#S8761, Sigma-Aldrich). All media were supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin, and 0.1 mg/mL streptomycin. The cell lines were incubated at 37ºC supplemented with 5% CO_2. The lines were passaged 1-2 times per week and routinely checked for mycoplasma contamination using MycoAlert Mycoplasma Detection Kit (#LT07-118, Lonza).

4.3.2. Generation of HT-29 knockout lines

HT-29 cells were electroporated with gRNA-Cas9 RNP complexes using the Neon Transfection System (#MPK10025, Invitrogen). Alt-R CRISPR-Cas9 crRNA targeting *JAK1*, *JAK2*, and *STAT1* and Alt-R CRISPR-Cas9 tracrRNA labelled with ATTO 550 (#1075928) were purchased from IDT (See Table 4.1 for target sequences). Briefly, 100 µM gRNA duplex was prepared by mixing equimolar amounts of crRNA, and tracrRNA in Duplex Buffer (#11-01-03-01, IDT) followed by incubation at 95 °C for 5 mins. gRNA-Cas9 RNP complexes were prepared by mixing 1.2 µL of gRNA

duplex, 1.7 µL of Atl-R Cas9 nuclease (#1081058, IDT) and 2.1 µL of phosphate buffered saline (PBS) for 20 mins at room temperature (RT). For electroporation, HT-29 cells were harvested by trypsinisation, washed with PBS, and resuspended in PBS at a concentration of $1.0*10^7$ cells/mL. 10 µL of cells were gently mixed with 2.5 µL of the gRNA-Cas9 RNP complexes and electroporated using the Neon Transfection System (Thermo Fisher Scientific) with the following parameters: 1650V, 10ms, 4 pulses. Immediately after electroporation, cells were transferred to 500 µL of prewarmed media and incubated overnight in a 24-well plate. The next day cells were trypsin-harvested, and single-cell sorted using FACSAria Fusion (BD Biosciences). Expanded clones were screened for the knockout by Sanger sequencing with validation by western blotting.

4.3.3. Generation of DLD-1 knockout lines

Single-cell knockout clones of DLD-1 cells were generated by reverse transfection of gRNA-Cas9 RNP complexes using Lipofectamine CRISPRMAX (#CMAX00008, Invitrogen). Alt-R® Cas9 Nuclease was diluted to a working concentration of 1 µM. crRNA and tracrRNA oligos fluorescently labelled with ATTO-550 were mixed in equimolar concentration to a final duplex concentration of 1 µM and heated at 95°C for 5 mins. RNP complex was formed by combining crRNA:tracrRNA complex, Cas9 nuclease, Cas9 Plus Reagent with OptiMEM. After 5 mins of incubation, the donor template was added. Further CRISPR Max reagent (#CMAX00008, Invitrogen) was added and incubated at room temperature (RT) for 20 mins to form the transfection complexes. The transfection complexes were added to the plates, and DLD-1 cells were added on top of the complexes. After 24 hrs of transfection, cells were trypsinised and mixed with FACS buffer (PBS with 2% FBS and 0.09% sodium azide). Single

cells were sorted, and expanded clones were screened for knockout using sanger sequencing and western blotting.

Table 4.1: crRNA sequence

Target	**crRNA sequence**	**Targeting Exon**
JAK1- for HT-29 KO	TCAACGGTGATGGTGCGATT	4
JAK1- for DLD-1 KO	TCGAGATATTCCATGCCGAT	4
JAK2	TCGAGATATTCCATGCCGAT	5
STAT1	TGTGATAGGGTCATGTTCGT	10

4.3.4. Colorectal cancer organoid line culture

The colorectal cancer organoid lines ISO38, ISO50, ISO57, and ISO68 were obtained from Cellesce®. Organoids were cultured using ENAS media prepared as follows: Advanced DMEM/F-12 supplemented with 10 mM HEPES, 2 mM glutamax, 1X N2 supplement (#17502001, Gibco), 1X B27 supplement (#17504044, Gibco), 1 mM N-acetylcysteine (#A9165, Sigma-Aldrich), 50 ng/mL human recombinant EGF (#100-47, PeproTech), 100ng/mL recombinant murine Noggin (#250-38, Peprotech), 10 μM Y-27632 (#S1049, Selleckchem), 10 μM SB-202190 (#S7067, Sigma-Aldrich), 500 nM A-83-01 (SML0788, Sigma-Aldrich), and filtered using the Steriflip vacuum system (Merck-Millipore).

5,000 organoids, in an undifferentiated state, on 10μL domes of Reduced Growth Factor Type 2 BME (#3533-010-02, AMS Biotechnology) were seeded in 96-well plates overlaid with ENAS media and left to recover for 3 days before treatment. Organoids were treated with either IFN-γ, TRAIL or the combination of both as

indicated in the figure. Supernatants were collected after 24 hrs for MSD immunoassay and cell viability was performed using Cell Titre Glo® (#G7573, Promega).

4.3.5. Reagents and Antibodies

Recombinant human IFN-γ (#300-02, Peprotech) and TRAIL (#AG-40T-0002, Adipogen) were used at final concentration of 10 ng/mL and 100 ng/mL respectively, unless otherwise mentioned. The following inhibitors were used: Z-VAD-FMK (Z-VAD) at 20 μM concentration (#14467, Cayman Chemicals), 7-Cl-O-Nec-1 (Nec1s) at 10 μM (#11657, Cayman chemicals), GSK'872 (G'872) at 10 μM (#530389, Merck), necrosulfonamide (NSA) at 1 μM (#20844, Cayman chemicals), upadacitinib at 1 μM (#HY-19569, MedChemExpress), ruxolitinib at 1 μM (#S1378, Selleckchem), baricitinib at 1 μM (#16707, Cayman chemicals) and tofacitinib was used at 10 μM (# T2398, TargetMol). Cells were treated with inhibitors for 30 mins prior to cytokine treatments. For western blotting, the following antibodies were used: anti-DR4 (#42533), anti-DR5 (#8074), anti-DcR1 (#4756), anti-DcR2 (#8049), anti-TRADD (#3684), anti-NFκB p65 (#3034), anti-FADD (#2782), anti-FLIP (#56343), anti-PARP (#9532), anti-RIPK3 (#95702), anti-JAK1 (#3332), anti-JAK2 (#3230), anti-STAT1 (#9172), anti-IRF1 (#8478), anti-Bcl-XL (#2764), anti-MCL1 (#4572) and all were obtained from Cell Signalling Technology and diluted at 1:1000; anti-RIPK1 (0.25 μg/mL, #610459, BD bioscience), anti-Caspase 8 (1 μg/mL,#ALX-804-242-C1, Enzolifesciences), anti-Caspase 10 (0.1 μg/mL, #M059-3, MBL), anti-IFNGR1 (0.1 μg/mL, #sc-12755, Santa Cruz), anti-IFNGR2 (0.58 μg/mL, #ab171081, Abcam), anti-Bcl2 (0.1 μg/mL, #sc-7382, Santa Cruz), anti-β-actin (diluted at 1:10000, #A1978, Sigma-Aldrich), anti-GAPDH (diluted at 1:80000, #am4300, Ambion), secondary antibodies: goat anti- Rabbit (diluted at 1:10000, #A6154), goat anti-mouse

(diluted at 1:10000, #A2554) were obtained from Sigma-Aldrich. All antibodies were diluted in 5% bovine serum albumin (BSA) diluted in Tris buffered saline (TBS) containing 0.1% Tween-20 (TBS-T).

4.3.6. Cell Viability

Cells were seeded in 96-well plates and incubated overnight (seeding densities are provided in table 4.2). Cells were treated with cytokines (alone or in combination) for 72 hrs, as described in figure legends. Cell viability was measured using Cell Titre Glo® (#G7573, Promega) following the manufacturer's instructions, and the luminescence was read using a Synergy plate reader (BioTek).

Table 4.2 Seeding densities of cell lines used

Cell Line	Cell Viability and ELISA- (96-well plate) cells/well	RNA extractions (48-well plate) cells/well	Protein extractions (6-well plate) cells/well	Protein extractions (60mm dishes) cells/well
HT-29	20,000	60,000	$7.5*10^5$	$1.5*10^6$
HT-29 KO lines	20,000	60,000	$7.5*10^5$	$1.5*10^6$
DLD-1	10,000	30,000	$3*10^5$	$0.75*10^6$
DLD-1 KO lines	10,000	30,000	$3*10^5$	$0.75*10^6$
HCT-15	10,000	-	-	$0.75*10^6$
COLO-205	20,000	-	-	$1.5*10^6$
HCT-116	10,000	-	-	$0.75*10^6$
SW-48	20,000	-	-	$1.5*10^6$
SW-948	20,000	-	-	$1.5*10^6$
LoVo	20,000	-	-	$1.5*10^6$
LS-174T	20,000	-	-	$1.5*10^6$
HCA-7	10,000	-	-	$0.75*10^6$
RKO	10,000	-	-	$0.75*10^6$

4.3.7. Chemokine Analysis by ELISA

Supernatants collected after treatment were stored at -20 °C until analysed by ELISA. Human DuoSet ELISA kits for CXCL10 (#DY266), CXCL8 (#DY208), and CXCL1 (#DY275) were purchased from R&D Systems, and the assay was performed according to the manufacturer's instructions. The plates were read using 1 volume of 1-Step™ Ultra TMB-ELISA Substrate Solution (#34028, ThermoFisher) and ½ volume of 1M sulphuric acid (#10325960, Fisher Chemical). The optical density at 450 nm and 570 nm was obtained using a Synergy plate reader (BioTek). The final concentration of the chemokine was obtained by subtracting $OD_{570\text{-blank}}$ from $OD_{450\text{-blank}}$ and extrapolating from the standard curve using the Gen 5 software (BioTek).

4.3.8. Meso Scale Discovery (MSD) immunoassay for chemokine analysis

Supernatants collected after treatment were stored at -20 °C until analysed by MSD. The MSD immunoassay was performed following the manufacturer's instructions. The plates were read in the MESO QuickPlex instrument, and analyte concentrations were extrapolated using the four-parameter logistic fit using the MSD workbench software (Meso scale discovery). The following assay kits were used for the experiments: U-PLEX U-PLEX 5-plex assay- CXCL10, CXCL8, CXCL1.

4.3.9. RNAi mediated gene knockdown

The reverse transfection method was used to perform knockdowns in the HT-29 and DLD-1 cell lines. The siRNAs were obtained from Dharmacon as ON-TARGETplus SMARTpools (see Table 4.3) for - si*JAK1* (#L-003145-00-0005), si*JAK2* (#L-

003146-00-0005), and si*STAT1* (#L-003543-00-005) and used at 25 nM final concentration. As control, ON-TARGETplus non-targeting pool- siCTRL (#D-001810-10-20) was used. An equal volume of 0.25 μM siRNA solution in Opti-MEM was combined with an equal volume of Lipofectamine RNAi Max transfection reagent (#13778150, Invitrogen) diluted 33.33 fold in Opti-MEM. After 20 mins of incubation at room temperature, the transfection solution was added to the plates. Cells were harvested by trypsinisation and resuspended in antibiotic-free media containing 10% FBS. Cells were added (5000 cells/well for 96-well plate, 50,000 cells/well for 12-well plate, and $1.5*10^5$ cells/dish for 6 cm^2 dish) into the wells containing the siRNAs and left for 48 hrs before protein or RNA isolation or treatment with cytokines. Knockdown levels were measured by RT-qPCR and western blotting.

4.3.10. RNA isolation and RT-qPCR

Following RNAi mediated knockdown in a 12-well plate, cells were washed with sterile ice-cold PBS, and total RNA was extracted using RNeasy Mini kit (#74106, Qiagen), including on-column DNA digestion (#79526, Qiagen) according to the manufacturer's protocol. 500 ng of total RNA was reverse transcribed using transcriptor reverse transcriptase (#3531287001, Roche) and random primer (#11034731001, Roche). RT-qPCR was performed on 2.5-10 ng of cDNA with 1X Sensi Mix II probe kit (#BIO-83005, Bioline), 500 nM forward and reverse primers (see Table 4.4) [designed using Probe Library System Assay Design platform (Roche) and purchased from Eurofin genomics] and 250 nM Probe (Roche) in a Light Cycler 480 instrument (Roche). The $2^{-\Delta\Delta CT}$ method was used to calculate fold changes in mRNA expression relative to the geometric mean of the two housekeeping genes- *ACTB* (β-actin) and *GAPDH* [81,82].

4.3.11. Western Blotting

Cells were seeded in 6-well plates or 60mm dishes. After 24 hrs, cells were washed with sterile ice-cold PBS and proteins extracted using RIPA lysis buffer containing 50 mM Tris, pH 8.0, 150 mM sodium chloride, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 1X HALT phosphatase, and protease inhibitor cocktail (#78440, Thermo Scientific) and 0.25 mM AEBSF-HCl serine protease (#S7378, Selleckchem). The protein concentration was determined using the Pierce® BCA assay (#23225, Thermo Scientific) using the manufacturer's protocol. 30 µg of protein were denatured in 1 × Bolt LDS Sample Buffer (#B0007, Invitrogen) supplemented with 1 × Bolt Sample Reducing Agent (#B0009, Invitrogen) by heating at 70 °C for 10 mins. They were separated in Bolt® 4-12% Bis-Tris gels (#NW04125BOX, Invitrogen) followed by transfer to 0.45 micron PVDF membrane (#IPVH00010, Millipore). The membranes were blocked for 1 hr with 5% BSA (#A7906, Sigma-Aldrich) dissolved in TBS-T. Primary antibodies diluted in 5% BSA in TBS-T were added to the membranes and left shaking overnight at 4 ºC. Membranes were washed thrice with TBS-T before adding secondary antibodies for 1 hr at room temperature. The blots were developed using the WesternBright Quantum HRP substrate (#K-12042-D10, Advansta) following the manufacturer's instructions. The images were captured using LAS-3000 imager (Fujifilm). Where required, the brightness/contrast was adjusted for the entire image using ImageJ-win64 software.

4.3.12. Homegenous Time Resolved Fluorescence (HTRF) assay

HTRF assays was performed according to the manufacturer's instructions to detect the following proteins: total STAT1 (#63ADK096PEG, CisBio) and phospho-STAT1

Tyr701 (#63ADK026PEG, CisBio). Colorectal cancer cell lines were seeded in 96 wells and incubated for 24 hrs in 100 µL of media before cytokine treatments. After cytokine treatments, the supernatants were removed, 50 µL of supplemented lysis buffer was added to each well, and the cells were lysed for 30 mins with shaking at room temperature. 16 µL of the lysed sample was transferred to a 384-well plate and mixed with 4 µL of antibody solution mix (containing an equal ratio of provided cryptate and a d2 STAT1 antibody). After overnight incubation of 16 hrs, the plate was read for fluorescence at two different wavelengths- 665 nM and 620 nM using a Synergy plate reader (BioTek). The normalised values were calculated by the following method:

- HTRF Ratio= (Signal at 665 nm * 10^4)/signal at 620 nm
- Normalized value= (phospho-Protein HTRF Ratio) *100/(Total Protein HTRF Ratio)

4.3.13. Statistics

Statistical analysis was carried out using GraphPad Prism version 5 as described in the figure legends. The p-value- $P<0.05$, $P<0.01$, and $P<0.001$ were considered to be statistically significant.

