

Investigating the Effect of SARS-CoV-2 upon the Anti-Viral and Immune Responses of Human Polymorphonuclear Leukocytes (PMNs)



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Table of Contents

Table of Contents	1
Declaration of Authorship	9
Acknowledgements	10
List of Figures	12
List of Tables	16
Abbreviations	17
Abstract	25
1. Introduction	28
1.1 Overview of the Immune System	28
1.2 Components of the Innate Immune System	28
1.3 Interaction Between Innate and Adaptive Immunity	29
1.3.1 Antigen Presentation by Innate Cells	29
1.3.2 TLRs and Danger Signals.....	31
1.3.3 Cytokine Signalling	31
1.4 Inflammation	32
1.4.1 The role of TLR activation in inflammation	32
1.4.2 Classification and Structure of TLRs	33
1.4.3 TLR Expression and Cellular Localization.....	34
1.4.4 Exogenous and Endogenous Ligands of TLRs	35
1.4.5 Signalling Pathways of TLRs.....	35
1.4.6 Adaptor Proteins for TLR Signalling.....	36
1.4.7 Function of TLRs	37
1.4.8 TLR2	38
1.4.8.1 TLR2 in viral infections.....	38
1.4.9 TLR4	39
1.4.9.1 TLR4 Ligands and Activation	39
1.4.9.2 MyD88-Dependent signalling	39
1.4.9.3 MyD88-Independent (TRIF-Dependent) Signalling.....	40
1.4.9.4 Endocytosis and Compartmentalized Signalling.....	40
1.4.9.5 Role of TLR4 in viral infections	40
1.4.10 TLR8: Structure, Activation, and Role in Viral Infections.....	42
1.4.10.1 Structure of TLR8.....	42
1.4.10.2 TLR8 Activation and signalling Pathway	43
1.4.10.3 Role of TLR8 in Viral Infections	43
1.4.11 TLR7	44
1.4.12 Cytosolic Receptors: RLRs.....	45
1.4.13 RIG-I-like receptors in viral infections	46
1.4.14 Cytosolic Receptors: NOD-like receptors (NLRs)	46
1.4.15 NF- κ B signalling	47
1.4.16 The mitogen-activated protein kinase (MAPK) signalling	48
1.4.17 Interferon Signalling in Antiviral Defence	49
1.4.18 JAK/STAT signalling.....	50
1.4.18.1 Molecular Mechanism of JAK/STAT Activation	50
1.4.18.2 JAK-STAT in Cytokine Signalling.....	51
1.4.18.3 Structural Components of JAK and STAT Proteins.....	51

1.4.18.4 Regulation of JAK-STAT Signalling	52
1.4.19 Viral Evasion and Induction of IFN Responses	53
1.4.20 ISGs and Their Functions	56
1.4.21 Proinflammatory Cytokines.....	58
1.4.21.1 IL-6.....	59
1.4.21.2 IL-8.....	60
1.4.21.3 TNF- α	61
1.4.21.4 IL-1 β	62
1.4.22 TGF- β	62
1.4.22.1 Canonical TGF- β Signalling (SMAD Pathway)	64
1.4.22.2 Non-SMAD TGF- β Signalling	65
1.4.22.3 TGF- β 1 in COVID-19.....	66
1.5 Neutrophils.....	67
1.5.1 Neutrophils recruitment to the site of infection “Neutrophil release from bone marrow and Neutrophil extravasation into tissue”	68
1.5.2 Mechanism of Neutrophil Activation	69
1.5.3 Neutrophil Surface Marker Changes During Infection	70
1.5.4 Neutrophils roles in innate immunity	71
1.5.5 Neutrophil Heterogeneity	75
1.6 SARS-CoV-2.....	77
1.6.1 COVID-19.....	77
1.6.2 Classification of COVID-19 Severity.....	78
1.6.3 Neutrophil Responses to SARS-CoV-2	78
1.6.4 Neutrophils as Mediators of Immunopathology in COVID-19	80
1.6.4.1 Damage to Alveolar-Capillary Barrier.....	80
1.6.4.2 Cytokine Storm Amplification.....	80
1.6.4.3 NETs and Immunothrombosis:	81
1.6.4.4 Neutrophil Heterogeneity and Dysfunction:	81
1.6.4.5 Multi-Organ Damage:.....	81
1.7 Overall Rationale Underpinning this Thesis.....	82
1.8 Importance of this Investigation	82
1.9 Hypothesis.....	83
1.10 Overall Objective	83
1.11 Specific Aims.....	84
2. Materials and Methods:.....	86
2.1 Ethics.....	86
2.2 Cell culture	86
2.3 PMN isolation.....	86
2.4 Cell counting.....	87
2.5 Propagation of SARS-CoV-2.....	87
2.6 Titration of SARS-CoV-2	87
2.7 SARS-CoV-2 Infection of PMNs.....	88
2.8 Stimulations and treatments	88
2.9 RNA extraction using Norgen Biotek corp	89
2.10 Reverse transcription (cDNA synthesis).....	89

2.11 Quantitative Real-time-Polymerase Chain Reaction (qRT-PCR)	89
Table 2. 3 PCR cycle.....	90
2.12 qRT-PCR data analysis	90
2.13 Protein extraction	91
2.14 Western Immunoblotting.....	91
2.14.1 SDS-PAGE	91
2.14.2 Transfer of proteins to the PVDF membrane	93
2.15 Flow cytometry staining	94
2.16 Identified PMNs population by flow cytometry:	95
2.17 Measurement of the ROS levels	95
2.18 ELISA Proinflammatory Cytokines	96
2.19 ELISA for TGF- β 1.....	96
2.20 ELISA for MPO and NE	97
2.21 NETosis Assay.....	97
2.22 Phagocytosis Assay	98
2.23 Apoptosis Assay Using Annexin V-FITC/PI Staining	98
2.24 Identification of potential TLR7/8-triggering ssRNA PAMPs	99
2.25 Statistics	99
Chapter 3:	100
<i>The Effect of SARS-CoV-2 SP on Activation and Inflammation in Primary Human PMNs</i>	100
3.1 Introduction.....	101
3.1.2 Immune Recognition and Responses to the SARS-CoV-2 SP	103
3.1.3 Innate Immune Recognition	103
3.1.4 Adaptive Immune Responses	105
3.1.5 Immune Evasion and Challenges	105
3.1.6 SARS-CoV-2 and TGF- β Signalling	106
3.1.7 TGF- β Regulation of Neutrophil Function	106
Table 3. 1 TGF- β and Neutrophil Interaction.....	107
3.2 Hypothesis	108
3.3 Specific Aims	108
3.4 Experiments & Discoveries:	112
3.4.1 CD66b and CD11b mRNA gene expressions in whole blood is associated with the severity of COVID-19	112
3.4.2 CD11b (and not CD66b) is increased in PMNs from COVID-19 patients	113
3.4.3 SARS-CoV-2 SP does not induce the expression of CD66b in human primary PMNs	113
3.4.4 SARS-CoV-2 SP Does Not Induce the Expression of CD11b in human primary PMNs	114
3.4.5 SARS-CoV-2 SP Does Not Induce Shedding of CD16 in human primary PMNs.....	115
3.4.7 SARS-CoV-2 SP Does Not Induce ROS Production in human primary PMNs	118
3.4.9 SARS-CoV-2 SP Does Not Significantly Induce Degranulation in human primary PMNs	121
3.4.10 SARS-CoV-2 SP does not Induce matrix metalloproteinase 9 (MMP-9) in human primary PMNs	122
3.4.11 SARS-CoV-2 SP Does Not Alter Phagocytosis of human primary PMNs	123

3.4.13 Human Primary PMNs Do Not Express ACE2 and Show Limited Induction Under Inflammatory Conditions	127
3.4.14 IL-10 and TGF- β treatments did not induce ACE2 mRNA in human Primary PMNs	128
3.4.15 SARS-CoV-2 SP enhances human primary PMNs Survival	129
3.4.16 SARS-CoV-2 SP Induces IL-8 but Not IL-1 β or IL-6 production in human Primary PMNs	131
3.4.17 SARS-CoV-2 SP-Induced IL-8 Production in Human Primary PMNs is Partially Reduced by TLR4 and TLR2 Inhibition	132
3.4.18 Role of NF- κ B, p38 MAPK, and ERK1/2 Pathways in SARS-CoV-2 SP-Induced IL-8 Production in Human Primary PMNs	134
3.4.19 TGF- β Induction by SARS-CoV-2 SP in Human Primary PMNs	136
3.4.20 SARS-CoV-2 SP Induces TGF- β Production in Human Primary PMNs via Integrin-Dependent Mechanisms	138
3.4.21 TGF- β does not induce the expression of Human Primary PMNs Activation Markers (CD66b, CD11b, CD16 and CD66b)	139
3.4.22 TGF- β Does Not Modulate Basal or tBuOOH-Induced ROS Production in Human Primary PMNs	140
3.4.23 TGF- β Does Not Modulate Basal nor PMA-Induced NETosis in Human Primary PMNs	142
3.4.24 TGF- β Does Not Significantly Alter Human Primary PMNs Phagocytosis of GFP-Expressing Staphylococcus aureus	143
3.4.25 TGF- β does not effect MPO and NE Release in PMNs	144
3.5 Discussion	146
3.5.1 Differential Expression of Neutrophil Activation Markers CD11b and CD66b in COVID-19 Disease Severity	146
3.5.2 Flow Cytometry Analysis of CD11b and CD66b Expression in COVID-19	146
3.5.3 SARS-CoV-2 SP Fails to Induce PMN Activation Markers	147
3.5.4 SARS-CoV-2 SP Does Not Induce ROS Production in PMNs	147
3.5.5 SARS-CoV-2 SP Alone Does Not Induce NETosis	148
3.5.6 SARS-CoV-2 SP Does Not Affect PMN Phagocytic Activity.	149
3.5.7 SARS-CoV-2 SP Selectively Induces IL-8 Production in PMNs via TLR2/TLR4-NF- κ B Signalling ...	149
3.5.8 SARS-CoV-2 SP Induces TGF- β Activation in PMNs Through Integrin-Dependent Mechanisms .	150
3.5.9 SARS-CoV-2 SP Inhibits PMNs Apoptosis and Promotes Prolonged Inflammatory Responses ...	150
3.5.10 TGF- β Has Minimal Direct Effects on PMNs Activation and Effector Functions	151
3.6 Future Directions	152
3.7 Conclusion	152
Chapter 4:	154
<i>TLR8 Recognition of SARS-CoV-2-Associated ssRNAs and Its Role in Inflammatory Responses in Human PMNs</i>	<i>154</i>
4.1 Introduction	155
4.1.1 Mechanisms of TLR7/8 Activation by ssRNAs	155
4.1.2 Role of MyD88 Signalling in Cytokine Production and Immune Activation	156
4.1.3 TLR7/8 in viral infections	157
4.1.4 Structural and Functional Properties of SARS-CoV-2 ssRNAs	158
4.1.5 The Role of TLR7 and TLR8 in Neutrophil Activation and Function	159
4.1.6 Neutrophil Apoptosis and Lifespan	161
4.1.8 Poly(I:C) as a Mimic of SARS-CoV-2 for Studying Neutrophil Responses	162
4.1.8 Rationale and Knowledge Gaps	163
4.2 Hypothesis	163
4.3 Specific Aims	164
4.4 Experiments & Discoveries:	167
4.4.1: TLR7 and TLR8 Expression and Transcriptional Activation in Whole Blood Are Associated with the Severity of COVID-19	167