Table 4.3 SMARTpool siRNA sequences

siRNA	**Gene ID**	**Gene Accession**	**siRNA sequence**
siCTRL	-	-	
si*JAK1*	3716	NM_002227.4	• CCACAUAGCUGAUCUGAAA • GACAUGAUAUUGAGAACGA • UUACAAGGAUGACGAAGGA • CGGAUGAGGUUCUAUUUCA
si*JAK2*	3717	NM_004972.3	• CGAAUAAGGUACAGAUUUC • UUACAGAGGCCUACUCAUA • AAUCAAACCUUCUAGUCUU • GGAAUGGCCUGCCUUACGA
si*STAT1*	6772	NM_007315.3	• AGAAAGAGCUUGACAGUAA • GAACCUGACUUCCAUGCGG • CUACGAACAUGACCCUAUC • GCACGAUGGGCUCAGCUUU

Table 4.4: Universal Probe Library Assays

Gene	**Gene ID**	**Probe**	**Forward Primer**	**Reverse Primer**
ACTB	60	11	ATTGGCAATGAGCGGTTC	CGTGGATGCCACAGGACT
GAPDH	2597	60	AGCCACATCGCTCAGACAC	GCCCAATACGACCAAATCC
JAK1	3716	72	CAGTTCATGCTGCTGAAAACC	TCTCTACTTTCCAAAGCTACTTCAGAG
JAK2	3717	50	ACCTGGTGAAAGTCCCATATTC	AGGCCACAGAAAACTTGCTC
STAT1	6772	74	GGATCAGCTGCAGAACTGGT	TTTCTGTTCCAATTCCTCCAA

4.4. Results

4.4.1. Differential sensitivity of a panel of colon cancer cell lines to TRAIL, IFN-γ or the combination of IFN-γ+TRAIL

Over the past 20 years, TRAIL has been shown repeatedly to preferentially kill cancer cells compared to normal untransformed cells[83,84]. We investigated the sensitivity of a panel of colon cancer cell lines to the cytotoxic effects of TRAIL, IFN-γ or the combination of both cytokines. Eleven colon cancer cell lines were screened and classified into four groups depending on their relative sensitivity to TRAIL alone, IFN-γ alone, or the combination of both cytokines. The first group (Group 1) of TRAIL-sensitive but IFN-γ-resistant cell lines consisted of six lines: DLD-1, HCT-15, COLO205, HCT-116, SW-48, SW-948 (Fig 4.1/4.2). Three lines - HCT116, SW-48 and SW-948, were sensitive only to higher concentrations of TRAIL from 25 ng/mL upwards. In HCT-116 and SW-48 cell lines, relative cell viability apparently increased with higher relative ATP levels in response to IFN-γ at 72 hrs only and not at 24 hrs. In DLD-1 and HCT-15 cell lines, co-treatment with IFN-γ and TRAIL reduced cell viability by about 10-20% more compared to the TRAIL treatment alone from a concentration of 12.5 ng/mL and upwards of both cytokines. LoVo was the only cell line screened belonging to Group 2- sensitive to both TRAIL at 24 hrs (Fig 4.3A) and IFN-γ at the longer treatment time of 72 hrs (Fig 4.4A). There was no further reduction in cell viability with IFN-γ+TRAIL in LoVo cells at both the time points investigated. Group 3 cell lines – cell lines where IFN-γ and TRAIL synergized to kill cells - consisted of two cell lines, LS-174T and HT-29. In this group, no effect on cell viability was observed with any individual cytokine treatment at 24 hrs (Fig 4.3B), but at 72 hrs, a synergistic reduction in cell viability upon co-treatment with both

cytokines was observed (Fig 4.4B). This synergism was more pronounced in the HT-29 cell line than in the LS-174T cell line. The last group of cell lines (Group 4) comprised TRAIL and IFN-γ -resistant cell lines, HCA-7 and RKO (Fig 4.3C and Fig 4.4B). These lines were resistant to IFN-γ, TRAIL and IFN-γ+TRAIL even at a higher concentration of 100 ng/mL of both cytokines.

Next, we investigated if the baseline expression of molecular components of the TRAIL and IFN-γ signalling pathways aligned with differential responsiveness of the cell lines to TRAIL, IFN-γ and the combination of IFN-γ+TRAIL in terms of cell viability and/or chemokine production. At the receptor level, the TRAIL-sensitive lines of Group 1 DLD-1 and HCT-15 expressed the highest level of TRAIL-R1 and R2 known for inducing death-receptor mediated apoptotic pathways (Fig 4.5-G1). The other lines in this group also expressed the TRAIL-R1 receptor. Three of the six lines in Group 1 also expressed the decoy receptor for TRAIL- TRAIL-R4/DcR2. In terms of other signalling components downstream of TRAIL/TRAIL-R such as TRADD, RIPK1, NFκB, FADD, caspase-8, caspase-10, FLIP and PARP, variable expression was observed in this group with no consistent pattern or association discernable. RIPK3 expression was detected in three lines - HCT-15, COLO205, and SW-948. The ligand-binding subunit of the receptor for IFN-γ - IFNGR1 - was expressed by all cell lines, with DLD-1 exhibiting the highest expression. HCT-116 and SW-48 lines had higher expression levels of the signalling subunit of the receptor for IFN-γ - IFNGR2 - compared to the other four lines. The expression of JAK1, JAK2, STAT1 and IRF1 differed across the cell lines. The expression of anti-apoptotic proteins, Bcl2, Bcl-X_L, and MCL, was also assessed, and all Group 1 cell lines expressed these proteins, with HCT-15 cells having the highest expression even though it is TRAIL-sensitive. LoVo, the sole member of Group 2, had detectable levels of TRAIL-R1 and TRAIL-R2 (Fig

4.5-G2). The decoy receptor TRAIL-R4/DcR2 was not detected. Expression of other components of the TRAIL pathway was detected in LoVo cells, with RIPK3 being the least detected. LoVo cells expressed both IFN-γ receptors, IFNGR1 and IFNGR2, and the downstream signalling components JAK1, JAK2, STAT1 and IRF1. It expressed higher levels of Bcl2 protein, while Bcl-X_L, and MCL, were at comparable levels with other groups. In Group 3 cell lines, while LS-174T cells expressed low TRAIL-R1 and TRAIL-R2 levels, HT-29 cells expressed higher TRAIL-R2 than the rest of the cell lines (Fig 4.5-G3). The expression of the decoy receptor TRAIL-R4/DcR2 was highest in LS-174T cells compared to all the other eleven cell lines, while HT-29 cells had no detectable amount of protein. Levels of RIPK1, NF-κB, and FADD were higher in HT-29 cells compared to LS-174T and other cell lines. Expression of TRADD, caspase-8, caspase-10, FLIP, PARP, and RIPK3 was also detected in cell lines belonging to this group. The receptor subunits for IFN-γ - IFNGR1 and IFNGR2 - were also detected in both these Group 3 lines. Expression of JAK1, JAK2, STAT1, and IRF1 was high in HT-29 compared with LS-174T or cell lines of other groups. While Bcl2 expression was not detected in HT-29 cells, other anti-apoptotic proteins were expressed in Group 3 cell lines. Interestingly, the TRAIL-resistant Group 4 cell lines expressed higher levels of the death receptor TRAIL-R1 (Fig 4.5-G4). However, these lines also expressed detectable levels of the decoy receptor TRAIL-R4, with RKO expressing the highest levels compared to the rest of the cell lines screened. These lines had no detectable levels of RIPK3, while all other components of the TRAIL pathway were expressed. While IFNGR-1 was not detected by WB, these lines expressed IFNGR2, JAK1, JAK2, STAT1, and IRF1. Both HCA-7 and RKO had the highest expression levels of the anti-apoptotic protein MCL-1.

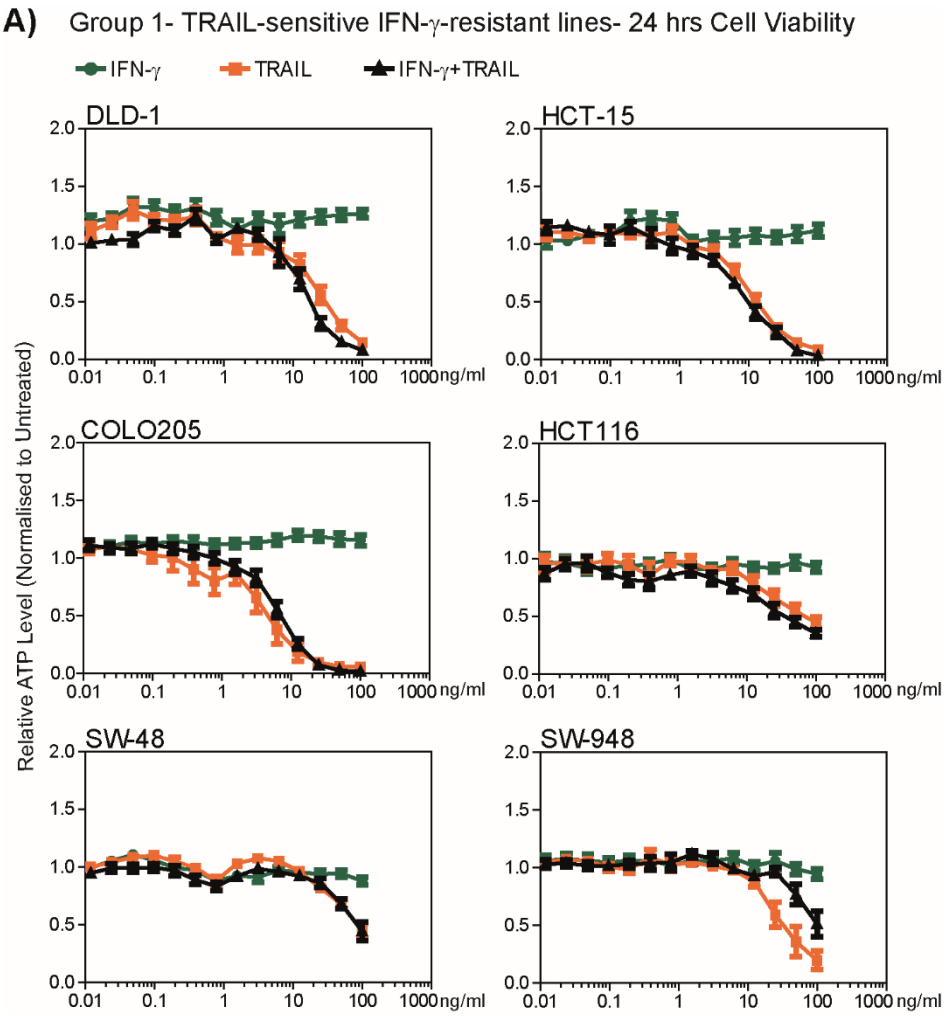


Figure 4.1: Colon cancer cell lines sensitive to TRAIL but resistant to IFN-γ at 24 hrs post-cytokine treatment

Human colon cancer cell lines belonging to **(A)** Group 1- TRAIL-sensitive IFN-γ resistant lines; cell lines were treated with different concentrations of IFN-γ, TRAIL, or the combination of both cytokines for 24 hrs. Relative cell viability to untreated cells (UT) was measured using CellTitre-Glo®. Data are represented as mean ± SEM from three independent experiments done in duplicates.

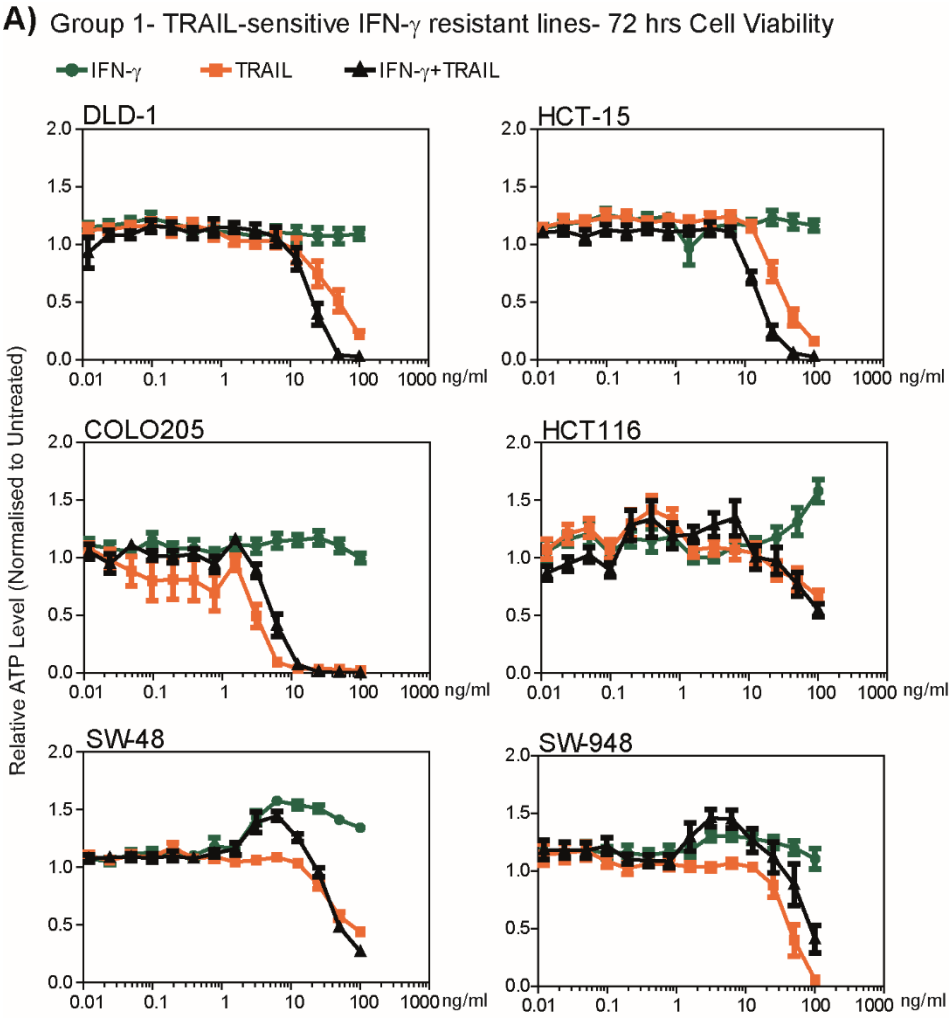


Figure 4.2: Colon cancer cell lines sensitive to TRAIL but resistant to IFN-γ at 72 hrs post-cytokine treatment

Human colon cancer cell lines belonging to **(A)** Group 1- TRAIL-sensitive IFN-γ resistant lines; cell lines were treated with different concentrations of IFN-γ, TRAIL, or the combination of both cytokines for 24 hrs. Relative cell viability to untreated cells (UT) was measured using CellTitre-Glo®. Data are represented as mean ± SEM from three independent experiments done in duplicates.

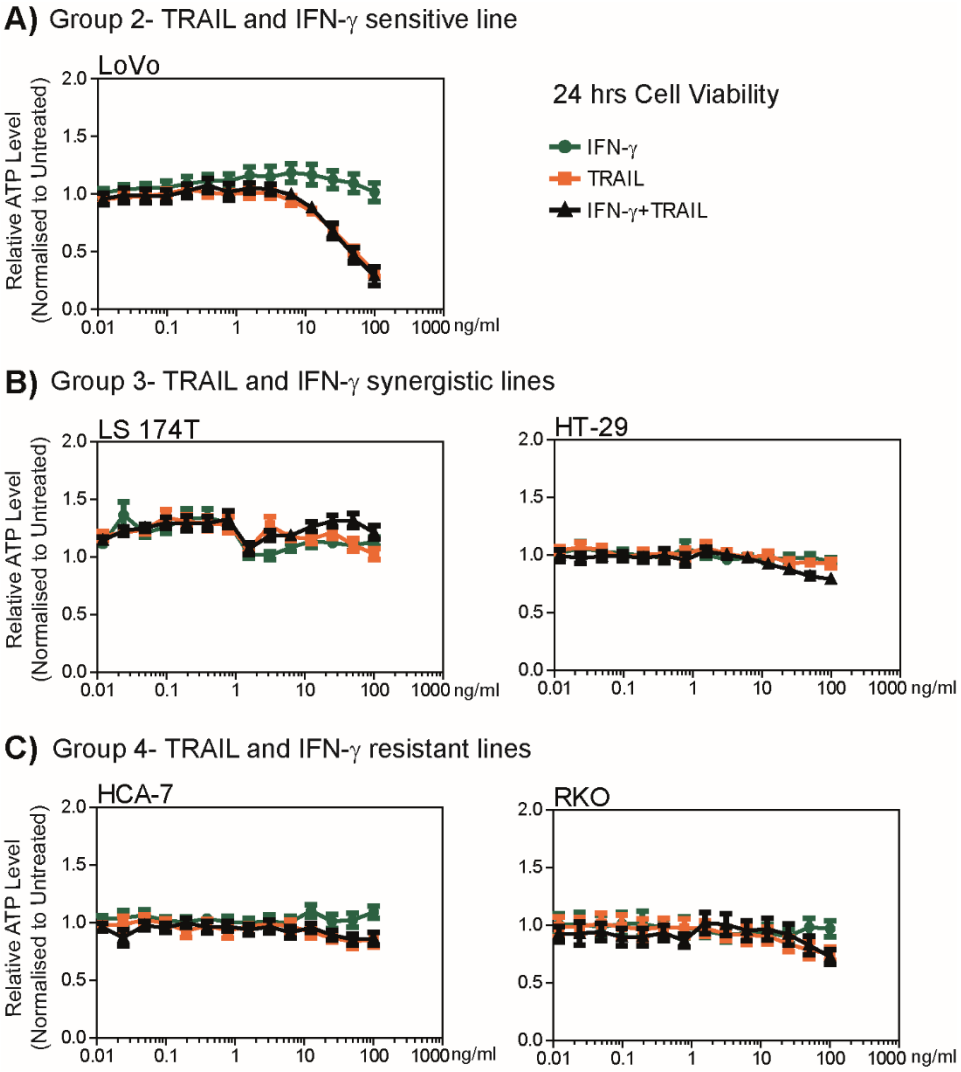


Figure 4.3: Colon cancer cell lines exhibit differential sensitivity to IFN-γ and TRAIL at 24 hrs post cytokine treatment

Human colon cancer cell lines were treated with different concentrations of IFN-γ, TRAIL, or their combination for 24 hrs. Relative cell viability to untreated cells (UT) was measured using CellTitre-Glo®, and the response to the cytokine treatment was divided into (**A**) Group 2- TRAIL and IFN-γ sensitive cell lines (**B**) Group 3- TRAIL and IFN-γ synergistic cell lines and (**C**) Group 4- TRAIL and IFN-γ-resistant cell lines. Data are represented as mean ± SEM from three independent experiments in duplicates.

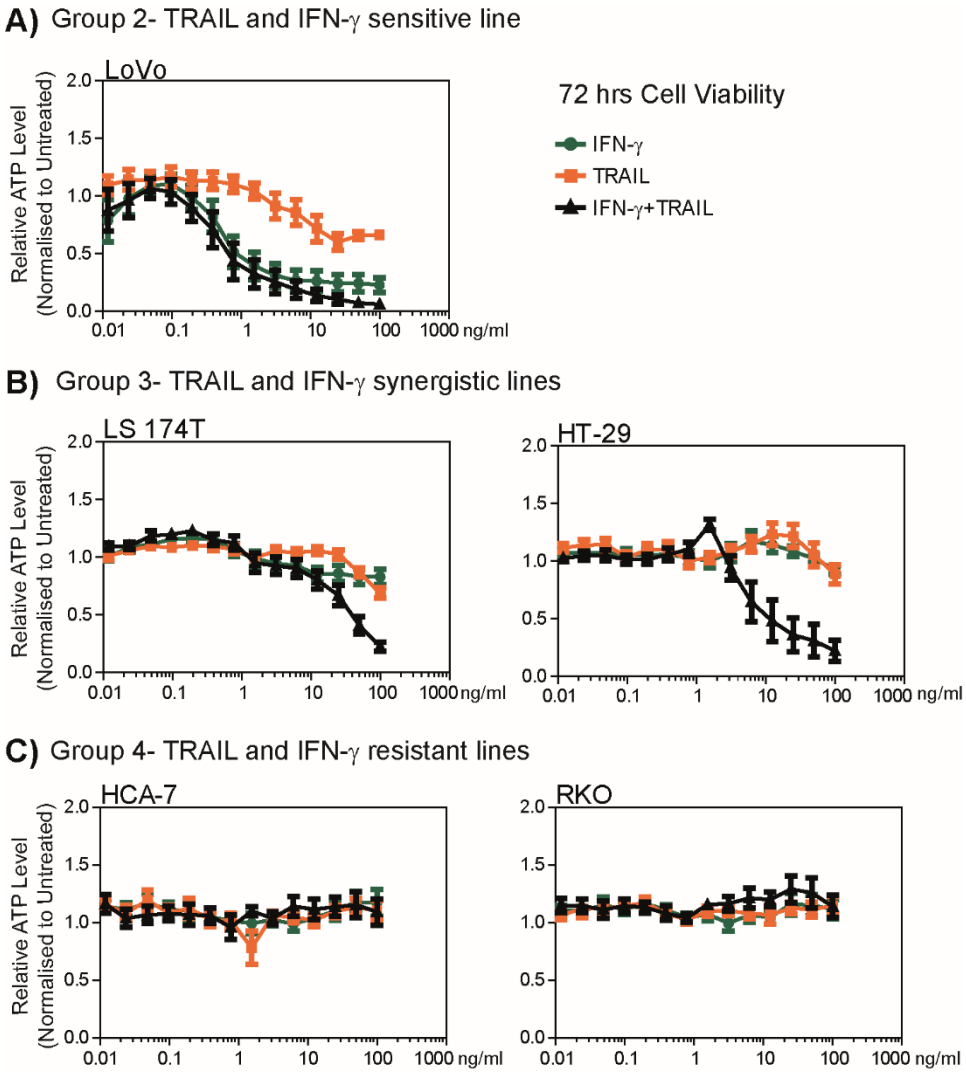


Figure 4.4: Colon cancer cell lines exhibit differential sensitivity to IFN-γ and TRAIL at 72 hrs post cytokine treatment

Human colon cancer cell lines were treated with different concentrations of IFN-γ, TRAIL, or their combination for 72 hrs. Relative cell viability to untreated cells (UT) was measured using CellTitre-Glo®, and the response to the cytokine treatment was divided into (**A**) Group 2- TRAIL and IFN-γ sensitive cell lines (**B**) Group 3- TRAIL and IFN-γ synergistic cell lines and (**C**) Group 4- TRAIL and IFN-γ-resistant cell lines. Data are represented as mean ± SEM from three independent experiments in duplicates.

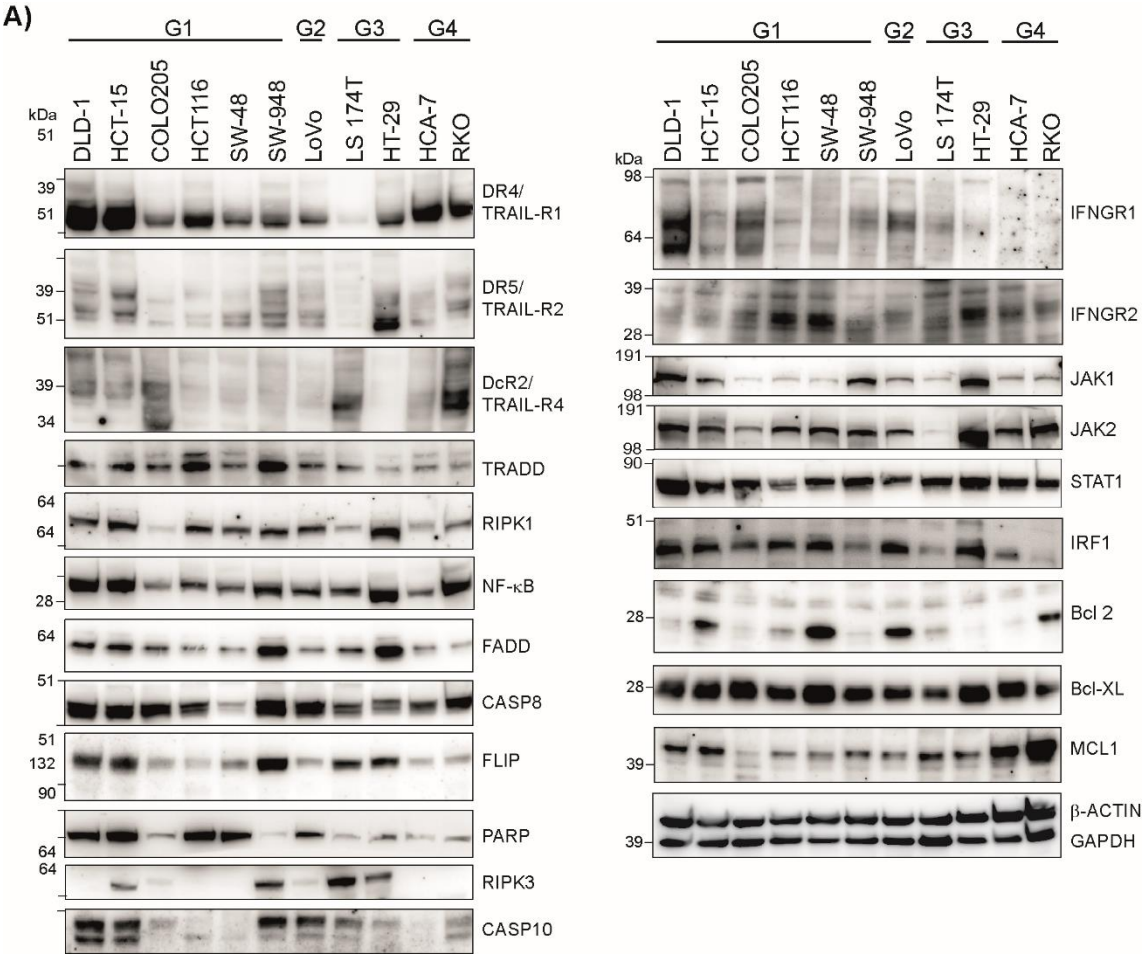


Figure 4.5: Baseline expression levels of components of the TRAIL and IFN-γ signalling pathways in a panel of colon cancer cell lines

Western blots for proteins from the IFN-γ and TRAIL signalling pathways at baseline in the human colon cancer cell lines in addition to anti-apoptotic proteins. 50 µg of total protein was loaded onto each well. β-Actin and GAPDH were used as the housekeeping markers. The blots shown are representative of two independent experiments.

4.4.2. TRAIL and IFN-γ induced inflammatory chemokine signature in colon cancer cell lines is not aligned with their effects on cell viability

Previous studies have reported that TRAIL can induce the production of inflammatory and pro-tumourigenic chemokines selective for myeloid cell and granulocyte cell populations in cancer cell lines[21,45]. We investigated if TRAIL alone or in combination with IFN-γ could induce the Th1 inflammatory and anti-tumourigenic chemokine-CXCL10 in addition to previously reported pro-tumorigenic chemokines - CXCL8 and CXCL1 - in these colon cancer cell lines. IFN-γ treatment induced CXCL10 production in six of eleven cell lines, falling into all four (Groups 1-4) cell viability sensitivity categories. These lines included the TRAIL sensitive HCT-15 and SW-948 lines from Group 1 and LoVo from Group 2; TRAIL-resistant LS-174T and HT-29 lines from Group 3 and HCA-7 from Group 4 (Fig 4.3A). In all these cell lines, co-treatment of TRAIL with IFN-γ synergistically increased the levels of CXCL10. In terms of CXCL8 production, IFN-γ did not affect the production of CXCL8 except in HCA-7, where IFN-γ reduced the levels of CXCL8 compared to untreated cells (Fig 4.6B). In contrast, TRAIL increased CXCL8 production in seven cell lines with a significant increase in 3 lines- HCT-116 from Group 1, LoVo from Group 2 and HT-29 from Group 3 (Fig 4.6B). In these same three lines, co-treatment of cells with IFN-γ and TRAIL resulted in decreased production of CXCL8. In TRAIL-resistant HCA-7, treatment with TRAIL reduced CXCL8 levels compared to untreated cells, and combined treatment of IFN-γ and TRAIL further reduced CXCL8 levels (Fig 4.6B). Investigating the levels of CXCL1, only TRAIL treatment of cell lines belonging to TRAIL-resistant Group 3- LS-174T and HT-29 resulted in increased production of

CXCL1 (Fig 4.6C). Treatment with IFN-γ either alone or in combination with TRAIL had no significant effects on the levels of CXCL1. In SW-948 cells belonging to Group 1, there was a synergistic induction of CXCL1 production when cells were treated with the combination of IFN-γ and TRAIL (Fig 4.6C).

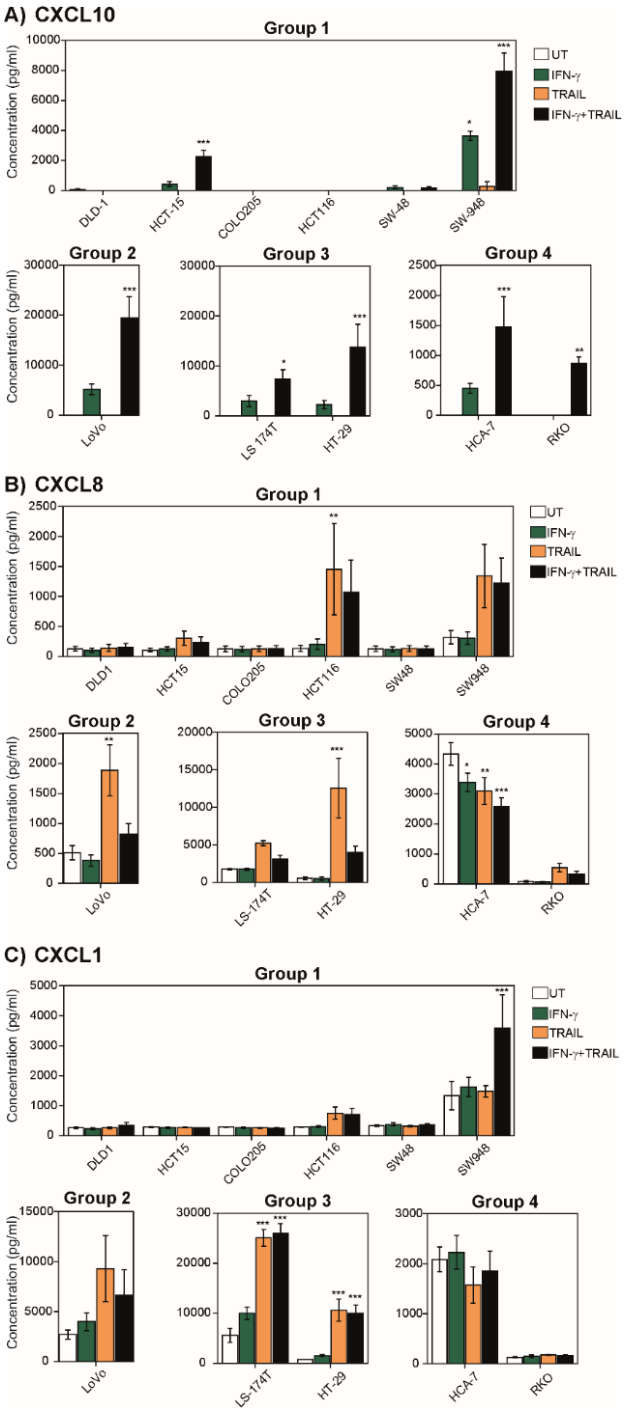


Figure 4.6: IFN-γ and TRAIL induce different inflammatory chemokine signatures in a panel of colon cancer cell lines

Human colon cancer cells were treated with 100 ng/mL of IFN-γ, TRAIL, or a combination of both for 24 hrs followed by measurement of the release of **(A)** CXCL10, **(B)** CXCL8, and **(C)** CXCL1 by ELISA. Group 1- TRAIL-sensitive IFN-γ resistant lines; Group 2- TRAIL and IFN-γ sensitive lines; Group 3- TRAIL and IFN-γ synergistic lines; Group 4- TRAIL

and IFN-γ-resistant lines. Data are represented as mean ± SEM from three independent experiments in duplicates. *p<0.05, **p<0.01, ***p<0.001, students t-test with untreated (UT).