4.4.2 The Role of Cytokines in COVID-19 Severity: Focus on IL-1 β , TNF- α , IL-6, and IL-8	168
4.4.3 SARS-CoV-2 GU-Rich ssRNAs does not induce the expression of CD66b in human primary PMNs:	169
4.4.4 SARS-CoV-2 ssRNA SCV2-RNA1 Induces CD11b Expression in human Primary PMNs:	171
4.4.5 CD16 Shedding in human primary PMNs Remains Unaffected by SARS-CoV-2 ssRNA SCV2-RNA1	172
4.4.6 SARS-CoV-2 ssRNA SCV2-RNA1 Induces TLR8 Expression in human Primary PMNs:	173
4.4.8 MPO Production in human primary PMNs Remains Unaffected by SARS-CoV-2 ssRNA SCV2-RNA1	176
4.4.9 NETosis Induction in human primary PMNs Remains Unaffected by SARS-CoV-2 ssRNA SCV2-RNA1	177
4.4.10 The TGF- β levels in human primary PMNs remain unchanged by SARS-CoV-2 ssRNA SCV2-RNA1.	178
4.4.11 SCV2-RNA2 has no Effects on human primary PMN Apoptosis and Cell Survival	179
4.4.12 SCV2-RNA2 has no Effects on human primary PMNs Phagocytosis	181
4.4.13 SARS-CoV-2 ssRNA SCV2-RNA1 Induces Proinflammatory Cytokine Production in human Primary PMNs	182
4.4.14 SCV2-RNA2-Induced Modulation of I κ B α Levels in PMNs: Activation and Recovery of NF- κ B Signalling	184
4.4.15 NF- κ B, MAPK, and TLR8 Inhibitors Do Not Significantly Reduce Viability in Primary Human PMNs	185
4.4.16 SCV2-RNA2 Induces Proinflammatory Cytokine Production in PMNs via a TLR8-Dependent Mechanism	187
4.4.18 SCV2-RNA2 Induces Proinflammatory Cytokine Production in PMNs via a NF- κ B -Dependent Mechanism	189
4.4.19 SCV2-RNA2 Induces Proinflammatory Cytokine Production in PMNs via a ERK -Dependent Mechanism	190
4.4.20 SCV2-RNA2 Induces Proinflammatory Cytokine Production in PMNs via a p38-Dependent Mechanism	191
4.4.21 SCV2-RNA2 Induces ISG Induction in PMNs Following SCV2-RNA2 Stimulation	192
4.4.22 dsRNA Does Not Upregulate Activation Marker Expression in Primary Human PMNs	193
4.4.23 dsRNA induces ROS production in Primary Human PMNs	194
4.4.24: Poly(I:C) does not induce Proinflammatory Cytokine Production in Primary Human PMNs ...	195
4.4.25: Poly(I:C) induces I κ B- α Degradation in primary human PMNs	196
4.4.26 Poly(I:C) induces ISGs in primary human PMNs	197
4.5 Discussion	198
4.5.1 TLR7/8 Expression in COVID-19 and Neutrophils	199
4.5.2 PMN Activation Markers	199
4.5.3 Pro-inflammatory cytokine production	200
4.5.4 ROS and Degranulation	201
4.5.5 NETosis	201
4.5.6 Anti-inflammatory Signalling and Neutrophil Lifespan	202
4.5.7 ISG induction by SARS-CoV-2 RNA in PMNs	203
4.5.8 Double-Stranded RNA (Poly I:C) Responses	203
4.6 Future Work	204
4.7 Conclusion	205
Chapter 5:	207
<i>PMNs and Their Role in SARS-CoV-2 Infection: Direct and Indirect Interactions</i>	<i>207</i>
5.1 Introduction	208
5.2 Neutrophil Responses to SARS-CoV-2 Variants: Implications for COVID-19 Severity	209
5.3 Specific Aims	210

5.4 Experiments & Discoveries	215
5.4.1 SARS-CoV-2 Delta Variant infection does not induce the expression of CD66b in Primary Human PMNs.....	215
5.4.2 SARS-CoV-2 Delta Variant infection decreased the expression of CD11b in Primary Human PMNs	216
5.4.3 SARS-CoV-2 Delta Variant infection does not induce shedding of CD16 in Primary Human PMNs	217
5.4.4 SARS-CoV-2 Delta Variant infection does not induce the shedding of CD62L in Primary Human PMNs.....	218
5.4.5 SARS-CoV-2 Delta Variant infection induce the expression of CD63 in Primary Human PMNs ..	220
5.4.6 SARS-CoV-2 Delta Variant infection does not induce the expression of CD64 in Primary Human PMNs.....	221
5.4.7: Gene Expression Analysis of Proinflammatory Cytokines in PMNs Increased IL-1 β mRNA Following SARS-CoV-2 Delta Variant Infection.....	222
5.4.8: SARS-CoV-2 Delta Variant Infection does not induce I κ B α Degradation in PMNs	223
5.4.9 Protein Expression Analysis of Proinflammatory Cytokines in PMNs Reveals Increased IL-1 β Levels Following SARS-CoV-2 Delta Variant Infection	224
5.4.10: TLR8 Signalling Contributes to IL-1 β Induction in PMNs During SARS-CoV-2 Delta Variant Infection	225
5.4.11: SARS-CoV-2 Delta Variant Modulates ISGs Expression in PMNs.....	226
5.4.12: SARS-CoV-2 Delta Variant Modulates STAT1 Phosphorylation in PMNs in a Viral Dose-Dependent Manner.....	227
5.4.13: SARS-CoV-2 Delta Variant Does Not Significantly Induce PMN Degranulation.....	228
5.4.14: Detection of SARS-CoV-2 Nucleocapsid Protein and RNA in PMNs Following SARS-CoV-2 Delta Variant Infection	229
5.4.15: SARS-CoV-2 Strain- and Time-Dependent Induction of IL-8 in A549-ACE2 Cells and Its Potential Role in PMNs Recruitment.....	230
5.4.16: Soluble Factors from SARS-CoV-2-Infected Epithelial Cells Modulate PMNs CD66b Expression in a Strain-Dependent Manner	231
5.4.17: PMNs Activation in Response to Soluble Factors from SARS-CoV-2-Infected Epithelial Cells: Time- and Strain-Dependent Effects on CD11b Expression	232
5.4.18: CD16 Expression and Shedding in PMNs Treated with Supernatants from SARS-CoV-2-Infected Epithelial Cells	233
5.4.19: Soluble Factors from SARS-CoV-2-Infected Epithelial Cells Modulate CD62L Shedding in PMNs in a Strain- and Time-Dependent Manner	234
5.4.20: Effect of SARS-CoV-2-Infected Epithelial Cell Supernatants on Neutrophil ROS Production ...	235
5.4.21: Effect of SARS-CoV-2-Infected Epithelial Cell Supernatants on PMNs NETosis.....	236
5.4.22: Effect of SARS-CoV-2-Infected Epithelial Cell Supernatants on PMNs MPO Release.....	237
5.4.23: Effect of SARS-CoV-2-Infected Epithelial Cell Supernatants on PMN NE Release.....	238
5.5 Discussion.....	240
5.5.1 Direct Effects of SARS-CoV-2 on PMNs	240
5.5.2 Indirect Effects via Epithelial Cell-Derived Soluble Mediators	244
5.5.4 Limitations and Future Directions.....	247
5.6 Conclusion	247
6. General Discussion	250
6.1 Introduction	250
6.2 Overview of Key Findings.....	250
6.4 SARS-CoV-2 SP Delays PMN Apoptosis and Promotes Cell Survival	253
6.5 SARS-CoV-2 SP Induces TGF-β Production in Human Primary PMNs via Integrin-Dependent Mechanisms (ATN-161)	254
6.6 TLR8 Recognizes GU-Rich ssRNA in SARS-CoV-2	255

6.7 SARS-CoV-2 Delta Variant Alters Neutrophil Activation Markers and Induces IL-1 β via TLR8-PKR-STAT1 Axis	256
6.8 IC-19-Infected Epithelial Supernatants Show a Trend Toward NE Release Without Significant Activation	257
6.9 Methodological Strengths and Innovations	257
6.10 Limitations of the Study	259
6.12 Concluding Remarks	261
7. References	264

Declaration of Authorship

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List of Figures

Figure 1.1 MHC Class I and II Pathways.	30
Figure 1.2 Cellular Locations of PRRs.	31
Figure 1.3 TLRs and Their Recognized PAMPs.	34
Figure 1.4 TLR Signalling Pathways: MyD88-Dependent and TRIF-Dependent Mechanisms.	37
Figure 1.5 TLR-Mediated NF- κ B and MAPK Signalling Pathways in Viral Infections.....	41
Figure 1.6 TLR8 Expression and Signalling Pathway Comparison with TLR7.....	45
Figure 1.7 JAK-STAT signalling Pathway in Type I, II, and III IFN Responses.	52
Figure 1.8 TGF- β Signalling Pathway: Canonical and Non-Canonical Pathways.	66
Figure 1.9 Neutrophil Development and Granulopoiesis.	68
Figure 1.10 Neutrophils in Viral Defence: Mechanisms of Phagocytosis, Degranulation, ROS Production, and NETosis.	75
Figure 1.11 Morphologically distinct subsets of neutrophils are observed in circulation. ..	76
Figure 2.1 Gating strategy for identification of PMNs.....	95
Figure 3.1 TLR-Mediated NF- κ B and MAPK Signalling Pathways in Response to SARS-CoV-2 SP.	104
Figure 3.2 CD66b and CD11b mRNA expression in COVID-19 patients with moderate, severe and critical disease and healthy individuals.....	112
Figure 3.3 Neutrophil activation marker expression in PMNs from COVID-19 and healthy individuals.....	113
Figure 3.4 CD66b expression in treated PMN with SARS-CoV-2 SP.....	114
Figure 3.5 CD11b expression in treated PMNs treated with SARS-CoV-2 SP.....	115
Figure 3.6 CD16 expression in treated PMNs with SARS-CoV-2 SP.	116
Figure 3.7: CD62L expression in PMNs treated with SARS-CoV-2 SP.....	117
Figure 3.8 ROS production in PMNs treated with SARS-CoV-2 SP.....	119
Figure 3.9 NETosis formation in treated PMNs with SARS-CoV-2 SP.	120
Figure 3.10 MPO and NE release in PMNs treated with SARS-CoV-2 SP.	122
Figure 3.11 MMP9 expression in PMNs treated with SARS-CoV-2 SP.....	123
Figure 3.12 Effect of SARS-CoV-2 SP on PMNs phagocytosis.	125
3.4.12 SARS-CoV-2 SP Does Not Induce Type I or Type III IFN mRNA Expression in human primary PMNs.....	125
Figure 3.13 mRNA expression of IFNs in PMNs treated with SARS-CoV-2 SP or poly I:C.	126
Figure 3.14 ACE2 expression in PMNs under baseline and inflammatory conditions.	128
Figure 3.15 Effect of IL-10 and TGF- β on ACE2 mRNA expression in PMNs.	129
Figure 3.16 SARS-CoV-2 SP Modulates Apoptosis in PMNs.....	131
Figure 3.17: SARS-CoV-2 SP Selectively Induces IL-8 Production in Human PMNs. ..	132
Figure 3.18 Effect of TLR4 and TLR2 Inhibition on IL-8 Production Induced by SARS-CoV-2 SP in Human Primary PMNs.	134
Figure 3.19 Effect of NF- κ B, p38 MAPK, and ERK1/2 Inhibition on IL-8 Production Induced by SARS-CoV-2 SP in Human Primary PMNs.	136
Figure 3.20 Induction of TGF- β by SARS-CoV-2 SP in Human Primary PMNs.....	137
Figure 3.21 Integrin-Dependent Mechanism of SARS-CoV-2 SP-Induced TGF- β Production Human Primary PMNs.	138
Figure 3.22 Assessment of TGF- β Effects on PMN Activation Marker Expression Human Primary PMNs.	140
Figure 3.23 Effect of TGF- β on Basal and tBuOOH-Induced ROS Production in Human Primary PMNs.	141

Figure 3.24 Effect of TGF- β on Basal and PMA-Induced NETosis in Neutrophils Assessed by Flow Cytometry.....	143
Figure 3.25 Effect of TGF- β on Neutrophil Phagocytosis of GFP-Expressing <i>S. aureus</i>	144
Figure 3.26: Effects of TGF- β on Human Primary PMN Degranulation.....	145
Figure 3.27: Selective Modulation of PMN Functions by SARS-CoV-2 SP.....	153
Figure 4. 1 SARS-CoV-2 Detection by TLR8 and Induction of Immune Responses.....	157
Figure 4. 2 TLR8 Activation in Neutrophils by SARS-CoV-2 ssRNA and Associated Cellular Responses	160
Figure 4.3 SARS-CoV-2 replication cycle showing dsRNA intermediate formation.....	162
Figure 4.4 TLR7 and TLR8 Expression and Their Downstream Signalling Molecules Correlate with Disease Severity in COVID-19 Patient.	168
Figure 4.5 Expression of Key Pro-Inflammatory Cytokines in Relation to COVID-19 Disease Severity.	169
Figure 4.6 Evaluation of CD66b Expression as a Marker of Neutrophil Activation Following Treatment with SARS-CoV-2 ssRNAs.....	170
Figure 4.7 Induction of CD11b Expression in PMNs by SARS-CoV-2 ssRNA SCV2-RNA1.....	172
Figure 4.8 Evaluation of CD16 Shedding in PMNs Following Treatment with SARS-CoV-2 ssRNA SCV2-RNA1.....	173
Figure 4.9 TLR8 expression in PMNs following treatment with CL075, SCV2-RNA2, and SCV2-RNA2A.....	174
Figure 4.10 Assessment of ROS Production in PMNs Treated with SARS-CoV-2 ssRNA and TLR7/8 Agonist CL075.....	175
Figure 4.11 Analysis of MPO Degranulation in PMNs Treated with SARS-CoV-2 ssRNA.....	176
Figure 4.12 Assessment of NETosis in PMNs Treated with SARS-CoV-2 ssRNA Using SYTOX Orange Fluorescence.....	178
Figure 4.13 Assessment of TGF- β production in PMNs Treated with SARS-CoV-2 ssRNA.....	179
Figure 4.14 Flow Cytometric Analysis of Apoptosis in PMNs Treated with SCV2-RNA2.....	180
Figure 4.15 Analysis of PMN Phagocytosis Following Treatment with SARS-CoV-2 ssRNA Sequences.....	182
Figure 4.16 SARS-CoV-2 ssRNA Induces IL-8, IL-6, IL-1 β and TNF- α Production in Human PMNs	184
Figure 4.17 SCV2-RNA2-Induced I κ B α Degradation at 2 Hours and Restoration at 4 Hours in Human PMNs	185
Figure 4.18 Impact of NF- κ B, MAPK, and TLR8 Inhibitors on PMN Viability After Stimulation with LPS or CL075.....	186
Figure 4.19 Proinflammatory Cytokine Production in PMNs Treated with SCV2-RNA2 and Inhibited by the TLR8 Inhibitor CU-CPT9a.....	188
Figure 4.20 TLR8 Inhibition by CU-CPT9a Reduces SCV2-RNA2-Induced NF- κ B Activation in Primary Human Neutrophils.....	189
Figure 4.21 NF- κ B Inhibition Blocks SCV2-RNA2-Induced IL-8, IL-6, TNF- α , and IL-1 β Production in Human PMNs.....	190
Figure 4.22 ERK Inhibition with PD98059 Reduces SCV2-RNA2-Induced Proinflammatory Cytokine Production in Primary Human PMNs.....	191
Figure 4.23 Effect of p38 Inhibition on SCV2-RNA2-Induced Proinflammatory Cytokine Production in Primary Human PMNs.....	192

Figure 4.24 SCV2-RNA2 Stimulation Upregulates ISG15, PKR, and Viperin Expression in Human PMNs.	193
Figure 4.25 Effect of Poly(I:C) Treatment on PMNs Activation Markers.....	194
Figure 4.26 Effect of Poly(I:C) Treatment on ROS Production in Human PMNs.....	195
Figure 4.27 Effect of Poly(I:C) on Proinflammatory Cytokine Production in Primary Human PMNs	196
Figure 4. 28 Time-Dependent Degradation of I κ B- α in PMNs Treated with Poly(I:C)....	197
Figure 4. 29 Time-Course Analysis of ISGs Expression in Poly(I:C)-Treated PMNs.....	198
Figure 4.26. Summary of TLR8-Mediated Neutrophil Responses to SARS-CoV-2 GU-Rich ssRNA	206
Figure 5. 1: CD66b Expression in PMNs Following SARS-CoV-2 Delta Variant Infection.	215
Figure 5. 2: CD11b Expression in PMNs Following SARS-CoV-2 Delta Variant Infection.	217
Figure 5. 3: CD16 Expression in PMNs Following SARS-CoV-2 Delta variant infection.	218
Figure 5. 4: CD62L Expression in PMNs Following SARS-CoV-2 Delta Variant Infection.	219
Figure 5. 5: CD63 Expression in PMNs Following SARS-CoV-2 Delta Variant Infection.	220
Figure 5. 6: CD64 Expression in PMNs Following SARS-CoV-2 Delta Variant Infection.	222
Figure 5. 7: qPCR Analysis of IL-6, IL-8, TNF- α , and IL-1 β Expression in PMNs Following SARS-CoV-2 Delta Variant Infection.....	223
Figure 5. 8: I κ B α Levels in PMNs Following SARS-CoV-2 Delta Variant Infection.	224
Figure 5. 9: SARS-CoV-2 Delta Variant Induces IL-1 β Production in Human PMNs.....	225
Figure 5. 10 SARS-CoV-2 Delta Variant Induces IL-1 β Expression in Neutrophils via TLR8-Dependent Signaling	226
Figure 5. 11 Induction of PKR in PMNs Following SARS-CoV-2 Delta Variant Infection.	227
Figure 5. 12: Phosphorylation of STAT1 in PMNs Following SARS-CoV-2 Delta Variant.	228
Figure 5. 13: MPO and NE Release in PMNs Following SARS-CoV-2 Delta Variant Infection.	229
Figure 5. 14 Detection of SARS-CoV-2 Nucleocapsid Protein in PMNs by Western Blot and qPCR.	230
Figure 5. 15: IL-8 Production in A549-ACE2 Cells Following Infection with SARS-CoV-2 Strains at Different Time Points.	231
Figure 5. 16: CD66b Expression in PMNs Treated with Supernatants from SARS-CoV-2-Infected A549-ACE2 Cells.....	232
Figure 5. 17: CD11b Expression in PMNs Treated with Supernatants from SARS-CoV-2-Infected A549-ACE2 Cells.	233
Figure 5. 18: CD16 Expression in Neutrophils Treated with Supernatants from SARS-CoV-2-Infected A549-ACE2 Cells.....	234
Figure 5. 19: CD62L Shedding in Neutrophils Treated with Supernatants from SARS-CoV-2-Infected A549-ACE2 Cells.....	235
Figure 5. 20: ROS Production in Neutrophils Treated with Supernatants from SARS-CoV-2-Infected A549-ACE2 Cells.....	236
Figure 5. 21 Effect of SARS-CoV-2-Infected Epithelial Cell Supernatants on Neutrophil NETosis.....	237

Figure 5. 22 MPO Release in PMNs Treated with Supernatants from SARS-CoV-2-Infected A549-ACE2 Cells.....	238
Figure 5. 23 NE Release in Response to Supernatants from SARS-CoV-2-Infected A549-ACE2 Cells.....	239
Figure 5. 24 Distinct Direct and Indirect Effects of SARS-CoV-2 Delta Variant on PMN Function.....	248
Figure 6.1. SARS-CoV-2-mediated innate immune signalling pathways in human PMNs.....	262

List of Tables

Table 1. 1 Components of the Innate Immune System.....	28
Table 1. 2 SARS-CoV-2 IFN Inhibition Mechanisms.....	54
Table 1. 3 Key ISGs and Their Functions.....	57
Table 1. 4 Summary of Neutrophil Surface Marker Expression Changes Across Different Viral Infections	70
Table 1. 5 Clinical Criteria for Classification of COVID-19 Severity Levels.....	78
Table 1. 6 Comparison of Neutrophil Responses in COVID-19: In Vivo Versus In Vitro Findings	79
Table 2. 1 List of intracellular signalling inhibitors	88
Table 2. 2 List of cell culture agonists and viruses.....	89
Table 2. 4 Primer sequences	91
Table 2. 5 Composition of Resolving Gel	92
Table 2. 6 Composition of Stacking Gel.....	92
Running Buffer	92
Table 2. 7 Composition of running buffer	92
Table 2. 8 Composition of 5x Sample Buffer.....	93
Table 2. 9 Composition of 10X transfer buffer.....	93
Table 2. 10 Antibodies used in Western immunoblotting.	93
Table 2. 11 Fluorescently conjugated antibodies and dyes.....	94

Abbreviations

A

ACE2 – Angiotensin-converting enzyme 2
ACK – Ammonium-Chloride-Potassium lysis buffer
ADCC – Antibody-dependent cellular cytotoxicity
AIM2 – Absent in melanoma 2
AP-1 – Activator protein-1
APC – Allophycocyanin
APCs – Antigen-presenting cells
ANOVA – Analysis of variance (statistical test)
ARDS – Acute respiratory distress syndrome
ATP – Adenosine triphosphate
AZU1 – Azurocidin 1

B

BAFF – B-cell Activating Factor
BAFFR – B-cell activating factor receptor
BMPs – Bone morphogenetic proteins
BSA – Bovine Serum Albumin
BST-2 – Bone marrow stromal cell antigen 2 (Tetherin)
 β -Actin – Beta-actin

C

CHIKV – Chikungunya virus
CAP – Community-acquired pneumonia
CARDs – Caspase activation and recruitment domains
CBV – Coxsackie B virus
CCL2 – C-C motif chemokine ligand 2
CH25H – Cholesterol-25-hydroxylase
CMV – Cytomegalovirus
COPD – Chronic obstructive pulmonary disease
COVID-19 – Coronavirus disease 2019

D

DAMPs – Damage-associated molecular patterns
DCs – Dendritic cells
DENV – Dengue virus

DMEM – Dulbecco’s Modified Eagle Medium

DNA – Deoxyribonucleic acid

dsRNA – Double-stranded RNA

DV2 – Dengue virus serotype 2

E

EBOV – Ebola virus

ECM – Extracellular matrix

EDTA – Ethylenediaminetetraacetic acid

ECL – Enhanced chemiluminescence

ELANE – Neutrophil elastase gene

ELISA – Enzyme-linked immunosorbent assay

EMT – Epithelial-to-mesenchymal transition

ER – Endoplasmic reticulum

EVD – Ebola virus disease

F

FACS – Fluorescence-activated cell sorting

FBS – Fetal bovine serum

FcγRI – Fc gamma receptor I

FGF-2 – Fibroblast growth factor-2

FITC – Fluorescein isothiocyanate

FSC – Forward scatter

G

G-CSF – Granulocyte colony-stimulating factor

GAS – Gamma-activated sequence

GDFs – Growth differentiation factors

GFP – Green Fluorescent Protein

GM-CSF – Granulocyte-macrophage colony-stimulating factor

GP – Glycoprotein

H

HA – Hemagglutinin

HBSS – Hank’s Balanced Salt Solution

HBV – Hepatitis B virus

HCV – Hepatitis C virus

HEK293T – Human embryonic kidney 293T cells

HEVs – High endothelial venules
HIV – Human immunodeficiency virus
HIV-1 – Human immunodeficiency virus type 1
HPV – Human papillomavirus
HMGB1 – High-mobility group box 1
HPEV-1 – Human parechovirus 1
HSCs – Hematopoietic stem cells
HSPs – Heat shock proteins
HSV – Herpes simplex virus
HSV-1 – Herpes simplex virus type 1
HSV-2 – Herpes simplex virus type 2

I

IAV – Influenza A virus
IVIg – Intravenous immunoglobulin therapy
IDT – Integrated DNA Technologies (company)
IFN – Interferon
IFN-I– Type I IFN
IFN- α – Interferon-alpha
IFN- β – Interferon-beta
IFN- γ – Interferon-gamma
IFNAR – Interferon-alpha/beta receptor
IFNGR – Interferon-gamma receptor
IFNs – Interferons
IgA – Immunoglobulin A
IgG – Immunoglobulin G
IgM – Immunoglobulin M
IKK – I κ B kinase
IL-1 – Interleukin-1
IL-1 β – Interleukin-1 beta
IL-2 – Interleukin-2
IL-6 – Interleukin-6
IL-8 – Interleukin-8
IL-10 – Interleukin-10
IRAKs – Interleukin-1 receptor-associated kinases

IRF3 – Interferon regulatory factor 3
IRF7 – Interferon regulatory factor 7
IRFs – Interferon regulatory factors
ISG15 – Interferon-stimulated gene 15
ISGs – Interferon-stimulated genes
ISRE – Interferon-stimulated response element
ISREs – Interferon-stimulated response elements

J

JAK – Janus kinase
JAKs – Janus kinases

K

KSHV – Kaposi's sarcoma-associated herpesvirus

L

LACV – La Crosse encephalitis virus
LAM – Lipoarabinomannan
LAPs – Latency-associated peptides
LBP – LPS-binding protein
LDGs – Low-density granulocytes
LDNs – Low density neutrophils
LMPPs – Lymphoid-primed multipotent progenitors
LPS – Lipopolysaccharide
LRR – Leucine-rich repeat
LTBP – Latent TGF- β binding protein
LT β R – Lymphotoxin β receptor

M

MAC – Membrane attack complex
MARV – Marburg virus
MAVS – Mitochondrial antiviral-signaling protein
MCP-1 – Monocyte Chemoattractant Protein-1
MDA5 – Melanoma differentiation-associated protein 5
MFI – Mean fluorescence intensity
MHC – Major histocompatibility complex
MIS – Müllerian-inhibiting substance
MMP-9 – Matrix metalloproteinase-9

MOI – Multiplicity of infection

MPO – Myeloperoxidase

MPP – Multipotent progenitor

mtDNA – Mitochondrial DNA

MV – Measles virus

Mx1 – Myxovirus resistance 1

MyD88 – Myeloid differentiation primary-response protein 88

N

NADPH – Nicotinamide adenine dinucleotide phosphate (reduced)