4.4.3. IFN-γ and TRAIL induced synergistic cell death is independent of canonical death-receptor mediated apoptotic and necroptotic pathways

Of the four groups of colon cancer cell lines, Group 3 (TRAIL and IFN-γ synergistic) cell lines were of interest to us for further study. These cell lines were resistant to individual cytokine treatment alone, even at the highest concentration of 100 ng/mL used but were sensitive to the combination of cytokines at a ten-fold lower concentration. In these cell lines, cytokines also synergistically affected inflammatory chemokine production. We investigated the mechanisms underpinning synergy between IFN-γ and TRAIL in the HT-29 cell line as our lab has previously investigated and published on the synergy between IFN-γ and TNF-α in this cell line and because we have also developed and acquired various HT-29 knockout cell lines for components of canonical cell death pathways[80]. Also, as a prominent death ligand, TRAIL has been shown to induce apoptosis in various cell lines through the caspase-8-mediated apoptotic pathway[85]. Initially, we investigated the role of caspase-8 canonical signalling in the response of HT-29 cells to IFN-γ and TRAIL by blocking caspase-8 activity using the pan-caspase inhibitor z-VAD. Z-VAD could block only 5% of the IFN-γ+TRAIL induced cell death and this was not significant (Fig 4.7A). Previous reports have also consistently shown that blocking caspase-8 enzymatic activity in the context of death receptor activation induces RIPK1/RIPK3/MLKL-necroptosis in response to TRAIL[86,87] as well as inducing IFN-γ dependent necroptosis through a RIPK3/ZBP1 signalling cascade[63]. Because of this, we also included the RIPK1 inhibitor- Nec1s, RIPK3 inhibitor GSK'872 and the MLKL inhibitor necro sulfonamide (NSA) in our perturbation experiments. Blocking of necroptosis using

these inhibitors either in the absence or presence of caspase inhibition with z-VAD did not have any effect on IFN-γ+TRAIL-induced cell death (Fig 4.7A). Even the combination of z-VAD, GSK'872 and NSA failed to block IFN-γ+TRAIL-induced cell death.

Analysing the chemokines produced, IFN-γ induced CXCL10 production in HT-29 cells (Fig 4.7B). IFN-γ+TRAIL increased the levels of CXCL10 by four-fold compared to the IFN-γ treatment alone. Blocking of RIPK1, RIPK3 or MLKL did not affect IFN-γ+TRAIL induced CXCL10. The combined blockade of RIPK3 and MLKL reduced the levels of CXCL10 at least by 50% compared to that produced by IFN-γ+TRAIL treatment. Inhibition of caspases has been shown to promote the recruitment of RIPK1 to TRAIL receptors as well as TRAIL-induced NF-κB activation leading to higher chemokine production[20,23]. Consistent with this, we found that pre-treatment of HT-29 with z-VAD along with the IFN-γ+TRAIL +/- the inhibitors further increased the level of CXCL10. The production of tumour promoting CXCL8 and CXCL1 were similar, with TRAIL treatment alone inducing their expression (Fig 4.7B). Co-treatment with IFN-γ did not alter the levels of TRAIL-induced CXCL8 or CXCL1. The inhibitors did not affect the levels of these chemokines except for z-VAD, which increased the expression of the chemokines. Interestingly though IFN-γ alone did not produce a significant amount of CXCL8 or CXCL1, IFN-γ+z-VAD had significantly higher levels of CXCL-8 and CXCL1.

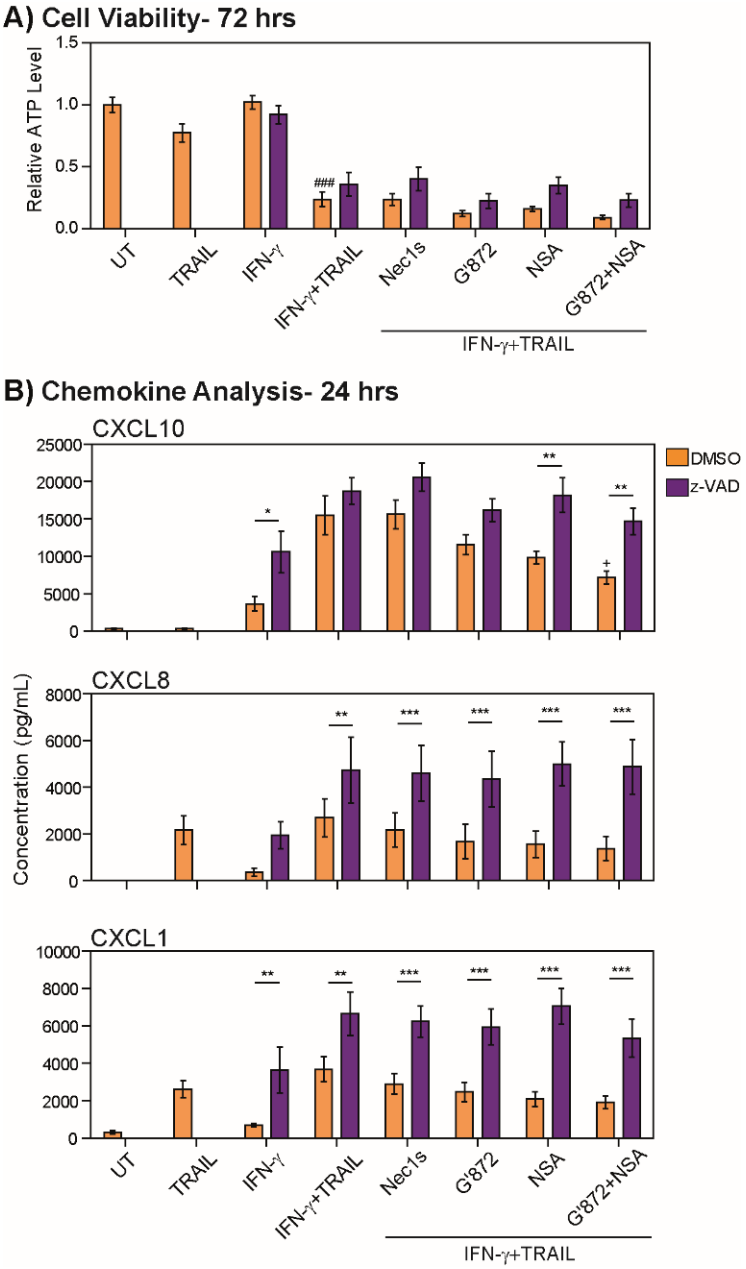


Figure 4.7: IFN-γ and TRAIL induced synergistic cell death is independent of canonical apoptotic or necroptotic cell death pathways

HT-29 cells were pre-treated with vehicle control (DMSO), 20 µM pan-caspase inhibitor z-VAD, 10 µM RIPK1 inhibitor Nec1s, 10 µM RIPK3 inhibitor GSK872, or/and 1 µM MLKL inhibitor necrosulfonamide (NSA) for 30 mins and stimulated with 10 ng/mL of IFN-γ, 100 ng/mL of TRAIL or a combination of both cytokines. **(A)** Cell viability was assessed after 72 hrs of cytokine treatment using CellTiter-Glo® **(B)** chemokine production was measured after 24 hrs of treatment using ELISA. Data are presented as mean ± SEM from three independent

experiments done in triplicates. *p<0.05, **p<0.01, ***p<0.001, #- one-way ANOVA with Tukey's multiple comparison test with UT (untreated), *- two-way ANOVA with Bonferroni post-test comparing DMSO vs z-VAD

To further investigate and validate these perturbation experiments with selective inhibitors, we obtained HT-29 knockout lines from Professor John Silke, The Walter and Eliza Hall Institute of Medical Research (WEHI), defective in key components of canonical death receptor apoptotic and necroptotic pathways. The knockout phenotype of the specific HT-29 derived cell lines was confirmed by western blotting (Fig 4.8A) and sequencing (data not shown).

The combination of IFN-γ+TRAIL reduced cell viability by about 90% in the HT-29 parental wildtype cells (Fig 4.8B). Knockout of caspase-8 (*CASP8*$^{-/-}$) partially blocked (40%) IFN-γ+TRAIL induced cell death. RIPK1 KO (*RIPK1*$^{-/-}$) made cells more susceptible to IFN-γ+TRAIL induced cell death. Knockout of caspase-10 (*CASP10*$^{-/-}$) or RIPK3 (*RIPK3*$^{-/-}$) had no additional effect on cell viability compared to the parental wildtype cells. Pre-treatment with z-VAD increased sensitivity to IFN-γ (20%) in all lines except *CASP8*$^{-/-}$ cells (Fig 4.8B). Cell death was inhibited by 30% in *RIPK1*$^{-/-}$ and *RIPK3*$^{-/-}$ HT-29 lines treated with z-VAD+IFN-γ+TRAIL.

With regards chemokine regulation, knockout of caspase-8, caspase-10, and RIPK1 significantly affected CXCL10 produced by IFN-γ+TRAIL but not CXCL-10 produced by IFN-γ treatment alone (Fig 4.8C). Pre-treatment with z-VAD increased CXCL10 levels produced by IFN-γ+TRAIL only in the *CASP10*$^{-/-}$ line. Knockout of RIPK1 significantly lowered the levels of CXCL8 produced by TRAIL alone or IFN-γ+TRAIL treatment. Pre-treatment with z-VAD increased levels of TRAIL-induced CXCL8 in wildtype, *CASP10*$^{-/-}$ and *RIPK3*$^{-/-}$ lines. Knockout of all components reduced the levels of CXCL1 in both TRAIL and IFN-γ+TRAIL treated cells, though significant only for the latter. Pre-treatment with z-VAD, lowered levels of CXCL1 in IFN-γ+TRAIL treated parental wildtype line but increased the levels of CXCL1 in IFN-γ+TRAIL treated *CASP10*$^{-/-}$ and *RIPK3*$^{-/-}$ lines. Overall, these results suggest

caspase-8 is required for IFN-γ and TRAIL induced synergistic cell death of HT-29 cells. The cell death is independent of canonical death receptor mediated apoptosis and necroptosis. Also, RIPK1 is required for TRAIL and IFN-γ+TRAIL induced pro-inflammatory chemokine production, especially for the neutrophil chemoattractants.

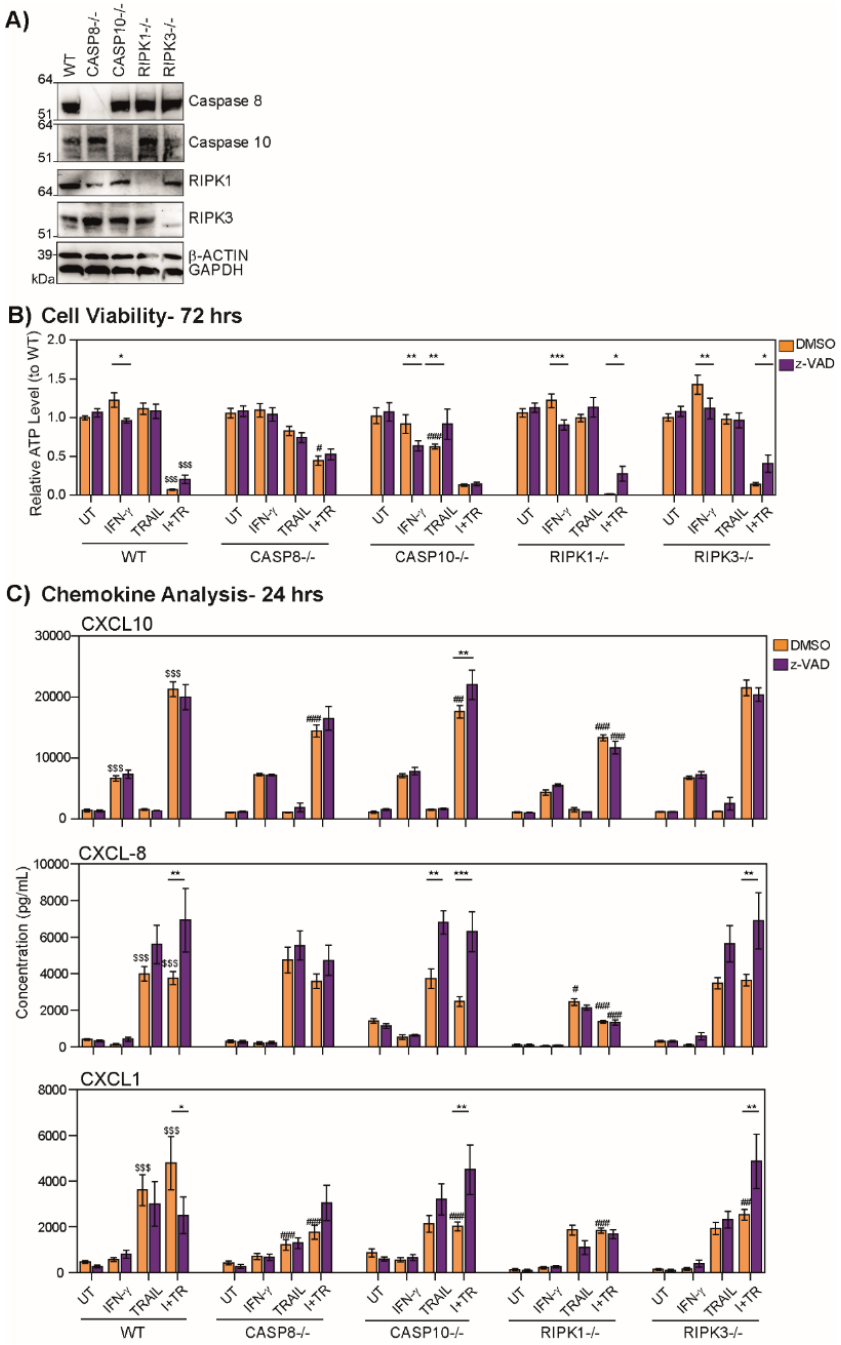


Figure 4.8: IFN-γ and TRAIL induced synergistic cell death is partially dependent on caspase-8

(A) Validation of HT-29 knockout cell lines *CASP8*$^{-/-}$, *CASP10*$^{-/-}$, *RIPK1*$^{-/-}$ and *RIPK3*$^{-/-}$ by western blotting. Knockout cell lines were pre-treated with the 20 μM pan-caspase inhibitor z-VAD or vehicle control DMSO for 30 mins and stimulated with 10 ng/mL of IFN-γ, 100 ng/mL of TRAIL or a combination of both cytokines. **(B)** Cell viability was assessed after 72 hrs of cytokine treatment using CellTiter-Glo® **(C)** chemokine production was measured after

24 hrs of treatment using ELISA. Data are represented as mean ± SEM from three experiments in triplicates. *p<0.05, **p<0.001, ***p<0.0001, $- one-way ANOVA with Tukey's multiple comparison test with UT; #- one-way ANOVA with Tukey's multiple comparison test compared to WT counterpart, *- two-way ANOVA with Bonferroni post-tests comparing DMSO vs z-VAD.

4.4.4. Synergistic cell death induced by the combination of IFN-γ and TRAIL is dependent on the JAK1/2-STAT1 pathway

Recent work in our lab identified and reported that TNF-α synergised with IFN-γ to induce cell death in HT-29 cells and primary colonic organoid lines via a caspase-8-JAK1/2-STAT1 pathway[80]. We, therefore, investigated if TRAIL also synergised with IFN-γ in a similar manner. Our initial analysis in HT29 KO lines indicated a dependency on caspase-8 for IFN-γ+TRAIL induced synergistic cell death, so next, we investigated the involvement of the JAK1/2-STAT1 pathway using selective small molecule inhibitors for JAK1/2 and *JAK1*, *JAK2*, and *STAT1* KO lines. Upadacitinib, targeting JAK1, blocked 70% of IFN-γ+TRAIL induced cell death at a concentration of 1 μM (Fig 4.9A). JAK1/2 inhibitors ruxolitinib and baricitinib blocked about 60% of IFN-γ+TRAIL induced cell death even at a concentration of 10 μM. The least effective JAK inhibitor was tofacitinib, targeting JAK1 and JAK3, with only 40% rescue even when used at 10 μM concentration. Inhibition of JAK1/2 reduced the production of IFN-γ+TRAIL induced Th1 chemokine CXCL10 in a dose-dependent manner (Fig 4.9B). In contrast, inhibiting JAK1/2 enhanced the expression of CXCL8 while there was no effect on CXCL1 levels.