NDNs – Normal density neutrophils

NE – Neutrophil elastase

NETs – Neutrophil extracellular traps

NF- κ B – Nuclear factor kappa-light-chain-enhancer of activated B cells

NK – Natural killer (cells)

NLR – NOD-like receptor

N-LR – Neutrophil-to-lymphocyte ratio

NLRP3 – NOD-, LRR- and pyrin domain-containing protein 3

NLRs – NOD-like receptors

NOD – Nucleotide oligomerization domain

Nox4 – NADPH oxidase 4

NS1 – Non-structural protein 1

O

ORF – Open reading frame

P

PAMPs – Pathogen-associated molecular patterns

PBMCs – Peripheral blood mononuclear cells

PBS – Phosphate-buffered saline

PDC – Plasmacytoid dendritic cell

pDCs – Plasmacytoid dendritic cells

PEDV – Porcine epidemic diarrhea virus

PFA – Paraformaldehyde

PI – Propidium iodide

PKC – Protein kinase C

PKR – Protein kinase R

PMA – Phorbol 12-myristate 13-acetate
PMN – Polymorphonuclear leukocyte
PMNs – Polymorphonuclear neutrophils
PRRs – Pattern recognition receptors
PRTN3 – Proteinase 3 or myeloblastin
PTK – Protein tyrosine kinase
PVDF – Polyvinylidene fluoride
Poly I:C – Polyinosinic:polycytidylic acid

Q

qPCR – Quantitative polymerase chain reaction
qRT-PCR – Quantitative real-time polymerase chain reaction

R

RANK – Receptor activator of NF- κ B
RANKL – Receptor activator of NF- κ B ligand
RBD – Receptor-binding domain
RdRp – RNA-dependent RNA polymerase
RIG-I – Retinoic acid-inducible gene I
RLRs – RIG-I-like receptors
RNA – Ribonucleic acid
ROS – Reactive oxygen species
RSV – Respiratory syncytial virus

S

SARS-CoV-2 – Severe acute respiratory syndrome coronavirus 2
SARS-CoV-2 SP – Spike Protein of SARS-CoV-2
SARM – Sterile α - and armadillo-motif-containing protein
SDF – Stromal cell-derived factor 1
SDS-PAGE – Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM – Standard error of the mean
SH2 – Src homology 2 (domain)
sGP – Secretory glycoprotein
sIL-6R – Soluble IL-6 receptor
SLC – Small latent complex
SOCS1 – Suppressor of cytokine signalling 1
SPR – Surface plasmon resonance

SSC – Side scatter (flow cytometry) (cells granularity measure)
ssRNA – Single-stranded RNA
S. aureus – Staphylococcus aureus
STAT – Signal transducer and activator of transcription
StIP – STAT-interacting protein

T

TAK1 – Transforming growth factor- β -activated kinase 1
TANs – Tumour-associated neutrophils
TAP – Transporter associated with antigen processing
Tat – Transactivator of transcription
TBK1 – TANK-binding kinase 1
tBuOOH – tert-Butyl hydroperoxide
TGF- β – Transforming growth factor-beta
TGFBR1 – Transforming growth factor- β receptor 1
T β Rs – Transforming growth factor- β receptors
Th1 – T helper type 1
Th2 – T helper type 2
Th17 – T helper type 17
TIR – Toll/IL-1 receptor (domain)
TLR – Toll-like receptor
TLR2 – Toll-like receptor 2
TLR3 – Toll-like receptor 3
TLR4 – Toll-like receptor 4
TLR7 – Toll-like receptor 7
TLR8 – Toll-like receptor 8
TLRs – Toll-like receptors
TMB – 3,3',5,5'-Tetramethylbenzidine
tmTNF- α – Transmembrane TNF- α
TNF- α – Tumour necrosis factor-alpha
TNFR1 – TNF receptor 1
TNFR2 – TNF receptor 2
TRAF6 – TNF receptor-associated factor 6
TRAM – TRIF-related adaptor molecule

Tregs – Regulatory T cells

TRIF – TIR domain-containing adaptor protein inducing IFN- β

V

VOC – Variant of concern

VZV – Varicella-zoster virus

W

WNV – West Nile virus

Z

ZIKV – Zika virus

Abstract

Human polymorphonuclear leukocytes (neutrophils) are critical innate immune cells that deploy antimicrobial and pro-inflammatory mechanisms, including reactive oxygen species (ROS) generation, cytokine secretion, degranulation, Neutrophil Extracellular Traps (NETs), and phagocytosis. While their functions against bacteria and fungi are well defined, their roles in viral infections are less clear. Severe Coronavirus disease 2019 (COVID-19) displays an atypical immune profile for a viral infection, marked by lymphopenia and neutrophilia, with disease severity closely associated with an elevated neutrophil-to-lymphocyte ratio. Massive neutrophil infiltration into the lungs is a hallmark of critical disease, yet the molecular pathways by which neutrophils sense Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and shape inflammation remain incompletely understood. Therefore, this study addressed three aims: (1) to assess the effects of purified SARS-CoV-2 spike protein (SP) on neutrophil activation, effector functions, and cytokine release; (2) to determine whether GU-rich viral single-stranded RNA (ssRNA) activates neutrophils via Toll-like receptor 8 (TLR8) and to characterize the downstream inflammatory responses; and (3) to compare neutrophil responses to direct infection with live SARS-CoV-2 (Delta variant) versus indirect stimulation by soluble mediators from infected lung epithelial cells. A combination of flow cytometry, functional assays, ELISA, qRT-PCR, and Western blotting was employed to assess neutrophil phenotypes, effector responses, cytokine production, and intracellular signalling pathways. Distinct responses emerged depending on the viral component encountered. The SARS-CoV-2 SP did not induce classical neutrophil activation, as shown by the absence of CD66b and CD11b upregulation, CD16 and CD62L downregulation, ROS production, or degranulation. Instead, SARS-CoV-2 SP selectively triggered IL-8 secretion via TLR2/4, promoted TGF- β release through integrin-dependent signalling, suppressed NET formation, and prolonged neutrophil survival. In contrast, GU-rich viral ssRNA elicited robust inflammatory activation through TLR8, driving CD11b upregulation, Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF- κ B) signalling, and strong secretion of TNF- α , IL-6, IL-1 β , and IL-8. These responses were abolished entirely by TLR8 inhibition. Direct infection with the Delta variant produced a distinct phenotype, downregulating CD11b while upregulating CD63 (a degranulation marker), inducing potent TLR8-dependent IL-1 β release, and activating antiviral PKR and STAT1 pathways. Finally, epithelial-cell-derived supernatants induced only modest and variable effects: Delta supernatants reduced ROS production, IC-19 supernatants enhanced neutrophil elastase release, and other viral strains

showed little activity. This study demonstrates that SARS-CoV-2 can modulate neutrophil function through distinct mechanisms, including selective anti-inflammatory responses to SP, TLR8-mediated inflammation triggered by ssRNA, and IL-1 β -driven inflammation following live virus infection. The findings indicate that neutrophils respond differently to individual viral components via distinct molecular pathways, which may help explain aspects of neutrophil dysfunction during COVID-19 and highlighting potential therapeutic targets for managing viral inflammatory responses while preserving beneficial immune functions.

Chapter 1

General Introduction

1. Introduction

1.1 Overview of the Immune System

The immune system consists of two main arms: innate immunity, which provides immediate, non-specific defence, and adaptive immunity, which develops more slowly and targets specific pathogens [1]. The innate immune system can be divided into three major components: physical barriers, cellular constituents, and humoral responses. The cellular and humoral components rely on pattern-recognition receptors (PRRs) to detect potential threats. These PRRs recognise a restricted set of evolutionarily conserved molecules shared by many microorganisms, such as lipopolysaccharides (LPS), peptidoglycans, unmethylated CpG DNA, and double-stranded RNA (dsRNA). These receptors are germline-encoded, allowing for immediate pathogen recognition without prior exposure, though their expression is often limited to specific cell types. Key examples of PRRs include the Toll-like receptor (TLR) family [1]. While this restricted recognition system provides rapid initial defence, it cannot address the vast diversity of potential pathogens. Unlike innate immunity, adaptive immunity generates pathogen-specific responses. Its specificity arises from the rearrangement of gene segments in T and B lymphocytes, enabling the production of unique antigen-binding molecules such as antibodies. Adaptive immunity also establishes immunological memory, allowing the body to respond more rapidly and effectively upon re-exposure to the same pathogen [2].

1.2 Components of the Innate Immune System

The innate immune system comprises several components, including physical and structural barriers, effector cells, cells that secrete antimicrobial peptides, soluble mediators, and surface receptors [3] (**Table 1.1**).

Table 1.1 Components of the Innate Immune System

Component	Function	Examples
Physical Barriers	Prevent pathogen entry	Skin, Mucous membranes, Tears, Saliva
Chemical Barriers	Destroy or inhibit pathogens	Stomach acid, Lysozymes in saliva and tears, Defensins
Cellular Defences	Detect, engulf, or kill pathogens	Macrophages, Neutrophils, Natural Killer (NK) Cells, Dendritic Cells (DCs)

Complement System	Enhances immune response via pathogen lysis	C3b (opsonization), C5a (inflammation), Membrane Attack Complex (MAC)
Cytokines & Chemokines	Regulate immune responses, recruit immune cells	Interferons (IFNs), TNF- α , Interleukins (Interleukin-1 (IL-1), IL-6)
Inflammatory Response	Localizes infection and recruits immune cells	Redness, Swelling, Heat, Pain (due to histamines, prostaglandins)
PRRs	Pathogen-associated molecular patterns (PAMPs)	TLRs, NOD-like Receptors (NLRs), RIG-I-like Receptors (RLRs)
Phagocytosis	Engulf and digest pathogens	Neutrophils, Macrophages, DCs
Fever Response	Inhibits pathogen growth and enhances immune activity	Pyrogens (IL-1, IL-6, TNF- α)

1.3 Interaction Between Innate and Adaptive Immunity

The adaptive immune response is activated and shaped by the innate immune system through several mechanisms, including:

1.3.1 Antigen Presentation by Innate Cells

DCs, macrophages, and B cells function as antigen-presenting cells (APCs), playing a crucial role in initiating adaptive immune responses. Using Major Histocompatibility Complex (MHC) molecules, APCs internalise pathogens, process their antigens, and present them to T lymphocytes. Distinct MHC classes elicit different T-cell responses: CD8⁺ cytotoxic T cells recognise antigens presented by MHC-I, whereas CD4⁺ helper T cells respond to antigens presented by MHC-II [4] (**Fig. 1.1**).

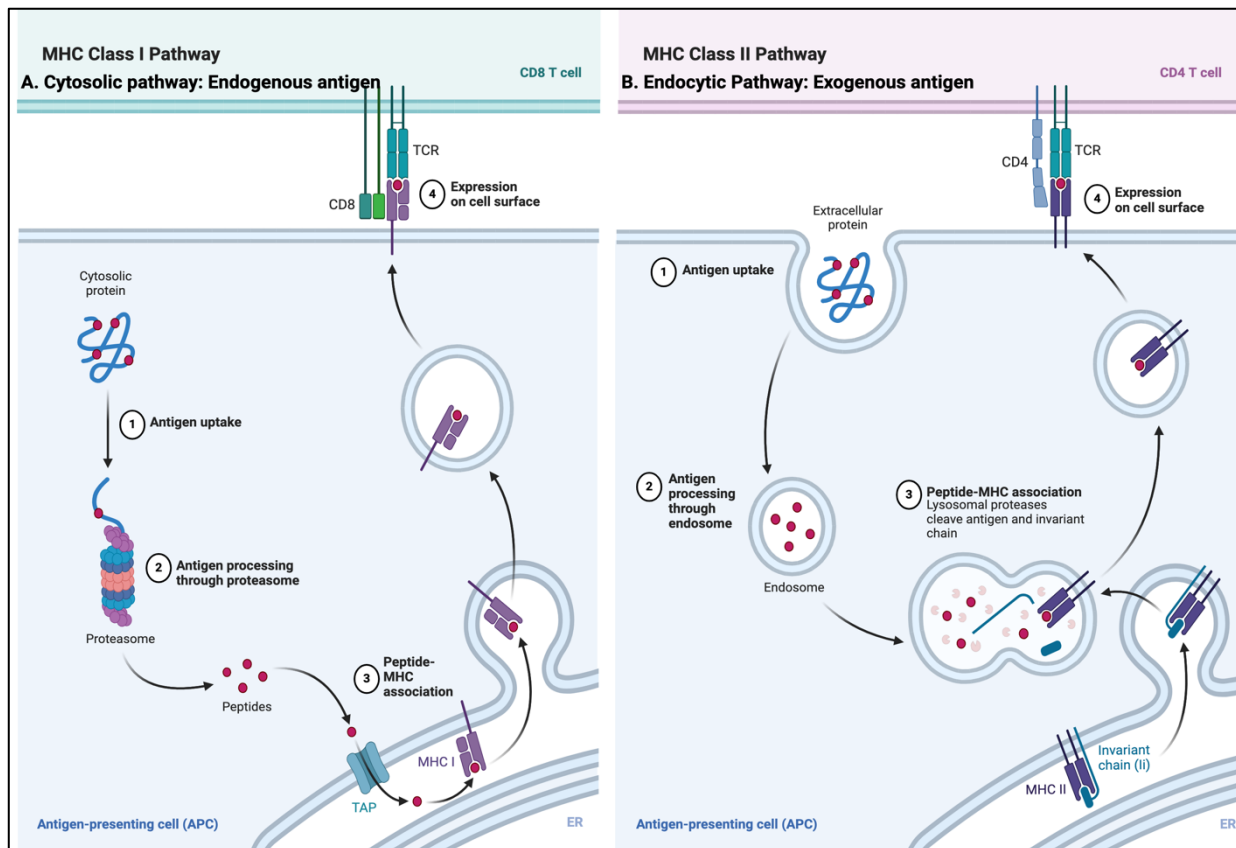


Figure 1.1 MHC Class I and II Pathways.