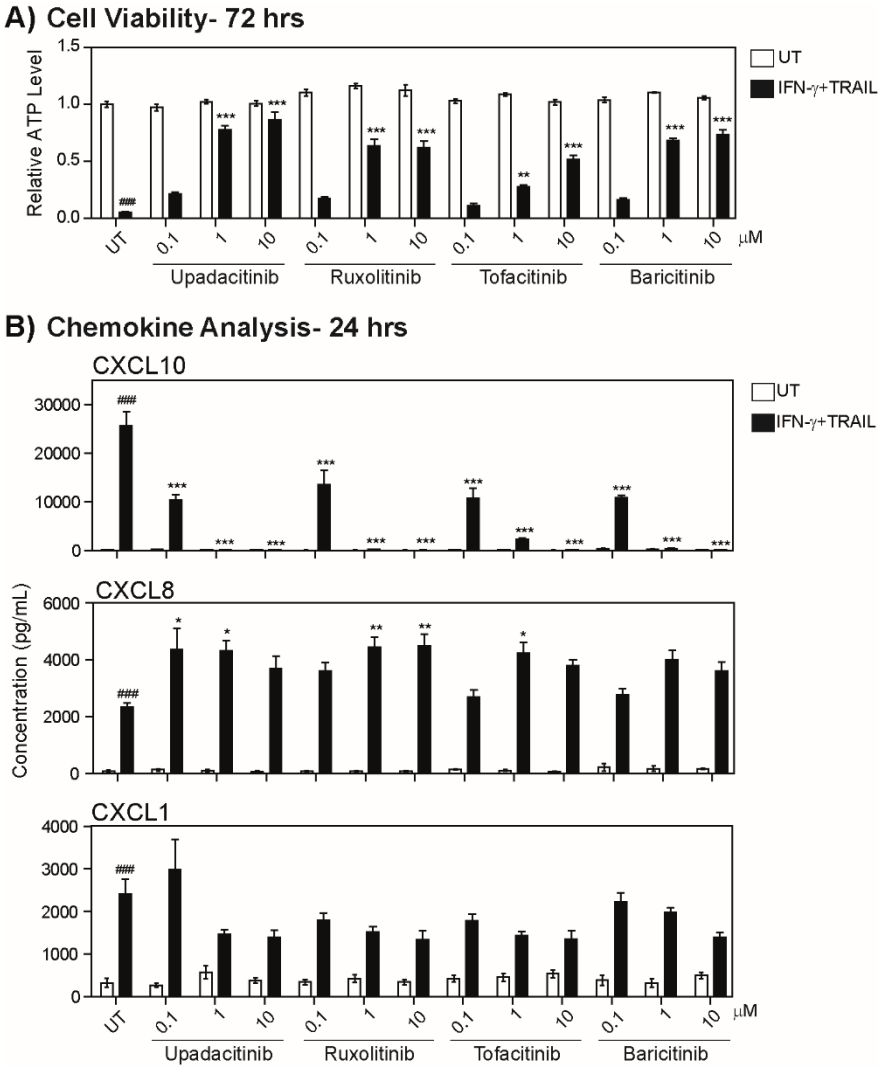


Figure 4.9: JAK inhibitors rescue HT-29 cells from IFN-γ and TRAIL induced cell death

HT-29 cells were pre-treated with different concentrations of JAK inhibitors (Upadacitinib, Ruxolitinib, Tofacitinib and Baricitinib) as indicated for 30 mins and treated with the combination of 10 ng/mL IFN-γ and 100 ng/mL TRAIL. **(A)** Cell viability was assessed after 72 hrs of cytokine treatment using CellTiter-Glo® **(B)** Chemokine production was measured after 24 hrs of treatment using ELISA. Data are represented as mean ± SEM from three experiments done in triplicates. *p<0.05, **p<0.01, ***p<0.001; # - one-way ANOVA with Tukey's multiple comparison test with untreated (UT); * - one-way ANOVA with Tukey's multiple comparison test with IFN-γ+TRAIL.

Next, we investigated the effect of individual knockdown of JAK1, JAK2, and STAT1 on IFN-γ+TRAIL induced responses. Knockdown of JAK1, JAK2, and STAT1 of more than 80% was achieved by reverse transfection of siRNAs and was confirmed by RT-qPCR (Fig 4.10A) and western blotting (Fig 4.10B). Interestingly, transfection of HT-29 cells with siCTRL non-targeting siRNAs made HT-29 cells sensitive to TRAIL, which was inhibited by the addition of the pan-caspase inhibitor z-VAD (Fig 4.10C). Knocking down JAK1, JAK2 and STAT1 made cells more sensitive to TRAIL, but this was blocked with the pretreatment of z-VAD. In contrast, the knockdown of JAK1, JAK2, STAT1 partially rescued cells from IFN-γ+TRAIL-induced synergistic cell death. The addition of z-VAD to these individual JAK1 and JAK2, knockdowns inhibited IFN-γ+TRAIL-induced cell death by 60%. STAT1 knockdown in combination with z-VAD could inhibit IFN-γ+TRAIL-induced synergistic cell death completely (Fig 4.10C).

Knocking down JAK1, JAK2 or STAT1 completely blocked IFN-γ-induced CXCL10 production and reduced IFN-γ+TRAIL-induced CXCL10 levels (Fig 4.10D). However, there was no significant effect on the levels of TRAIL or IFN-γ+TRAIL induced CXCL8 or CXCL1 (Fig 4.10D).

We then performed experiments with knockout lines for JAK1, JAK2 and STAT1 to see if the RNAi knockdown results could be confirmed by gene knockout. The knockout lines were created inhouse using the CRISPR-cas9 method and the phenotype was confirmed by western blotting (Fig 4.11A) and sanger sequencing (data not shown). HT-29 parental lines were sensitive to TRAIL with 30% reduction in cell viability (Fig 4.11B). Knockout of JAK1, JAK2, STAT1 further sensitised cells to TRAIL and had 40% reduction in cell viability compared to the parental line. Pre-treatment with z-VAD alone could completely block cell death induced by TRAIL

alone. About 20% increase in cell viability was achieved in *JAK1*$^{-/-}$ and *JAK2*$^{-/-}$ cells treated with IFN-γ+TRAIL compared to that of the parental line (Fig 4.11B). A higher increase in cell viability (about 60%) was observed in *STAT1*$^{-/-}$ compared to parental when treated with IFN-γ+TRAIL (Fig 4.11B). z-VAD addition completely blocked IFN-γ+TRAIL induced cell death in *STAT1*$^{-/-}$ and about 80% in *JAK1*$^{-/-}$ and 90% in *JAK2*$^{-/-}$ compared to parental (Fig 4.11B).

As seen with the knockdowns, knock out of the JAK1/2-STAT1 pathway in *JAK1*$^{-/-}$, *JAK2*$^{-/-}$ and *STAT1*$^{-/-}$ HT-29 lines completely blocked IFN-γ-induced CXCL10 production (Fig 4.11C). In *JAK2*$^{-/-}$ and *STAT1*$^{-/-}$ but not *JAK1*$^{-/-}$ lines, CXCL10 was also completely blocked in response to IFN-γ+TRAIL. Similar to knockdowns, knockout of JAK1, JAK2 and STAT1 did not significantly affect the levels of TRAIL-induced CXCL8 (Fig 4.11C). Knockout of JAK1/2-STAT1 pathway in *JAK1*$^{-/-}$, *JAK2*$^{-/-}$ and *STAT1*$^{-/-}$ lines in combination with z-VAD reduced levels of IFN-γ+TRAIL induced CXCL1 compared to the parental line (Fig 4.11C).

We then investigated the effect of JAK1/2-STAT1 pathway inhibition using JAK inhibitors - upadacitinib, ruxolitinib, tofacitinib and baricitinib - in cells devoid of canonical TRAIL signalling pathway components – caspase-8, caspase-10, RIPK1 and RIPK3. Interestingly, we observed complete inhibition of cytokine-induced cell death in *RIPK1*$^{-/-}$ and *RIPK3*$^{-/-}$ lines in the presence of JAK inhibitors, whereas in *CASP8*$^{-/-}$ and *CASP10*$^{-/-}$, this could not be achieved even in the presence of JAKinibs (Fig 4.12A). In all lines, CXCL10 production by IFN-γ+TRAIL was blocked entirely by the JAK inhibitors, no effect was observed on the production levels of pro-tumorigenic CXCL8 and CXCL1 (Fig 4.12B).

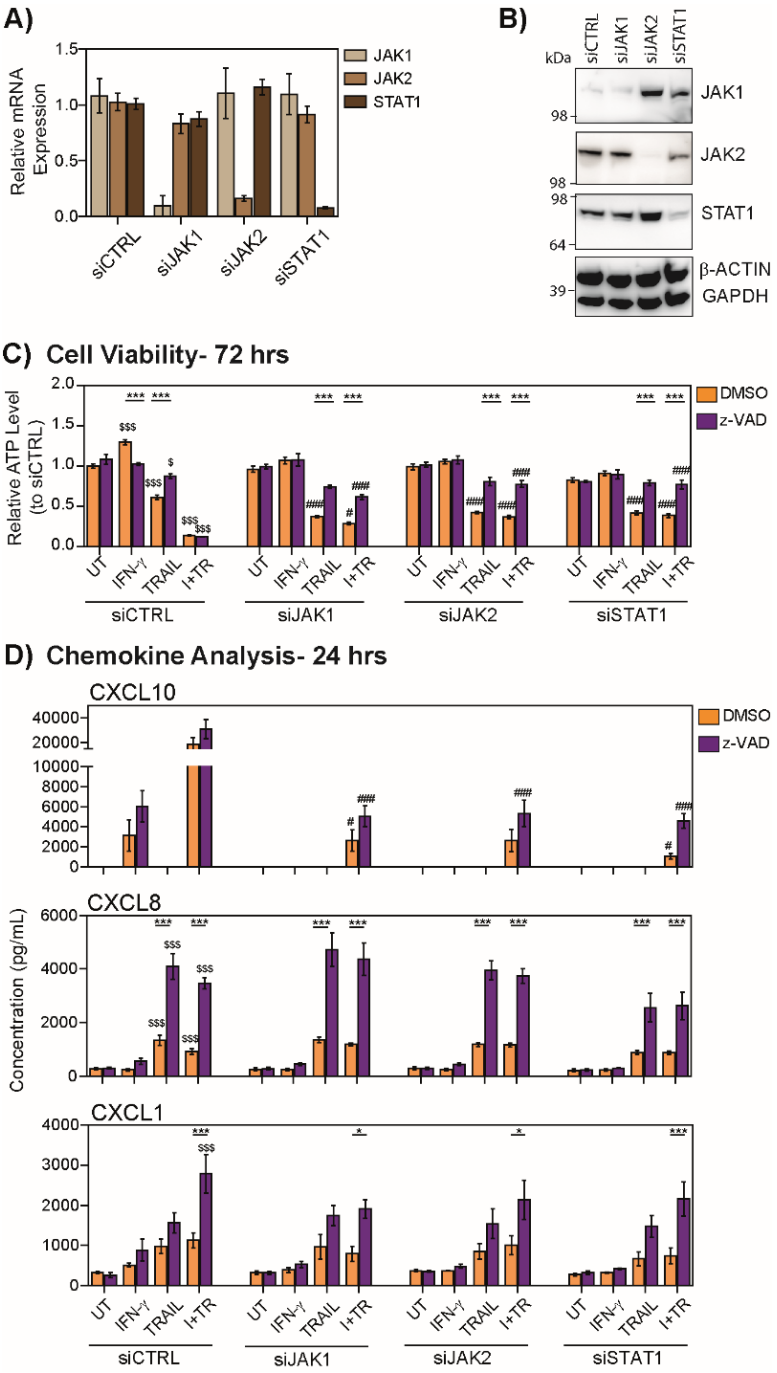


Figure 4.10: Knockdown of JAK1, JAK2 or STAT1 inhibits IFN-γ+TRAIL induced cell death and CXCL10 chemokine production

Validation of *JAK1*, *JAK2*, and *STAT1* knockdowns by **(A)** qRT-PCR and **(B)** western blotting. HT-29 knockdown cells (si*JAK1*, si*JAK2*, and si*STAT1)* and control (siCTRL) were treated with either vehicle control DMSO or 20 µM z-VAD for 30 mins and further challenged with 10 ng/mL IFN-γ, 100 ng/mL TRAIL, or a combination thereof. **(C)** Cell viability was

assessed after 72 hrs cytokine treatment by CellTitre-Glo® **(D)** Chemokines were analysed after 24 hrs of cytokine treatments by ELISA. Data are represented as mean ± SEM from three experiments done in triplicates. *p<0.05, **p<0.01, ***p<0.001, $- one-way ANOVA with Tukey's multiple comparison test with untreated (UT); # - one-way ANOVA with Tukey's multiple comparison test compared to siCTRL counterpart; * - two-way ANOVA with Bonferroni post-tests comparing DMSO vs z-VAD. Blots are representative of three independent experiments

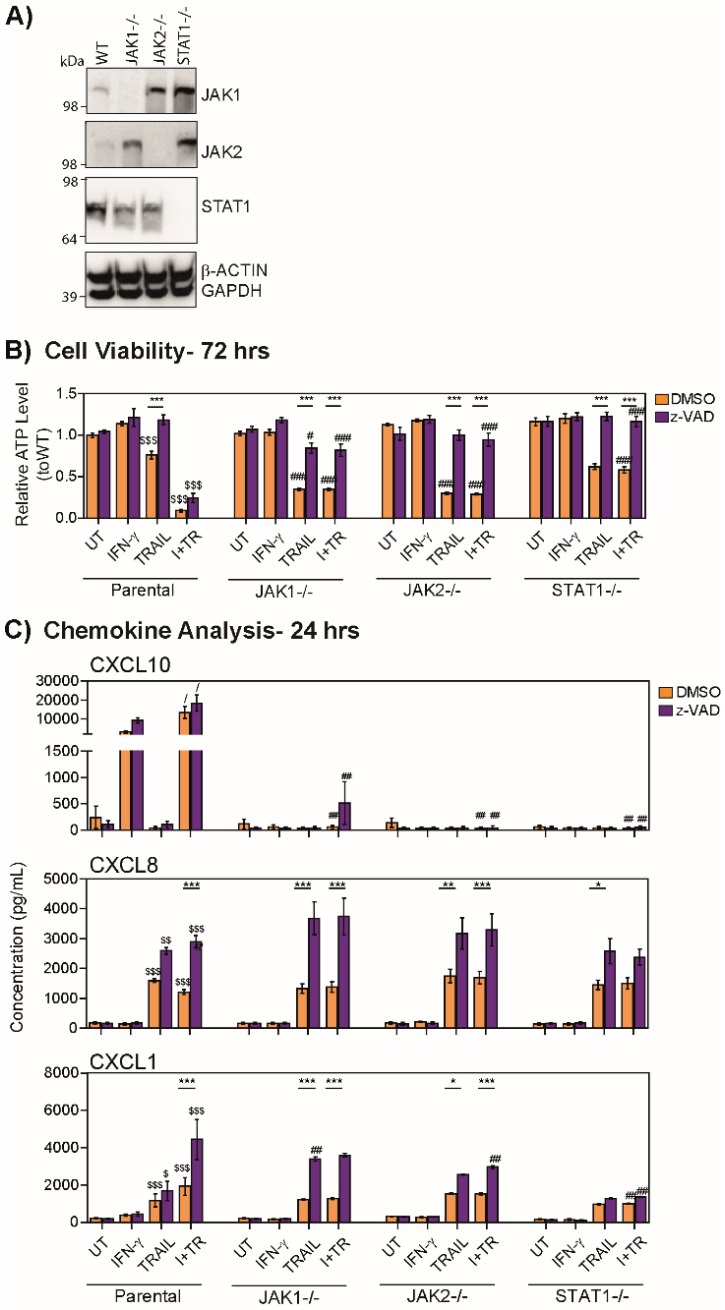


Figure 4.11: JAK1, JAK2 and STAT1 knockout cell lines are resistant to IFN-γ and TRAIL induced cell death

(A) Validation of Knockout lines (JAK1$^{-/-}$, JAK2$^{-/-}$ and STATI$^{-/-}$) by western blotting. HT-29 knockout and parental lines were pre-treated with vehicle control DMSO or 20 μM z-VAD for 30 mins and further treated with 10 ng/mL IFN-γ, 100 ng/mL TRAIL, or a combination of both. **(B)** Cell viability was assessed after 72 hrs cytokine treatment by CellTitre-Glo® **(C)** Chemokines were analysed after 24 hrs of cytokine treatments by ELISA. Data are represented

as mean ± SEM from three experiments in triplicates. *p<0.05, **p<0.01, ***p<0.001, $- one-way ANOVA with Tukey's multiple comparison test with UT; #- one-way ANOVA with Tukey's multiple comparison test compared to parental counterpart, *- two-way ANOVA with Bonferroni post-tests comparing DMSO vs z-VAD.