A. MHC Class I Pathway (Endogenous Antigen Processing)

The MHC Class I pathway presents intracellular antigens, including viral and abnormal self-proteins. Cytosolic proteins are first degraded into peptides by the proteasome. These peptides are then transported into the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP), where they bind to MHC Class I molecules. The resulting peptide–MHC I complexes are exported to the cell surface for recognition by CD8⁺ cytotoxic T cells. This interaction initiates targeted killing of infected or abnormal cells.

B. MHC Class II Pathway (Exogenous Antigen Processing)

The MHC Class II pathway presents extracellular antigens internalised by APCs such as DCs cells, macrophages, and B lymphocytes. Internalised antigens are degraded in endosomal compartments by lysosomal proteases. Meanwhile, the invariant chain (Ii), which initially occupies the peptide-binding groove of MHC Class II, is degraded, allowing antigenic peptides to load onto MHC Class II molecules. The peptide–MHC II complexes are then transported to the cell surface, where they are recognised by CD4⁺ helper T cells. Activated helper T cells enhance the activity of macrophages and stimulate B cells, orchestrating a coordinated immune response against extracellular pathogens [5].

1.3.2 TLRs and Danger Signals

PRRs, including NLRs, RLRs, TLRs, and C-type lectin receptors (CLRs), detect PAMPs from microbes as well as danger-associated molecular patterns (DAMPs) released by stressed or damaged host cells. Upon recognition of these signals, PRRs trigger specific intracellular pathways that drive the production of cytokines and co-stimulatory molecules essential for effective immune activation. In this way, PRR signalling bridges innate and adaptive immunity by enhancing antigen presentation, promoting T- and B-cell activation, and regulating inflammatory responses to ensure coordinated host defence [6].

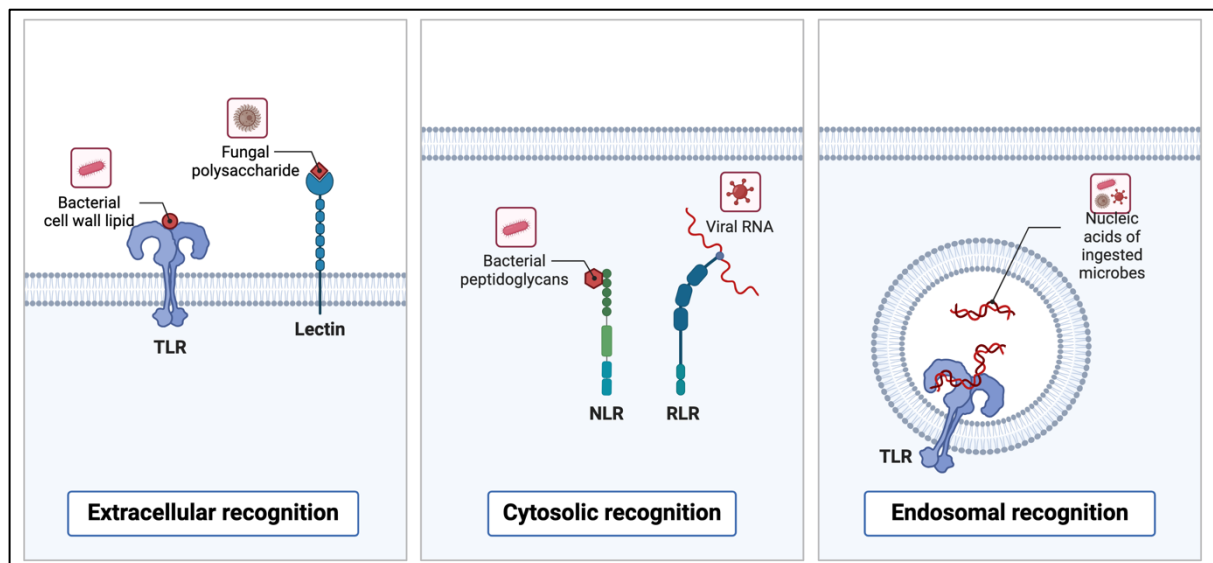


Figure 1.2 Cellular Locations of PRRs.

This diagram illustrates the cellular localisation of PRRs involved in pathogen detection and immune activation. PRRs are strategically positioned at different cellular locations to maximize pathogen detection. Cell-surface TLRs and C-type lectins mediate extracellular recognition of microbial components such as bacterial lipopolysaccharides and fungal β -glucans. This recognition activates signalling pathways that promote inflammation and immune responses. Cytosolic recognition is mediated by NLRs, which sense bacterial peptidoglycans, and RLRs, which detect viral RNA, thereby initiating responses to intracellular pathogens. Endosomal TLRs detect nucleic acids from phagocytosed microbes, enabling immune cells to recognise viral or bacterial genetic material. Through this strategic positioning, these receptors allow the innate immune system to detect a broad spectrum of pathogens and mount appropriate defensive responses.

1.3.3 Cytokine Signalling

Innate immune cells, including macrophages, dendritic cells, NK cells, and neutrophils, play a pivotal role in shaping adaptive immunity by producing cytokines such as IL-1, IL-6, TNF- α , and type I IFN, along with IFN- γ from NK cells. These cytokines drive T helper (Th) cell differentiation and thereby influence the type of adaptive immune response tailored to the nature of the invading pathogen. The Th1 response promotes cell-mediated immunity by activating cytotoxic T cells, which are essential for eliminating viruses and other intracellular pathogens. Conversely, the Th2 response supports B cell activation and antibody production to clear extracellular pathogens. The Th17 response contributes to mucosal immunity and inflammation, providing protection at barrier sites such as the gastrointestinal and respiratory tracts. Through this cytokine-based regulation, innate immunity ensures the adaptive response is appropriately coordinated and targeted against diverse infections [7].

1.4 Inflammation

Inflammation is the body's normal response to harmful stimuli, such as pathogens, cellular injury, or toxins. It occurs in vascularised tissues and aims to eliminate the cause of damage, repair injured tissue, and initiate healing. The innate immune system plays a central role in this process, which can be categorised by duration and resolution into acute and chronic inflammation [8]. Acute inflammation is a rapid, short-lived response characterised by vasodilation, increased vascular permeability, and neutrophil infiltration. It is triggered when PRRs such as TLRs recognise PAMPs or DAMPs, leading to the activation of pro-inflammatory transcription factors like NF- κ B. This, in turn, drives the release of key cytokines, including TNF- α , IL-1 β , and IL-6, which regulate vascular changes and coordinate leukocyte recruitment to sites of injury or infection [9]. Chronic inflammation, in contrast, represents a fundamentally different process: a prolonged immune response driven by persistent antigens, unresolved infections, or immune dysregulation. It is characterised by sustained infiltration of mononuclear cells, primarily macrophages and lymphocytes, along with fibroblast activation. This ongoing process involves continuous cytokine release and oxidative stress, ultimately [10]. Transforming Growth Factor-Beta (TGF- β) serves as a key mediator in chronic inflammation, modulating immune activity and driving tissue remodelling that can progress to fibrotic disease [11].

1.4.1 The role of TLR activation in inflammation

TLRs are a large family of PRRs in the innate immune system that detect PAMPs and DAMPs to initiate immune responses. Different immune cells express distinct sets of TLRs,

allowing for specialized recognition of specific molecular signatures and tailored immune responses [12].

1.4.2 Classification and Structure of TLRs

To date, 13 TLRs have been identified in mice and 10 in humans, although mouse TLR10 is non-functional. The first human TLR to be characterised was TLR4, which was shown to induce the expression of genes mediating inflammatory responses. The critical role of TLR4 in immune signalling was confirmed when a point mutation in the TLR4 gene was discovered in a mouse strain that failed to respond to LPS [13, 14].

Based on their localisation and function (Fig. 1.3), TLRs are functionally subdivided into two major groups:

- A- Cell membrane TLRs, which include heterodimers of TLR2 with TLR1 and TLR6, and TLR4, TLR5 and TLR10 that are expressed on the cell surface.
- B- Intracellular TLRs or nucleic acid sensors, including TLR3, TLR7, TLR8 and TLR9, which are expressed in the ER, endosomes and lysosomes [15].

TLRs are type I transmembrane proteins containing 20–27 extracellular leucine-rich repeats (LRRs) that mediate recognition of PAMPs and DAMPs, a transmembrane domain, and an intracellular Toll–interleukin-1 receptor (TIR) domain essential for downstream signal transduction. In addition to the LRRs, their extracellular domains also carry glycan moieties that contribute to protein stability and may influence ligand recognition, although the precise mechanisms of glycan-mediated ligand recognition remain unclear [16].

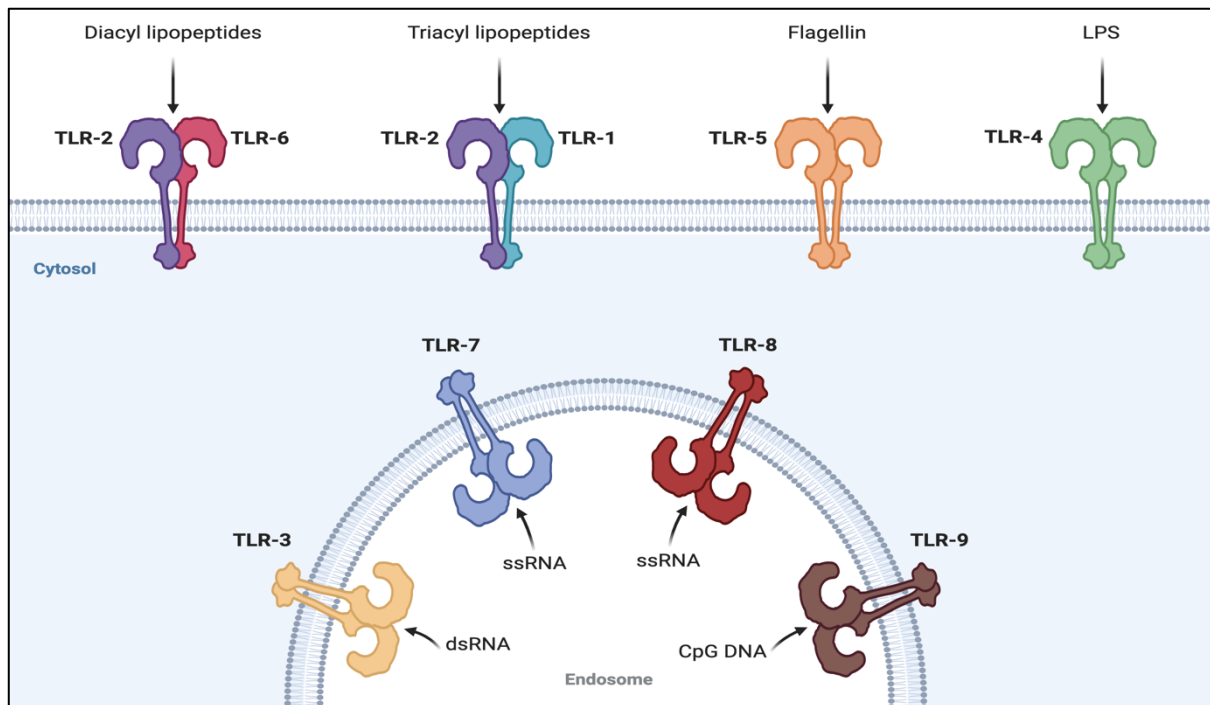


Figure 1.3 TLRs and Their Recognized PAMPs.

The figure illustrates the subcellular localisation and ligand specificity of different TLRs within the innate immune system. TLRs are located either on the plasma membrane or in endosomal compartments, where they act as detectors of PAMPs. On the plasma membrane, TLR2 associates with TLR1 or TLR6 to recognise diacyl and triacyl lipopeptides from bacterial lipoproteins, while TLR5 detects flagellin and TLR4 specifically senses LPS from Gram-negative bacteria. In endosomes, TLR3 dsRNA, a hallmark of viral infection, whereas TLR7 and TLR8 detect ssRNA, primarily from viral sources, and TLR9 recognises unmethylated CpG DNA from bacterial and viral origins. Upon ligand binding, these receptors activate intracellular signalling cascades that converge on NF- κ B, AP-1, and IRFs, driving the production of inflammatory cytokines, type I IFNs, and antimicrobial peptides. These responses are essential for pathogen clearance and host protection.

1.4.3 TLR Expression and Cellular Localization

Unlike many immune receptors restricted to specialised organs, TLRs are broadly expressed across multiple tissues. Their presence is not confined to immune sites such as the spleen, thymus, tonsils, lymphatic vessels, and lymph nodes but also extends to peripheral blood leukocytes, heart, liver, pancreas, gastrointestinal tract, lung, kidney, reproductive organs, skeletal muscle, and brain [17-19]. In terms of cellular distribution, TLRs are expressed on all innate immune cells, including macrophages, NK cells, DCs, and circulating leukocytes such as monocytes and neutrophils [20]. TLRs are also expressed on adaptive immune cells,

including T and B lymphocytes, as well as on non-immune cells such as epithelial cells, endothelial cells, and fibroblasts. This broad distribution underscores their central role in immune surveillance and the maintenance of tissue homeostasis [21-23].

1.4.4 Exogenous and Endogenous Ligands of TLRs

A- Exogenous Ligands of TLRs

PAMPs, acting as exogenous ligands, include various microbial molecular structures such as:

Flagellin from bacterial flagella.

Lipoteichoic acid (LTA) and peptidoglycan (PGN) from Gram-positive bacteria.

LPS from Gram-negative bacteria.

Lipoarabinomannan (LAM), lipopeptides, lipoglycans, and lipomannans from mycobacteria.

Zymosan from yeast.

Double-stranded (ds) RNA from viruses.

Bacterial and viral Deoxyribonucleic acid (DNA) [16, 24, 25].

B- Endogenous Ligands of TLRs

DAMPs, or endogenous ligands, are host-derived molecules that signal tissue injury and cellular stress. These ligands, which are typically produced during cellular damage or non-physiological cell death, include:

Extracellular matrix components (e.g., hyaluronan and fibrinogen).

Plasma membrane constituents.

Nuclear and cytosolic proteins include high-mobility group box protein 1 (HMGB1) and heat shock proteins (HSPs).

Elements of damaged organelles, such as mitochondrial DNA (mtDNA) [26-28].

1.4.5 Signalling Pathways of TLRs

The activation of TLRs triggers intracellular signalling cascades, leading to immune activation and inflammatory responses. These pathways produce pro-inflammatory cytokines and type I IFN, shaping immune responses against specific pathogens. The choice of signalling pathway depends on the kind of stimulus, the activated TLR, and the downstream adaptor molecule (**Fig. 1.4**) [29].

There are two distinct signalling pathways:

The MyD88-dependent pathway, utilised by all TLRs except TLR3, results in NF- κ B activation and the production of inflammatory cytokines.

The TRIF-dependent pathway, utilised by TLR3 and TLR4, is primarily associated with the stimulation of type I IFN (IFN- α , IFN- β) [30].

Each pathway coordinates an appropriate immune response, ensuring the clearance of pathogens, tissue repair, and immune regulation. However, dysregulated TLR activation can contribute to chronic inflammatory diseases, autoimmune disorders, and immune dysfunction.

1.4.6 Adaptor Proteins for TLR Signalling

TLRs rely on intracellular TIR domain-containing adaptor proteins to mediate downstream signalling. There are five major adaptor proteins:

- 1- Myeloid differentiation primary-response protein 88 (MyD88).
- 2- TIR domain-containing adaptor protein (TIRAP or MAL).
- 3- TIR domain-containing adaptor protein inducing IFN- β (TRIF).
- 4- TRIF-related adaptor molecule (TRAM).
- 5- Sterile α - and armadillo-motif-containing protein (SARM) [31].

Among these, the MyD88-dependent signalling cascade is crucial for TLR2, TLR4, TLR5, TLR7, TLR8, and TLR9 activation [32]. TIRAP is required for MyD88-dependent signalling, specifically in TLR2 and TLR4 activation [33]. TRIF, on the other hand, functions independently of MyD88, mediating TLR3 and TLR4 activation [34]. TRAM mediates TLR4 signalling in a MyD88-independent, TRIF-dependent manner [35], while SARM acts as a negative regulator of TRIF signalling, controlling TLR3 and TLR4 activation [36].

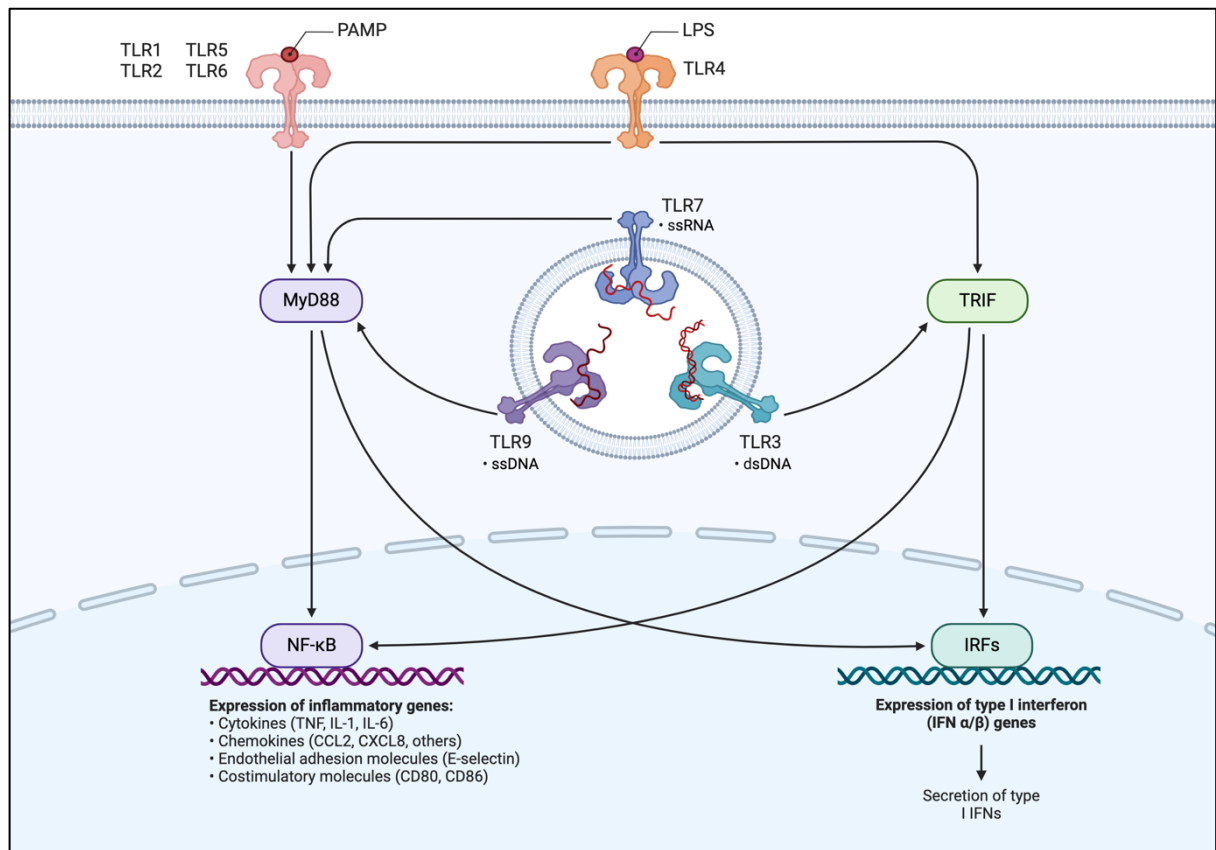


Figure 1.4 TLR Signalling Pathways: MyD88-Dependent and TRIF-Dependent Mechanisms.

This figure depicts the two major TLR signalling pathways: the MyD88-dependent and TRIF-dependent cascades, both of which are essential for innate immune responses. The MyD88-dependent pathway is used by most TLRs and activates NF-κB to induce the production of inflammatory cytokines (TNF-α, IL-1, IL-6), chemokines (CCL2, CXCL8), adhesion molecules (E-selectin), and costimulatory molecules (CD80, CD86), thereby driving inflammation and immune activation. The TRIF-dependent pathway is utilised by TLR3 exclusively and by TLR4 in addition to MyD88 signalling, and activates IRFs to promote the secretion of type I IFN (IFN-α and IFN-β), which are crucial for antiviral defence.

1.4.7 Function of TLRs

TLRs are crucial for innate immunity, as they trigger antimicrobial activities and promote the production of inflammatory cytokines [37]. Upon activation by PAMPs or DAMPs, TLRs recruit adapter proteins that act as signalling platforms to activate IL-1 receptor-associated kinases (IRAK1, IRAK2, IRAK4, and IRAK-M), TAB2, and TNF receptor-associated factor 6 (TRAF6). This signalling cascade leads to the activation and nuclear translocation of key transcription factors, such as NF-κB, AP-1, and interferon regulatory

factor 3 (IRF3). Once in the nucleus, these transcription factors control the expression of multiple immune mediators, including pro-inflammatory cytokines (TNF- α , IL-1 β , IL-6), type I IFN (IFN- α , IFN- β), chemokines (CXCL8, CXCL10), and antimicrobial peptides [38, 39].

1.4.8 TLR2

TLR2 recognises a broad range of PAMPs from bacteria, fungi, viruses, and parasites. Unlike other TLRs, TLR2 forms functional heterodimers with TLR1 and TLR6, expanding its ligand recognition capabilities. It interacts with lipoproteins, peptidoglycans, lipoteichoic acids, and glycolipids, enabling the detection of Gram-positive bacteria, mycobacteria, fungi, and certain viruses [40]. Additionally, accessory molecules like CD14, CD36, and vitronectin enhance TLR2 signalling by facilitating ligand delivery and receptor activation [40]. TLR2 also responds to endogenous DAMPs such as heat shock proteins, HMGB1, and extracellular matrix components like hyaluronan fragments, which are released during tissue damage. These endogenous ligands trigger inflammatory responses via TLR2 and contribute to the pathogenesis of autoimmune diseases, including rheumatoid arthritis, systemic lupus erythematosus, and multiple sclerosis [41]. Upon activation, TLR2 initiates MyD88-dependent signalling, leading to the activation of NF- κ B and MAPK pathways. This drives the production of pro-inflammatory cytokines (TNF- α , IL-1 β , IL-6), chemokines, and antimicrobial peptides. This response plays a crucial role in host defence, but can also contribute to chronic inflammation and immune pathology [40].

1.4.8.1 TLR2 in viral infections

TLR2 plays a significant role in recognising and responding to enveloped viruses, contributing to the innate immune system's defence. Unlike other TLRs that detect viral nucleic acids (e.g., TLR3, TLR7, TLR9), TLR2 recognises structural proteins present on viral envelopes, allowing it to detect and initiate immune responses against viruses such as cytomegalovirus (CMV), respiratory syncytial virus (RSV), measles virus (MV), hepatitis C virus (HCV), and herpes simplex virus (HSV) [42]. For example, RSV's G protein interacts with the TLR2/TLR6 heterodimer, triggering an inflammatory response that aids viral clearance [43]. TLR2 can contribute to type I IFN (IFN- α , IFN- β) production in response to viral infections, although this role is less dominant than TLR3- and TLR7-mediated pathways. Studies have demonstrated that TLR2 is required for IFN- β and IFN- α 4 output in response to vaccinia virus, independent of nucleic acid-sensing TLRs [44], and TLR2 priming enhances macrophage resistance to viral infections by upregulating TRAF3,

leading to increased IFN- β production [45]. Nevertheless, TLR2's role in IFN production is context-dependent, and it does not typically serve as a primary driver of this response [46]. Despite its protective role, several viruses exploit TLR2 to evade immune detection. HSV employs multiple evasion mechanisms: HSV-1 inhibits TLR2-driven NF- κ B activation through its immediate-early protein ICP0, which promotes the degradation of MyD88 and Mal adapter proteins, while HSV-2 uses its virion host shutoff (vhs) protein to selectively inhibit TLR2-mediated antiviral pathways, suppressing IFN- β responses in human epithelial cells [47]. Varicella-zoster virus (VZV) manipulates TLR2 signalling in dendritic cells to block IL-12 release, thereby weakening Th1 immune responses [48], and CMV modulates TLR2 signalling via miRNAs to evade immune detection [49]. In HCV infection, TLR2 contributes to chronic inflammation that worsens liver disease progression, as HCV's core protein triggers TLR2-mediated monocyte activation, leading to IL-10 production that promotes plasmacytoid dendritic cell apoptosis and inhibits IFN- α production, facilitating viral persistence [50, 51]. This demonstrates how TLR2 activation can sometimes be detrimental to host defence, contributing to chronic disease pathogenesis.