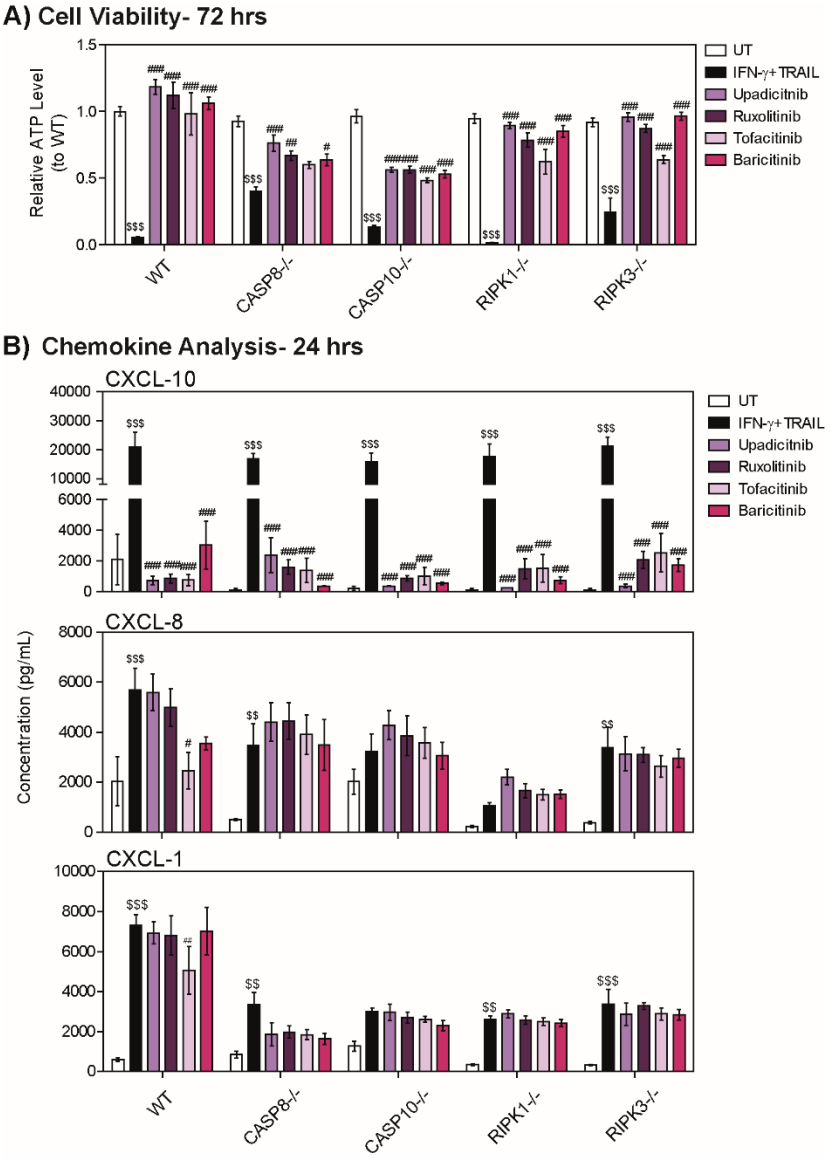


Figure 4.12: JAK inhibitors can block IFN-γ and TRAIL induced cell death and CXCL10 production in caspases and RIPK deficient HT-29 cells

HT-29 knockout lines (CASP8$^{-/-}$, CASP10$^{-/-}$, RIPK1$^{-/-}$ and RIPK3$^{-/-}$) and parental (WT) cells were pre-treated with 1 µM JAKinib (Upadacitinib, ruxolitinib, tofacitinib and baricitinib) for 30 mins and stimulated with both 10 ng/mL IFN-γ and 100 ng/mL TRAIL. **(A)** Cell viability was assessed after 72 hrs cytokine treatment by CellTitre-Glo® **(B)** Chemokines were analysed after 24 hrs of cytokine treatments by ELISA. Data are represented as mean ± SEM from three experiments done in triplicates. *p<0.05, **p<0.01, ***p<0.001, $- one-way ANOVA with Tukey's multiple comparison test with untreated (UT); #- one-way ANOVA with Tukey's multiple comparison test compared to IFN-γ+TRAIL for the respective cell line.

4.4.5. IFN-γ and TRAIL induced cell death and CXCL10 production is dependent on caspases and the JAK1/2-STAT pathway in the TRAIL-sensitive DLD-1 cell line

Next, we investigated whether the JAK-STAT pathway also plays a role in cytokine-induced cell death in the TRAIL sensitive cell line, DLD-1. While caspase inhibition with z-VAD completely blocked TRAIL-induced DLD-1 cell death, it could only partially block (up to 60%) IFN-γ+TRAIL induced cell death (Fig 4.13A). In these lines, treatment with JAK inhibitors, upadacitinib and baricitinib alone only marginally blocked (10%) cytokine IFN-γ+TRAIL induced cell death. However, the combination of z-VAD with the JAK inhibitors completely blocked IFN-γ+TRAIL induced DLD-1 cell death (Fig 4.13A).

IFN-γ alone or IFN-γ+TRAIL did not induce any CXCL10 production but pre-treatment with z-VAD followed by IFN-γ+TRAIL treatment produced high levels of CXCL10 (Fig 4.13B). However, this increase was inhibited with the addition of JAK inhibitors. z-VAD pretreatment resulted in TRAIL-induced CXCL8 production, which could not be inhibited by the JAKinibs (Fig 4.13B). No effect was seen on the expression levels of CXCL1 from treatments of DLD-1 with cytokines, z-VAD, the JAKinibs or their combinations (Fig 4.13B).

To validate the inhibitor experiment, we generated DLD-1 knockout clones for JAK1, JAK2 and STAT1, which were validated by western blotting (Fig 4.14A) and validated by sanger sequencing (data not shown). JAK1/2-STAT1 pathway *JAK2*$^{-/-}$ and *STAT1*$^{-/-}$ DLD-1 cell lines were found to be more sensitive to TRAIL compared to their parental wild-type; however, caspase inhibition completely blocked the TRAIL-

induced cell death in these lines (Fig 4.14B). The *JAK1*$^{-/-}$ line was partially resistant to IFN-γ+TRAIL-induced cell death, and the addition of z-VAD resulted in complete inhibition of cell death (Fig 4.14B). Knock out of *JAK2* and *STAT1* did not affect IFN-γ+TRAIL-induced cell death; however, in the presence of z-VAD, the cytokine-induced cell death was completely blocked (Fig 4.14B).

As before, CXCL10 produced after treatment with z-VAD+IFN-γ+TRAIL in the parental line was completely blocked in the *JAK1*$^{-/-}$, *JAK2*$^{-/-}$ and *STAT1*$^{-/-}$ lines (Fig 4.14C). In the *STAT1*$^{-/-}$ line z-VAD+TRAIL increased the levels of CXCL8, while knockout of *JAK1* and *JAK2* did not have any significant effect (Fig 4.14C). TRAIL increased the expression of CXCL1 in the *JAK2*$^{-/-}$ line, and this was inhibited by the pan-caspase inhibitor z-VAD (Fig 4.14C).

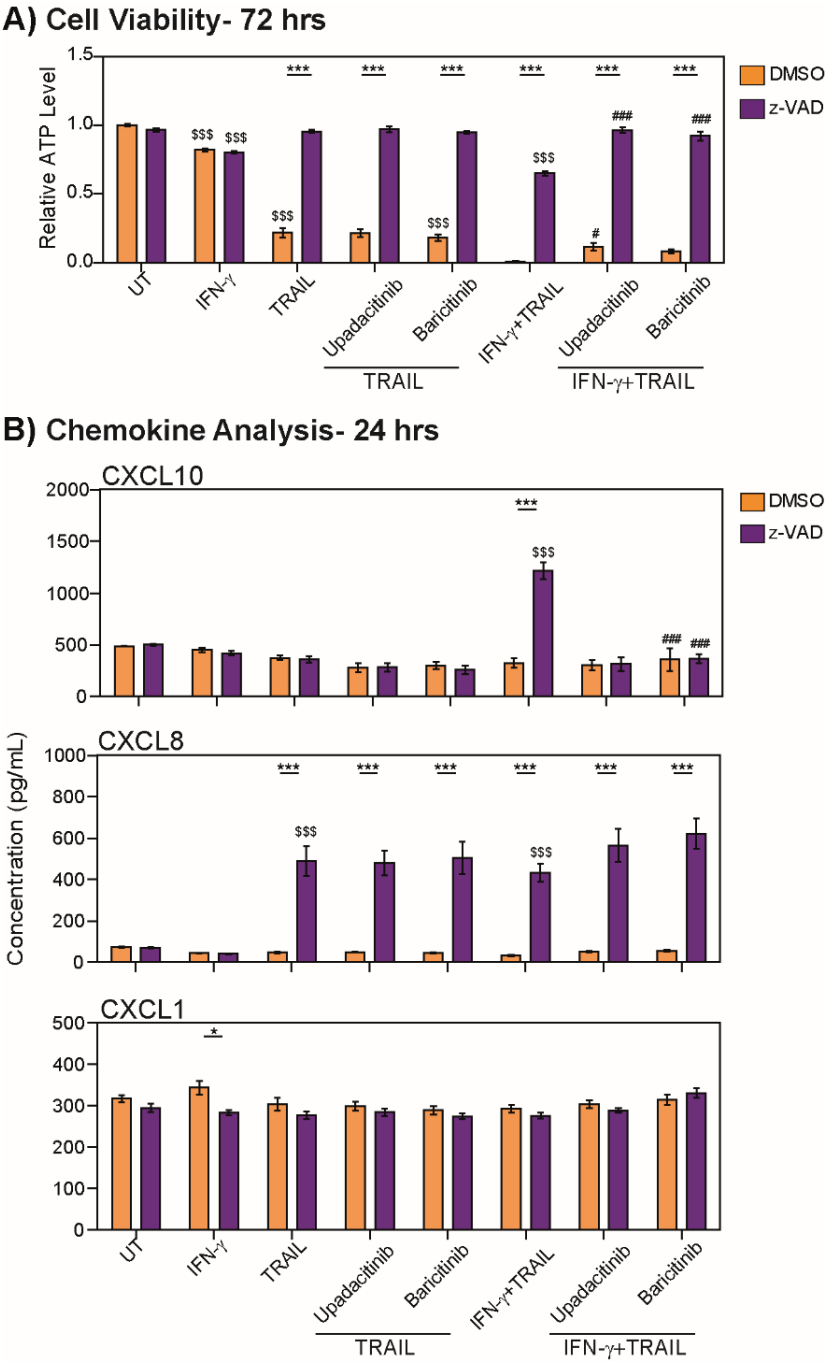


Figure 4.13: Inhibition of both caspase and JAKs blocked IFN-γ and TRAIL mediated cell death in the TRAIL sensitive DLD-1 line

DLD-1 cells were pre-treated with vehicle control DMSO or 20 µM z-VAD in the presence or absence of 1µM JAKinib- -upadacitinib and baricitinib for 30 mins and stimulated with 10 ng/mL IFN-γ, 25 ng/mL TRAIL, or a combination of both as indicated. **(A)** Cell viability was assessed after 72 hrs cytokine treatment by CellTitre-Glo® **(B)** Chemokines were analysed after 24 hrs of cytokine treatments by ELISA. Data are represented as mean ± SEM from three

experiments done in triplicates. *p<0.05, **p<0.01, ***p<0.001, $- one-way ANOVA with Tukey's multiple comparison test with untreated (UT); # - one-way ANOVA with Tukey's multiple comparison test compared to IFN-γ+TRAIL, *- two-way ANOVA with Bonferroni post-tests comparing DMSO vs z-VAD.

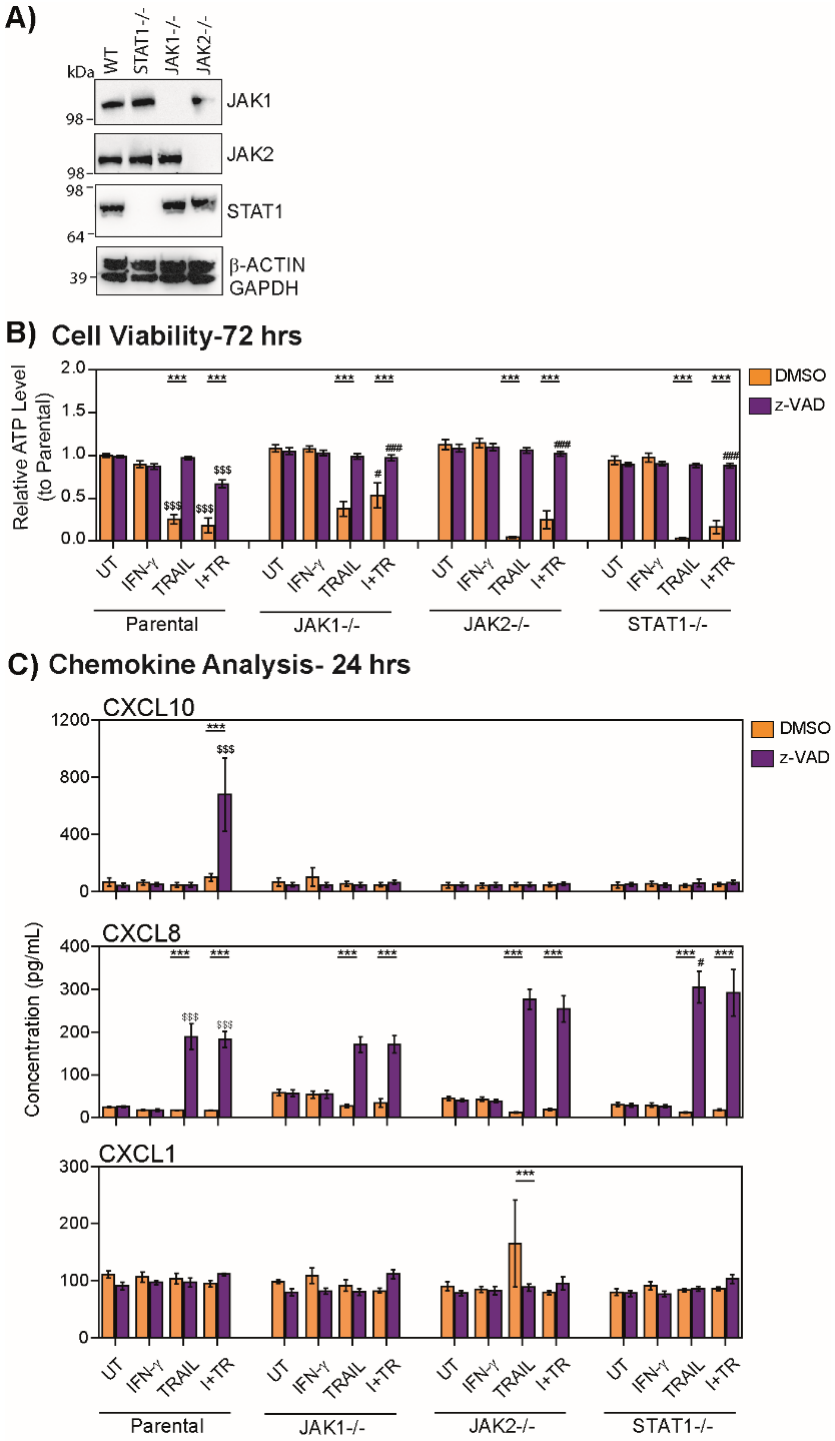


Figure 4.14: Caspase inhibition in JAK1, JAK2 and STAT1 knockout cell lines completely blocks IFN-γ+TRAIL DLD-1 cell death

(**A**) Western blot validation of DLD-1 KO lines generated by the CRISPR-Cas9 method. The KO lines cells were then stimulated with 10 ng/mL IFN-γ, 25 ng/mL TRAIL, or both in the presence or absence of 20 μM z-VAD. **(B)** Cell viability was assessed after 72 hrs cytokine treatment by CellTitre-Glo® **(C)** Chemokines were analysed after 24 hrs of cytokine

treatments by ELISA. *p<0.05, **p<0.01, ***p<0.001, $ - one-way ANOVA with Tukey's multiple comparison test with untreated (UT); # - one-way ANOVA with Tukey's multiple comparison test compared to parental counterpart, * - two-way ANOVA with Bonferroni post-tests comparing DMSO vs z-VAD.