1.4.9 TLR4

TLR4 is a PRR in the innate immune system, primarily responsible for detecting LPS from Gram-negative bacteria. TLR4 activates both MyD88-dependent and TRIF-dependent pathways, allowing for a complex immune response that produces both inflammatory cytokines and type I IFNs. This dual signalling capability, facilitated by accessory molecules such as myeloid differentiation factor-2 (MD-2) and cluster of differentiation 14 (CD14), makes TLR4 particularly effective at mounting comprehensive antimicrobial responses [32].

1.4.9.1 TLR4 Ligands and Activation

TLR4 recognises a broad range of microbial components, with LPS being its most well-characterised ligand. LPS recognition requires additional molecules such as LPS-binding protein (LBP), CD14, and MD-2, which together form a complex that facilitates TLR4 activation. In addition to bacterial LPS, TLR4 can also recognise endogenous DAMPs, including heat shock proteins, HMGB1, and oxidised phospholipids, linking it to inflammatory diseases and tissue damage. This broad ligand recognition makes TLR4 important in both infectious and sterile inflammation [52, 53].

1.4.9.2 MyD88-Dependent signalling

At the plasma membrane, TLR4 can signal through the MyD88-dependent pathway. Upon ligand binding, the TIR domain of TLR4 recruits MyD88 via the bridging adaptor protein

TIRAP (also known as MAL). This leads to the recruitment of interleukin-1 receptor-associated kinases (IRAKs) (IRAK1, IRAK2, IRAK4, and IRAK-M), which activate TRAF6. TRAF6, in turn, triggers a signalling cascade involving transforming growth factor- β -activated kinase 1 (TAK1), leading to the activation of NF- κ B and mitogen-activated protein kinases (MAPKs). This pathway activation produces pro-inflammatory cytokines such as TNF- α , IL-6, and IL-1 β , which coordinate inflammatory responses and antimicrobial defence [33].

1.4.9.3 MyD88-Independent (TRIF-Dependent) Signalling

TLR4 can also signal through the MyD88-independent pathway, mediated by the adaptor protein TRIF (TIR-domain-containing adapter inducing interferon- β). This pathway is activated after TLR4 is internalised into early endosomes, where TRAM facilitates TRIF recruitment. The TRAM-TRIF pathway activates IRF3, resulting in the production of type I IFN (IFN- α and IFN- β). This antiviral response enhances antimicrobial defence and shapes adaptive immunity. The TRIF-dependent pathway can also activate NF- κ B and MAPKs, leading to delayed cytokine production that creates a characteristic biphasic response pattern [54].

1.4.9.4 Endocytosis and Compartmentalized Signalling

One of the defining characteristics of TLR4 signalling is its ability to function from different cellular locations. While MyD88-dependent signalling occurs at the plasma membrane, TLR4 undergoes endocytosis where TRAM then facilitates TRIF-dependent signalling from endosomes. This spatial separation ensures balanced inflammatory and antiviral responses, allowing the immune system to effectively adapt to different threats [55].

1.4.9.5 Role of TLR4 in viral infections

TLR4 activation represents a double-edged sword in viral infections, as it can both facilitate immune responses and exacerbate viral pathogenesis through excessive inflammation.

Respiratory Viral Infections: In RSV infections, the viral fusion (F) protein interacts with TLR4, triggering immune responses that, while designed to combat infection, can contribute to excessive inflammation and tissue damage, ultimately worsening disease severity [56, 57]. SARS-CoV-2 exploits TLR4 signalling more extensively, with the SARS-CoV-2 SP (S1 domain) directly binding to TLR4 and triggering cytokine storms characteristic of severe Coronavirus disease 2019 (COVID-19). This creates a feedback loop where increased TLR4 expression amplifies inflammatory responses, contributing to disease severity [58, 59] .

Vector-Borne Viral Infections: Chikungunya virus (CHIKV) utilizes its envelope protein E2 to facilitate attachment and entry into macrophages via TLR4. Inhibition of TLR4 using TAK-242 significantly reduced viral load and pro-inflammatory cytokine expression, including TNF- α , IL-6, and Monocyte Chemoattractant Protein-1 (MCP-1) [60]. Similarly, Dengue virus non-structural protein 1 (NS1) interacts with TLR4 to activate inflammatory pathways that contribute to severe dengue pathogenesis. NS1-TLR4 engagement disrupts endothelial integrity, leading to increased vascular permeability and the characteristic vascular leak syndrome of severe Dengue [61, 62]. Importantly, TLR4 inhibition has been shown to mitigate NS1-induced vascular leakage, highlighting its therapeutic potential [63].

Chronic and Oncogenic Viral Infections: HCV engages TLR4 to establish a pro-fibrotic microenvironment that promotes chronic liver inflammation. Activation of MyD88-dependent pathways leads to persistent IL-6 and TNF- α production, while modulation of TRIF signalling maintains chronic inflammation, ultimately contributing to fibrosis and cirrhosis development [64]. In human papillomavirus (HPV) infections, TLR4 facilitates immune evasion and cancer progression, demonstrating its complex role in oncogenic viral pathogenesis [65].

Severe Acute Viral Infections: Ebola virus glycoprotein (GP) acts as a potent TLR4 activator, contributing to the excessive inflammatory responses characteristic of Ebola virus disease. GP-TLR4 interactions on antigen-presenting cells drive production of inflammatory cytokines such as TNF- α and IL-6, exacerbating disease pathology [66].

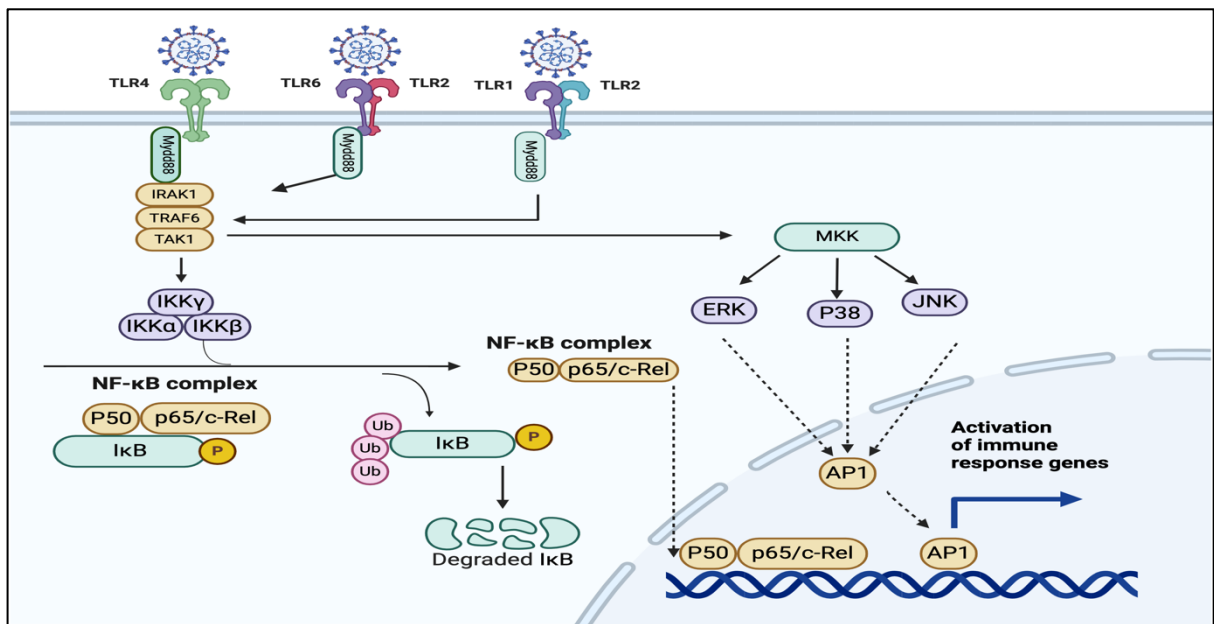


Figure 1.5 TLR-Mediated NF- κ B and MAPK Signalling Pathways in Viral Infections.

The diagram illustrates the activation of NF- κ B and MAPK signalling pathways through TLRs in response to viral infections, highlighting their role in innate immune defence. TLR4, TLR2, TLR1, and TLR6 recognise viral components and initiate signalling cascades that lead to immune activation.

TLR4 (green) engages MyD88, recruiting IRAK1, TRAF6, and TAK1 to activate downstream pathways. In the NF- κ B pathway, these components activate the I κ B kinase (IKK) complex (IKK α , IKK β , IKK γ), leading to phosphorylation and degradation of I κ B. This releases the NF- κ B complex (p50 and p65) to translocate into the nucleus and drive pro-inflammatory gene expression. TLR2/TLR6 (red) and TLR2/TLR1 (purple) heterodimers signal through similar MyD88-dependent pathways to amplify these inflammatory responses. Concurrently, TRAF6 activates the MAPK signalling cascade through TAK1, which phosphorylates specific MAP kinase kinases: MKK3/6 activate p38, MKK4/7 activate JNK, and MKK1/2 activate ERK. These activated kinases stimulate the AP-1 transcription factor, which enhances expression of inflammatory mediator genes. The convergence of NF- κ B and AP-1 pathways promotes the production of pro-inflammatory cytokines and chemokines essential for viral clearance. However, excessive activation of these pathways can contribute to tissue damage in severe infections, such as those caused by RSV, SARS-CoV-2, and Dengue Virus.

1.4.10 TLR8: Structure, Activation, and Role in Viral Infections

TLR8 is a PRR primarily expressed in monocytes, macrophages, neutrophils, myeloid dendritic cells, and regulatory T cell [67-71] (**Fig.1.6**). It plays a vital role in antiviral immunity by detecting ssRNA from viruses and initiating immune responses. Despite its similarity to TLR7, TLR8 has distinct activation mechanisms, cytokine responses, and structural features, making it a critical player in antiviral defence [72].

1.4.10.1 Structure of TLR8

Understanding TLR8's structure is essential for appreciating its unique functional capabilities in viral RNA recognition. TLR8 is a type I transmembrane receptor with a distinct structural organisation that plays a crucial role in the recognition of ssRNA from viral pathogens. Its structure consists of three main domains: an extracellular domain with LRRs that facilitate ligand recognition, a transmembrane domain that anchors the receptor within endosomal membranes, and a TIR domain responsible for downstream signalling. Unlike other TLRs, TLR8 undergoes proteolytic cleavage at a conserved Z-loop region between LRR14 and LRR15, which is necessary for its activation and signal transduction

[73]. The receptor exists as a preformed inactive homodimer, and upon ligand binding, a conformational rearrangement brings the C-terminal regions of the two monomers closer together, initiating the recruitment of adaptor proteins and triggering intracellular signalling cascades (**Fig.1.6**) [74].

1.4.10.2 TLR8 Activation and signalling Pathway

Building on its structural organisation, TLR8's activation mechanism reflects its specialisation for viral RNA detection. TLR8 is activated upon recognition of viral ssRNA, which is characteristically enriched in AU- and GU-rich sequences found in viral genomes. Once activated, TLR8 undergoes a conformational change that enables signal transduction through the adaptor protein MyD88. This initiates a signalling cascade involving IRAKS (IRAK1, IRAK2, IRAK4, and IRAK-M) and TRAF6, leading to the activation of NF- κ B, AP-1, and IRFs [75]. NF- κ B activation promotes the production of pro-inflammatory cytokines, including IL-6, IL-1 β , and TNF- α , whereas IRF3/7 activation induces the production of type I IFN, particularly IFN- β . Compared to TLR7, TLR8 triggers a stronger inflammatory response due to its potent NF- κ B activation, while TLR7 favours IFN- α production [76].