4.4.6. Reduced STAT1 activation in colon cancer cell lines is associated with resistance to IFN-γ and TRAIL induced cell death

Because activation of the JAK1/2-STAT1 pathway plays an important role in cytokine induced cell death[80,88], we investigated the kinetics of STAT1 activation in representative cell lines from each of the groups (Groups 1-4) classified based on their relative sensitivity or resistance to cytokine induced killing. We used DLD-1 cells from Group 1- TRAIL sensitive, LoVo cells from Group 2 - TRAIL and IFN-γ sensitive, HT-29 cells from Group 3- TRAIL and IFN-γ synergistic, and RKO from Group 4- TRAIL-resistant. Across all four lines, there was no difference in the activation of STAT1 in terms of phospho-STAT Tyr701, between IFN-γ and IFN-γ+TRAIL treated groups. TRAIL treatment alone did not activate STAT1 phosphorylation. In DLD-1 cells, STAT1 was phosphorylated for 30 mins, and the levels of phosphorylation were maintained upto 16 hrs (Fig 4.15A Group 1). In LoVo cells, there was an increase in phosphorylation from 30 mins which was maintained up to 4 hrs of treatment. However, phosphorylation levels of STAT1 reduced as the treatment time progressed to 8 hrs and continued to decrease up to 16 hrs of treatment (Fig 4.15A Group 2). In HT-29 cells, IFN-γ induced phosphorylation of STAT1 as early as 30 mins, but phosphorylation levels reduced as the treatment time progressed up to 12 hrs. However, unlike LoVo, there was an increase in the levels of phospho-STAT1 when cells were treated with IFN-γ+TRAIL observed at 16 hrs - suggesting sustained activation of STAT1 in this line. In TRAIL and IFN-γ - resistant RKO cells, STAT1 phosphorylation was induced by IFN-γ within 30 mins of treatment. However, phosphorylation levels decreased very quickly by 2 hrs of treatment, and there was no

further increase observed until 16 hrs (Fig 4.15A- Group 4). These HTRF results were further validated by western blotting (Fig 4.15B). IFN-γ- or IFN-γ +TRAIL induced p-STAT1 Tyr701 was rarely detectable in RKO of Group 4 compared to other lines after 8 and 16 hrs of treatment.

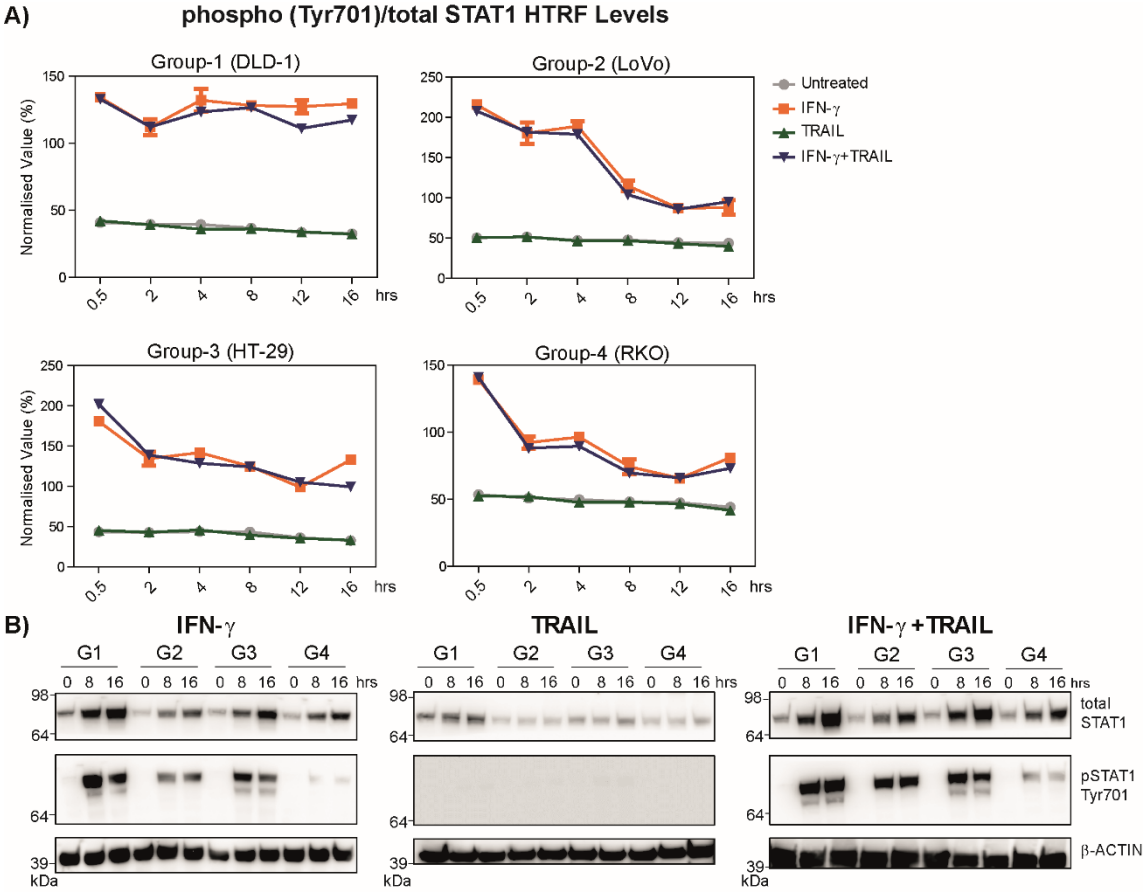


Figure 4.15: IFN-γ and TRAIL resistant RKO cancer line** has **reduced activation of STAT1 upon treatment with IFN-γ+TRAIL

Cells were treated with 100 ng/mL of IFN-γ, 100 ng/mL of TRAIL or a combination of both for the indicated times (**A**) Relative p-STAT1 Tyr 701 levels compared to total STAT1 levels measured by HTRF assay. Data are representative from one run of three independent experiments **(B)** western blots measuring total STAT1, p-STAT1 Tyr 701 with β-Actin as the housekeeping proteins.

4.4.7. Colorectal cancer organoids are resistant to IFN-γ and TRAIL induced cell death

In our cell line work, we identified that in certain cancer cell lines, cell death induced by the combination of IFN-γ and TRAIL was dependent on both caspase-8 activity and the JAK1/2-STAT1 pathway. We then investigated if these findings were also seen in human primary colorectal cancer (CRC) organoid lines. All four CRC organoid lines tested were resistant to IFN-γ, TRAIL or a combination of both cytokines, and these CRC lines were similar in lack of response to cytokines to the Group 4 colon cancer cell lines – i.e. the TRAIL and IFN-γ resistant lines (Fig 4.16-left panel). IFN-γ induced CXCL10 production in all four CRC organoid lines tested, and this was dose-dependent. Co-treatment with both IFN-γ and TRAIL did not further increase CXCL10 levels as observed previously in some cell lines before (Fig 4.16). Interestingly, TRAIL did not induce the production of inflammatory pro-tumorigenic CXCL8 in these CRC organoid lines. Further investigation is required to understand the role of these cytokine signalling pathways in CRC.

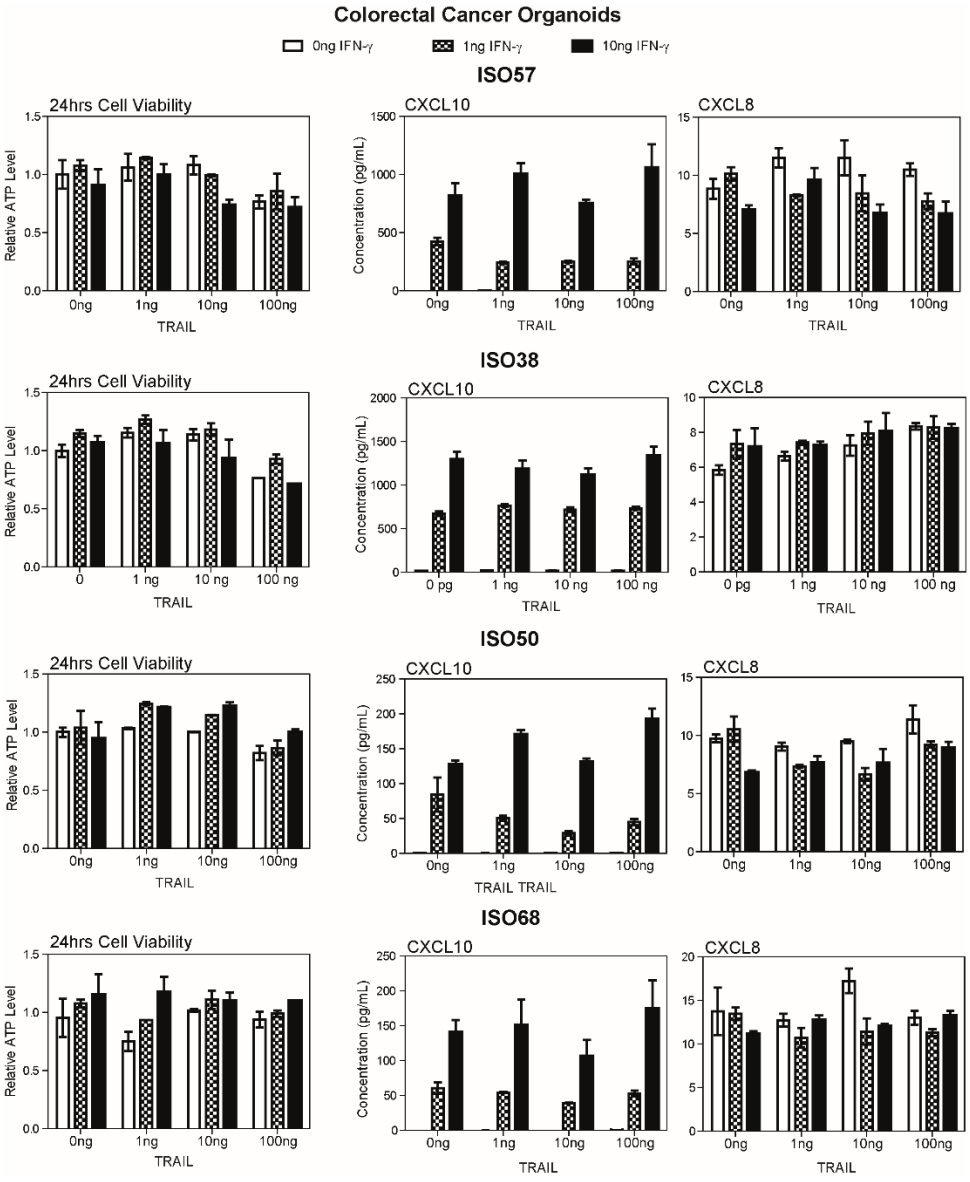


Figure 4.16: Colorectal cancer organoids are resistant to IFN-γ and TRAIL to IFN-γ and TRAIL-induced cell death.

Colorectal cancer organoids- ISO57, ISO38, ISO50, and ISO68, were treated with varying concentrations of IFN-γ and TRAIL as indicated in the figure for 24 hrs. Cell viability was assessed using CellTitre-Glo® (left panel). Chemokines were analysed from supernatants collected by MSD assay.

4.5. Discussion

The TNFSF member and death receptor ligand TRAIL, has been investigated as a promising candidate anti-cancer therapy for decades now as it triggers apoptosis selectively in cancer cells but not in normal untransformed cells. However, TRAIL-receptor agonists have failed in human clinical trials to produce evidence of efficacy, and only a few drugs have entered phase II clinical trials[83]. Many cancer cells develop resistance to TRAIL and in KRAS-mutated cancer, TRAIL can lead to tumour growth and induce metastasis[88,89]. IFN-γ, a tumour suppressor cytokine which exhibits a variety of anti-tumorigenic effects, can sensitise cancer cell lines to TRAIL[58]. In this study, we show that in certain TRAIL-resistant cancer cell lines, TRAIL can synergise with IFN-γ to induce cell death and production of the anti-tumorigenic Th1 chemokine, CXCL10. In addition, we found that the synergistic killing effects of IFN-γ+TRAIL was dependent on both caspase-8 enzymatic activity and the JAK1/2-STAT1 signalling pathway. While blocking caspase activity alone with the pan-caspase inhibitor z-VAD did not significantly affect cell viability in parental HT-29 cells treated with IFN-γ+TRAIL, it increased production of pro-inflammatory chemokines. In contrast, knockdown or deletion of caspase-8 partially blocked IFN-γ+TRAIL-induced cell death and reduced chemokines levels. Perturbation of RIPK1/RIPK3 involved in TRAIL-induced necroptosis did not affect cell viability suggesting that IFN-γ+TRAIL-induced cell death may not be the classical TRAIL-receptor induced apoptotic or necroptotic pathway. Inhibition of the JAK1/2-STAT1 pathway using either JAK inhibitors or by knockdown/knockout of JAK1, JAK2 or STAT1 completely blocked IFN-γ+TRAIL-induced Th1 chemokine secretion but only partially blocked cytokine-induced cell death. Cell death was blocked entirely by inhibition of caspase-8 expression and activity in combination with perturbation of the

JAK1/2-STAT 1 pathway. Also, TRAIL-resistant cells which were resistant to IFN-γ sensitization had lower and transient activation of STAT1 compared to TRAIL-sensitive and IFN-γ+TRAIL sensitive cell lines. These results suggest that there might be crosstalk or interaction between caspase-8 and the JAK1/2-STAT1 pathway which determines sensitivity to these cytokines.

TRAIL has been extensively studied for its role in inducing caspase-8 dependent apoptosis[7,29,90–93]. To this end, the mode of action of TRAIL includes the binding of TRAIL to its receptors TRAIL-R1 and TRAIL-R2 resulting in the recruitment of FADD. FADD contains a death effector domain (DED), enabling the binding of caspase-8 or caspase-10, promoting their activation and resulting in apoptotic cell death[93]. Previous reports have shown that various resistance mechanisms can block TRAIL-mediated apoptosis. One mechanism includes the overexpression of decoy receptors DcR1 and DcR2, which can bind to TRAIL and form inactive heteromeric complexes with DRs[94]. Elevated levels of anti-apoptotic proteins such as Bcl-2, Bcl-X_L are also observed in TRAIL-resistant cancer cell lines[26,34,82]. Consistent with these observations, in our study, we found that TRAIL-resistant HCA-7 and RKO cell lines exhibited higher TRAIl-R3 (DcR2) and expression of the anti-apoptotic proteins Bcl-2 and MCL-1.

TRAIL has also been shown to induce necroptosis in the context of caspase-inhibition in certain cell lines, including the HT-29 cell line used in this study through a RIPK1/RIPK3 dependent pathway[84–86]. In our study, we show that the TRAIL-resistant HT-29 cell line is not rescued by the addition of the caspase inhibitor z-VAD from IFN-γ+TRAIL induced cell death, suggesting the activation of the necroptotic pathway[86]. However, the addition of necroptotic inhibitors such as RIPK inhibitor Nec1s, RIPK3 inhibitor GSK’872, or MLKL inhibitor NSA could not block IFN-

γ+TRAIL-induced cell death, indicating that the cell death pathway is different from classical TRAIL-R-induced apoptotic or necroptotic cell death. We found that knockout of *caspase-8* partially blocked IFN-γ+TRAIL-induced cell death while *RIPK1*$^{-/-}$ and *RIPK3*$^{-/-}$ did not. Recent work from our lab evaluating IFN-γ+TNF-α-induced cell death and work by Henry and Martin, 2017 have found similar findings suggesting that caspase-8 can have a scaffolding role in signalling pathways in addition to its canonical enzymatic role as an initiator caspase for death-receptor signalling pathways[20,79]. In the DLD-1 cell line, deficient in RIPK3 and RIPK3-involved necroptosis[95,96] inhibiting caspases by z-VAD completely blocked TRAIL-induced cell death but not IFN-γ+TRAIL-induced cell death, further providing indirect evidence of the activation of a non-canonical cell death pathway by the cytokine combination.

Evaluating IFN-γ+TNF-α induced cell death previously in our lab, we identified that the JAK1/2-STAT1 pathway played an essential role in cytokine-induced cell death[79]. Interestingly, we also found that inhibiting JAKs with small molecule inhibitors completely blocked HT-29 cells from IFN-γ+TRAIL-induced cell death. However, depletion of JAK1/2-STAT1 could only partially block these cells from IFN-γ+TRAIL. In contrast, the depletion of JAK-STAT resulted in higher sensitivity to TRAIL-induced cell death due to unknown mechanisms but was rescued by additional caspase inhibition. Another novel finding was that in the TRAIL-resistant RKO cancer line, we found transient phosphorylation of STAT1 at the Tyr 701 residue compared to TRAIL-sensitive and IFN-γ+TRAIL synergistic cell lines. Phosphorylation of STAT1 tyrosine residue 701 is required for homodimerization of STAT1 and translocation of transcriptionally competent STAT1 to the nucleus and binding to gamma-activated sequence (GAS) of target genes[97]. Therefore, the transient

activation of STAT1 in RKO cells might result in the generation of different transcriptional profiles in TRAIL-resistant cell lines compared to others which are yet to be evaluated. Overall, these results suggest that the interaction between caspase-8 and JAK1, JAK2, STAT1 plays a major role in IFN-γ+TRAIL-dependent cell death and that the two cytokines together rewire cell death pathways depending on the presence and absence of caspase-8, JAK1/2 or STAT1. However, further work is required to identify molecular mechanisms of pathway crosstalk and rewiring.

Recently published works on TRAIL biology have been focused on understanding the mechanisms of TRAIL-induced chemokine production, particularly in the context of the TME[44]. It has been shown that TRAIL can induce pro-tumorigenic, neutrophil attracting chemokines such as CXCL8, CXCL1, and CCL2 through a 'FADDosome' complex consisting of FADD, caspase 8, and RIPK1, which activates the NF-κB pathway[19,20,23,44]. This signalling through caspase-8 is dependent on its oligomerisation but independent of its catalytic activity[98]. Consistently, our study also showed that in HT-29 cells, while inhibition of caspase-8 increased TRAIL or IFN-γ and TRAIL induced chemokine production, the depletion of caspase-8 reduced it. Inhibition of caspases has been shown to promote the recruitment of RIPK1 to TRAIL receptors resulting in TRAIL-induced NF-κB activation leading to higher chemokine production observed[20,23]. Similar to the findings by Henry and Martin, 2017, we observed that the depletion of RIPK1 affected the secretion of the pro-tumorigenic chemokines[20]. IFN-γ co-treatment with TRAIL did not modify secreted levels of pro-tumorigenic CXCL8 or CXCL1 in HT-29 or DLD-1 cell lines.

CXCL10 is a Th1 chemokine exhibiting anti-tumorigenic properties resulting from the migration of CXCR3 expressing T-cells, NK cells and NKT cells towards CXCL10 chemokine gradients in the TME, resulting in immune-mediated tumour

regression[53]. CXCL10 is predominantly an IFN-γ induced chemokine[54], and in our study, we found that IFN-γ induced CXCL10 production in seven out of eleven cancer lines tested irrespective of their sensitivity towards the cytokines - IFN-γ or TRAIL in terms of cell death. Our study identified that IFN-γ synergised with TRAIL, further increasing levels of CXCL10 compared to IFN-γ treatment alone in HT-29 cells. Interestingly in the DLD-1 cell line, with IFNγ, TRAIL alone or in combination, we could not detect any CXCL10 induction but with the addition of caspase inhibition, increased CXCL10 levels were observed. This was in contrast with our findings in the previous chapter (Chapter 3), where we reported that CXCL-10 was produced by TRAIL in combination with IFN-γ. We reason this might be due to higher cell death observed at 24 hrs in this study, which limits the number of cells available to secrete the chemokines. The difference between the two studies was from the fact that the recombinant TRAIL used in both studies were different (sourced from R&D systems for chapter 3 and Adipogen for this chapter). IFN-γ or IFN-γ+TRAIL induced CXCL10 was effectively blocked with inhibition of JAK1, JAK2 and STAT1. For the first time we report that IFN-γ+TRAIL can modulate levels of anti-tumorigenic CXCL10 and this may increase Th1-mediated immunity in the context of the TME.

Overall, this study shows that in certain colon cancer cell lines, IFN-γ can synergise with TRAIL to induce the Th1 chemokine CXCL10 and induce cell death. This synergism is regulated by caspase-8 and the JAK1/2-STAT1 pathway and suggests that there might be crosstalk or direct interaction between the two pathways, which needs to be further investigated. We evaluated our findings in primary human CRC organoids; however, we found that IFN-γ+TRAIL could not increase levels of IFN-γ-induced CXCL10 or induce cell death in the organoid lines tested. This might be due to the low number of organoid lines tested or that CRC organoids might fall

into the category similar to Group 4 cell lines which are TRAIL and IFN-γ-resistant. However, further investigation is required to identify molecular mechanisms underpinning these initial observations.

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Chapter 5：General Discussion and Future Directions

5.1. Discussion

Inflammatory bowel diseases (IBD), comprising of two major forms, Crohn's disease and ulcerative colitis, are chronic inflammatory diseases of the gastrointestinal tract. With a prevalence of 0.5% in the western world, IBD is now emerging as a global disease with significantly high morbidity rates[1–3]. Immunopathogenesis of IBD is characterised by increased intestinal epithelial cell death, epithelial barrier disruption, infiltration by innate and adaptive immune cell populations, and increased levels of inflammatory mediators, including cytokines and chemokines[4,5]. One main factor in the pathogenesis of IBD is the dysregulation and rewiring of immune cells, stromal cell and cytokine networks, which can promote the breakdown of intestinal homeostasis leading to IBD[6]. Two Th1 cytokines - interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) are detected at elevated levels in gut mucosal biopsy tissue of patients with IBD, especially in CD[7–10]. Infliximab, the first anti-TNF, was approved for the treatment of CD in 1998 by the FDA. Almost 25 years later, anti-TNFs, are still one of the main top-line therapies used clinically for the treatment of IBD[11]. However, 30% of patients have no initial response to anti-TNFs (primary non-responders), while 30-50% of patients lose their response over the course of anti-TNF treatment (secondary non-responders)[12–14]. Various omics approaches have been used to identify molecular signatures that would distinguish anti-TNF responders from non-responders and vice versa. These include other inflammatory cytokines such as oncostatin M, IL-23 and IL-1 driven pathways driving resistance to anti-TNFs[15–17]. Also, targeting IFN-γ by Fontolizumab did not provide significant efficacy in a phase 2 trial for CD[18]. Hence, there is a need to understand the complexity of cytokine networks involved in the pathogenesis of IBD and identify molecular mechanisms underpinning the cytokine driven chronic inflammation.

In the first research study (Chapter 2), we investigated the molecular mechanism underpinning IFN-γ and TNF-α induced synergistic cell death in colonic epithelial lines. Synergism between IFN-γ and TNF-α was initially reported in 1983[19], and many studies have investigated their synergistic effect in the context of anti-viral immunity, tumourigenesis, IBD and other inflammatory diseases[20–23]. Focussing on IBD and intestinal epithelial cell (IEC) death, various mechanisms have been put forth depending on the cell line investigated, but definitive mechanisms have still not been reported. Investigating the effects of IFN-γ+TNF-α on colonic epithelial cells to identify key genes that are regulated by the combination of the cytokines, we performed a microarray experiment and analysed the differentially expressed genes by weighted gene co-expression network analysis (WGCNA) to identify key module genes[24]. Interestingly, we found that of 14 modules identified by WGCNA - two modules- yellow- upregulated by TNF-α, and magenta - upregulated by IFN-γ, were of importance. In the two modules, receptor genes of one cytokine were closely associated with signalling components of the other cytokine, i.e., *IFNGR1* and *IFNGR2* were associated in the same yellow module with *TNF*, *NF-κB,* and *TNFR1* and *TNFR2* were associated in the magenta module with *JAK2* and *IRF1*. Such pathway cross-regulation - TNF-α increasing IFNGR1 and IFNGR2 expression and IFN-γ inducing TNFR1 and TNFR2 expression was confirmed by *in vitro* studies on HT-29 cells and in colonic epithelial organoid lines for the first time. We further identified that the two main transcription factors - NF-κB and STAT1 of the TNF-α and IFN-γ pathways, respectively, are required for cross-regulation of receptor expression, but only STAT1 played a critical role in IFN-γ+TNF-α induced cell death of HT-29 cells. This is in agreement with previous studies that reported the JAK/STAT pathway playing a critical role in IFN-γ+TNF-α induced cell death of colon cancer

lines[20,22]. We also report that though genes belonging to canonical TNFR receptor-induced apoptotic and necroptotic processes were enriched in functional analysis of global differential gene expression, IFN-γ+TNF-α induced cell death could not be blocked by inhibitors targeted towards these canonical cell death pathways. Our data collectively support a model whereby IFN-γ and TNF-α cross-regulate genes for components of each other's pathway and activate a positive feedback loop that boosts the response of cells to the cytokines over time, leading to STAT1-mediated cell death.

However, more research is required to identify downstream effector genes and molecules of NF-κB or STAT1 that might play a role in this feedback mechanism. Also, we did not evaluate if receptor knockdowns inhibited cytokine-induced cell death. Another future direction might be to investigate the involvement of autophagic cell death. Functional enrichment analysis identified increased enrichment of genes and modules associated with unfolded protein response and ER stress. UPR-ER-stress can lead to induction of autophagy by modulating calcium channels leading to a beclin-1-mTOR associated autophagic pathway[25]. It would be interesting to see if inhibitors of autophagy such as bafilomycin, either alone or in combination with other cell death inhibitors, could rescue cells from IFN-γ+TNF-α induced cell death. This study provides a mechanistic explanation for the observed therapeutic effects of JAK inhibitors in the treatment of IBD. With Tofacitinib, a JAK1 and JAK3 inhibitor, approved by the FDA for the treatment of ulcerative colitis in 2018[26–28] and Upadacitinib, a JAK1 inhibitor, approved recently in 2022 by the FDA for the treatment of UC [29], it is clear that the JAK-STAT pathway plays a critical role in the pathogenesis of IBD. In our study, we also found that pan-JAK inhibitors could rescue primary colonic organoid lines from IFN-γ and TNF-α induced cell death.

In our next study (Chapter 3), we utilised the identified synergism phenotypes of IFN-γ+TNF-α induced cell death and chemokine production in cell-based phenotypic screens to identify TNF-α-like molecules that could be potential alternative therapeutic targets for the treatment of IBD. We screened the TNF superfamily of ligands as TNF-α is also part of the same family. We used two cell line models for screens and validated our hits in primary colonic organoids generated from crypts isolated from colonic mucosal pinch biopsies of patients with Crohn's disease. TRAIL was identified as the most TNF-α like molecule with respect to its ability to synergise with IFN-γ to induce cell death and inflammatory chemokines such as CXCL10 and CXCL11 in cell lines. TWEAK and LIGHT were also identified as hits, as they synergised with IFN-γ to induce inflammatory chemokines but not cell death. Future validation of these hits in pre-clinical models is required. They can be investigated for their role in IBD by either targeting the molecule alone or in combination with anti-TNFs.

An antibody targeting LIGHT - CERC-002 - developed by the company Cerecor, has now given positive phase I trial results in patients with CD who had previously failed therapy with anti-TNF treatment[30]. Clinical trials targeting TWEAK and TRAIL have not yet been reported, but various animal studies have focussed on their role in chemically-induced colitis models[31]. Our study has limitations that have to be addressed too. Our screening model positively identified LIGHT as a potential target but failed to identify TL1A and RANKL, which are susceptible genes for IBD[32], as targets. This might be due to a lack of receptor expression in the cell lines tested; however, this must be confirmed. Future experimental work investigating the expression of receptors in non-treated and cytokine-treated cells and organoids might partly answer why certain ligands failed to be identified as TNF-α-like molecules.

Though there are limitations, our study is a good example of how cell-based phenotypic screens can be used to identify molecules that are TNF-α-like. This protocol can be used in other ways, i.e., to identify molecules that are IFN-γ like with respect to synergism with TNF-α. Since complex cytokine networks are involved in IBD immunopathogenesis, such a screening process based on cytokine synergism might help identify targets that can be used in combinatorial therapies. Our lab has screened about 600+ recombinant proteins that are associated with CD in colonic epithelial cell lines, colonic fibroblasts and human macrophages using the screening strategy applied in this study to identify more ligands that can be validated as potential targets for the treatment of IBD.

In our final study (Chapter 4), we investigated the effect of TRAIL and mechanisms underpinning its synergism with IFN-γ, as it was identified to be the most TNF-α-like molecule, at least in cell lines. We took a tumour immunology focused perspective and approach, as TRAIL, though initially reported as anti-tumorigenic, is now being reported to have pro-tumorigenic effects as well[33,34]. Hence, we wanted to test whether IFN-γ, a pleiotropic cytokine exhibiting anti-tumourigenic effects, can interact with TRAIL to induce tumour suppressing effects. For this, we initially used eleven colon cancer cell lines to identify cell lines that were resistant to TRAIL, but with IFN-γ and TRAIL, cell death was induced profoundly. One such cell line was HT-29, and we used it as a model to investigate potential mechanisms underpinning IFN-γ+TRAIL killing. Also, in the same model, our lab previously investigated mechanisms responsible for IFN-γ+TNF-α induced synergistic cell death[20]. Investigating the role of receptor-associated cell death pathways such as apoptosis and necroptosis, we identified that IFN-γ+TRAIL induced cell death could not be blocked by known inhibitors of canonical cell death pathways. Interestingly, knockout of the

components rather than enzymatic inhibition offered partial protection to cytokine-induced cell death. Similar to the finding previously that the JAK1/2-STAT1 pathway played an important role in IFN-γ+TNF-α induced cell death[20,22], we found that the JAK1/2-STAT1 pathway was also involved in IFN-γ+TRAIL induced cell death. However, to achieve complete inhibition of IFN-γ+TRAIL induced cell death, both caspases and the JAK1/2-STAT1 pathway had to be blocked. Also, blocking the JAK1/2-STAT1 pathway using inhibitors or RNAi perturbation completely blocked CXCL-10 production in HT-29 cells. These results suggest that IFN-γ and TRAIL together can kill colon cancer cell lines and modulate their inflammatory chemokine profile by inducing more anti-tumourigenic CXCL-10 with no effect on the pro-tumourigenic CXCL8 or CXCL1.

Even though our study was successful in identifying the contribution of the JAK1/2-STAT1 pathway to IFN-γ+TRAIL induced cell death, more research is required to identify the exact downstream mechanisms underpinning this atypical mode of transcriptionally mediated cell death. Our study did not address if caspase-8 is directly associated with the JAK1/2-STAT1 pathway or through any other downstream molecule. We identified that RIPK3 was required for the production of inflammatory chemokines, but its role in the cell pathway/s needs to be explored further. Our study also did not investigate the cell death mechanism of autophagy, pyroptosis or ferroptosis. Our work with colorectal cancer organoids did not reproduce findings from the cell line models, as these organoids were completely resistant to both IFN-γ and TRAIL. However, further investigation is required to identify molecular mechanisms underpinning this apparent resistance to cytokine induced cell death. Overall, these results are relevant to the role of both IFN-γ and TRAIL in tumour immunology and immunopathogenesis of IBD . TRAIL was upregulated in

intestinal epithelial cells in inflamed colonic and ileal tissues[35]. Since there is a dependency on the JAK1/2-STAT1 pathway in IFN-γ+TRAIL induced cell death, the success of JAKinibs in the treatment of IBD may partly be attributed to the inhibition of IFN-γ+TRAIL induced cell death of the epithelium, but this has to be further investigated.

Overall, my PhD research has been very significant in 1) identifying new TNF-like targets for inflammatory bowel diseases, 2) understanding the molecular mechanism underpinning cell death induced by IFN-γ+TNF-α and IFN-γ+TRAIL. In addition, my PhD research supports the fact that the JAK1/2-STAT1 pathway plays a crucial role in cytokine synergism and is a very good target pathway for the treatment of IBD.

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