1.4.10.3 Role of TLR8 in Viral Infections

Several viruses effectively activate TLR8 signalling to induce robust immune responses. RNA viruses, including influenza A virus subtype H3N2 and Coxsackie B virus, activate TLR8 to induce cytokines like IL-8 and IFN- β , contributing to viral clearance [77]. Interestingly, even DNA viruses such as vaccinia virus can stimulate TLR8-mediated IFN production [78] demonstrating the receptor's broad antiviral capacity.

Viral Evasion and Exploitation Strategies: Many viruses have evolved mechanisms to evade or exploit TLR8 signalling. Hepatitis B virus (HBV) suppresses TLR8 expression, weakening immune responses and facilitating persistent infection [79]. Similarly, Zika virus actively downregulates both TLR8 and MyD88 expression, compromising antiviral defences [79, 80]. HIV-1 presents a complex case, exploiting TLR8 activation in CD4⁺ T cells to enhance viral replication [81], while simultaneously disrupting TLR8 colocalisation in dendritic cells to reduce immune effectiveness [81]. West Nile virus infections demonstrate increased severity in TLR8-deficient models, highlighting the receptor's protective role [79, 80].

Clinical and Genetic Significance: Clinical studies have established correlations between TLR8 expression levels and disease outcomes. Reduced TLR8 activity correlates with

diminished TNF- α production in both RSV infections and chronic HBV patients, suggesting impaired immune responses contribute to disease severity [79, 82]. Genetic variations in TLR8, particularly single-nucleotide polymorphisms (SNPs), significantly influence antiviral responses. Some SNPs impair immune defences against HCV while offering protective effects against HIV [83, 84]. However, investigations into TLR8 SNPs in SARS-CoV-2 infections revealed no significant association with COVID-19 disease severity [85], indicating virus-specific genetic influences on TLR8-mediated immunity.

1.4.11 TLR7

TLR7 is an endosomal PRR that detects ssRNA and initiates antiviral immune responses. It is predominantly expressed in plasmacytoid dendritic cells and B cells, where it binds GU rich ssRNA sequences (such as those from RNA viruses) in acidified endosomes [86]. Upon ssRNA recognition, TLR7 dimerizes and recruits MyD88, leading to activation of IRAK4 and IRAK1 and the E3 ubiquitin ligase TRAF6 [87]. This MyD88-dependent pathway culminates in the nuclear translocation of transcription factors NF- κ B and IRF7, inducing the production of pro-inflammatory cytokines and type I IFN (notably IFN- α) [87]. TLR7 also contributes to the detection of retroviral RNA such as HIV-1, which can activate TLR7 and drive interferon- α responses during infection [88]. In SARS-CoV-2 infection, TLR7 sensing of the coronavirus's genomic RNA activates antiviral interferon signalling in DCs [89], and rare loss-of-function mutations in the TLR7 gene that abrogate this pathway have been linked to severe COVID-19 due to impaired interferon immunity [87]. Recent in vivo evidence further demonstrates that Tlr7 deficiency markedly increases viral replication and pulmonary pathology, concomitant with diminished type I and II IFN signalling and defective humoral responses [90].

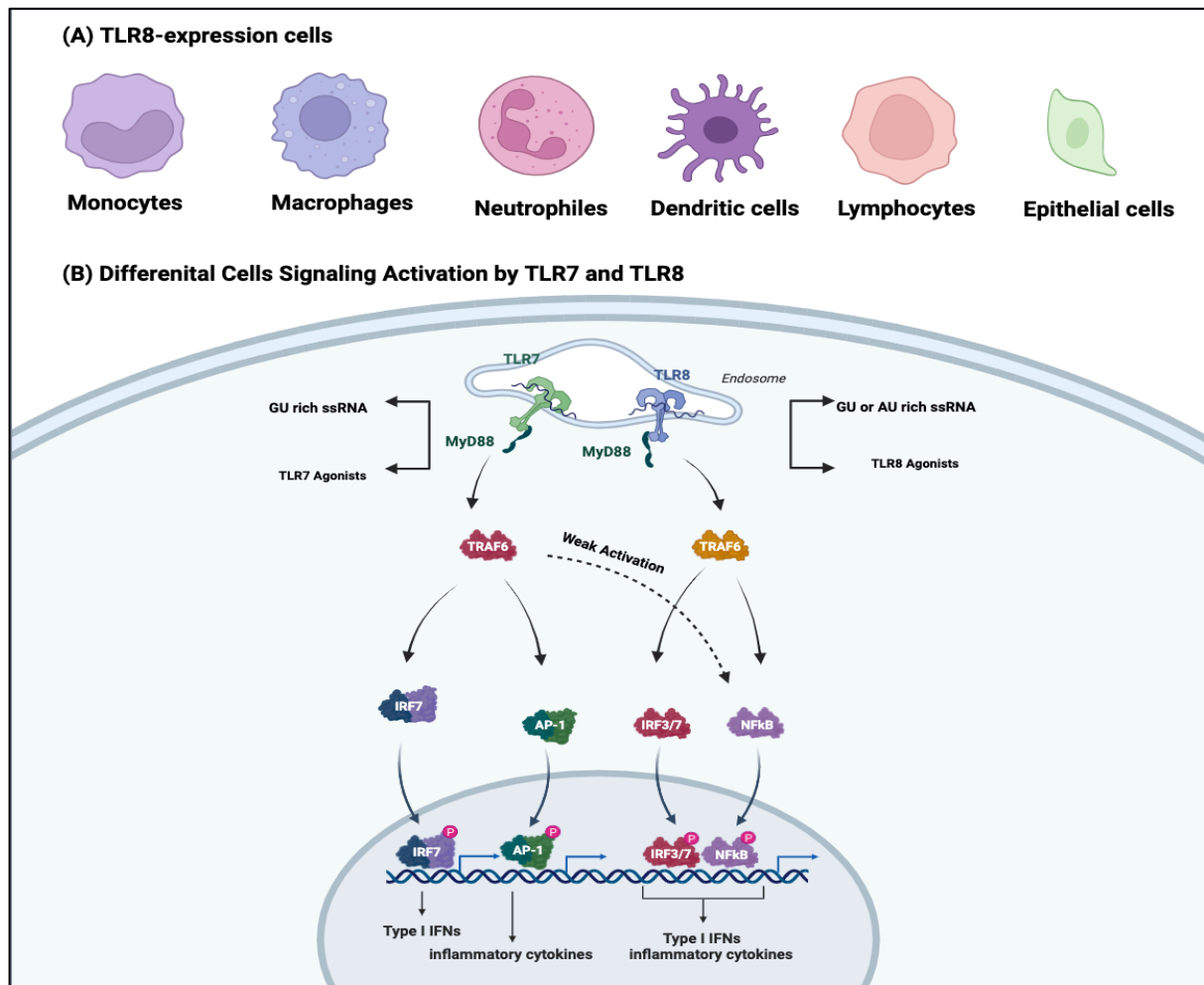


Figure 1.6 TLR8 Expression and Signalling Pathway Comparison with TLR7.

(A) Various immune cells, including monocytes, macrophages, neutrophils, myeloid dendritic cells, and regulatory T cells, have been identified to express TLR8 constitutively.

(B) Both TLR7 and TLR8 utilise the MyD88 adaptor protein for signalling, but they differ in their downstream pathways. TLR7-mediated IFN- α production occurs through IRF7 activation downstream of MyD88-TRAF6 signalling. TRAF6 also triggers AP-1 activation and weakly stimulates NF- κ B, resulting in the production of inflammatory cytokines. In contrast, TLR8 signalling differs markedly in its downstream effects by robustly activating the TRAF6-NF- κ B pathway, while IFN- β production occurs through the IRF3/7 signalling cascade.

1.4.12 Cytosolic Receptors: RLRs

In addition to endosomal PRRs such as TLR3, TLR7/8, and TLR9, the RIG-I-like receptor (RLR) family plays a crucial role in recognising viral RNA within the cytoplasm. The RLR family consists of retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated protein 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2) [91].

These receptors detect viral dsRNA and ssRNA with distinct structural features, initiating antiviral responses independently of TLR signalling. Structurally, all three RLRs contain a DExD/H-box helicase core and a C-terminal domain (CTD) responsible for RNA recognition. However, RIG-I and MDA5 uniquely possess caspase activation and recruitment domains (CARDs), which are essential for downstream signalling, while LGP2 lacks CARDs and serves a regulatory function [92].

RIG-I primarily detects uncapped 5'-triphosphate and 5'-diphosphate RNA structures commonly found in viral genomes, while MDA5 recognises longer dsRNA molecules. Following viral RNA recognition, RIG-I undergoes ATP-dependent conformational changes, facilitating its interaction with the adaptor protein mitochondrial antiviral signalling protein (MAVS), also known as IPS-1, VISA, or CARDIF. MAVS is anchored to mitochondria and peroxisomes, serving as a key signalling hub that recruits downstream kinases, such as TANK-binding kinase 1 (TBK1) and I κ B kinase- ϵ (IKK ϵ). This activation leads to phosphorylation and nuclear translocation of IRF3/IRF7, culminating in the production of type I IFN. Additionally, MAVS signalling activates NF- κ B, driving the expression of pro-inflammatory cytokines such as IL-6 and TNF- α . MDA5 utilises similar MAVS-dependent signalling pathways upon recognising its dsRNA ligands [93-95].

1.4.13 RIG-I-like receptors in viral infections

Many viruses have evolved mechanisms to evade RLR-mediated detection and suppress IFN production. For instance, HCV encodes the NS3/4A protease, which cleaves MAVS at the mitochondrial membrane to prevent IFN induction [96]. Similarly, the VP35 protein of Ebola virus (EBOV) and Marburg virus (MARV) binds dsRNA and inhibits RIG-I activation [97-99]. Influenza A virus (IAV) expresses the NS1 protein, which interacts with the E3 ubiquitin ligase TRIM25 to block the ubiquitination of RIG-I, thereby preventing RIG-I activation and downstream IFN signalling [100]. Additionally, SARS-CoV-2 has been shown to suppress MAVS signalling through its open reading frame 9b (ORF9b) protein, contributing to immune evasion and delayed IFN responses [101].

1.4.14 Cytosolic Receptors: NOD-like receptors (NLRs)

In addition to RLRs, NLRs play a pivotal role in detecting intracellular pathogens, including viruses, and initiating innate immune responses. A key function of NLRs is their involvement in inflammasome assembly, a multiprotein complex that senses PAMPs and DAMPs within the cytoplasm. Inflammasome activation leads to the recruitment of caspase-1, which subsequently cleaves pro-IL-1 β and pro-IL-18 into their mature, biologically active forms, promoting inflammation and antiviral responses [102]. Several NLR family members

are known to activate inflammasomes, including NLRP1, NLRP3, and NLRC4, as well as non-NLR proteins such as absent in melanoma 2 (AIM2) and pyrin. Among these, NLRP3 is the most extensively studied in the context of viral immunity, with its activation linked to infections by IAV, HIV-1, and RSV, leading to IL-1 β and IL-18 secretion that amplifies immune responses and drives inflammation [103]. NLRP3 activation typically occurs through a two-step process: first, a priming signal increases inflammasome component expression, followed by a secondary activation signal that induces inflammasome assembly and caspase-1 activation [103]. Beyond cytokine release, NLRs contribute to immune cell recruitment, with neutrophils expressing NOD2 and NLRP3 that respond to muramyl dipeptide (MDP) by secreting IL-8, downregulating CD62L, and enhancing migration toward inflammatory sites [104]. However, excessive or dysregulated inflammasome activation can contribute to immunopathology, as demonstrated in COVID-19, where NLRP3-driven IL-1 β and IL-6 release exacerbates disease severity through amplified epithelial-immune interactions [105].

1.4.15 NF- κ B signalling

NF- κ B Structure and signalling Pathways: NF- κ B is a family of essential transcription factors that control immune responses, inflammation, cell survival and apoptosis [106]. This family consists of NF- κ B1 (p50), NF- κ B2 (p52), RelA (p65), RelB, and c-Rel, which function as homo- or heterodimers by binding to κ B enhancers to regulate gene transcription [107]. NF- κ B signalling is mediated via two primary pathways: the canonical and non-canonical pathways. The canonical pathway is rapidly activated by PRRs such as TLRs and cytokine receptors, including TNF receptor 1 (TNFR1) and IL-1R. Upon activation, I κ B proteins (e.g., I κ B α) are phosphorylated by the I κ B kinase (IKK) complex, leading to their ubiquitination and proteasomal degradation, allowing NF- κ B dimers such as p65/p50 to translocate into the nucleus and drive the expression of proinflammatory genes [108]. For example, LPS activation of TLR4 leads to the NF- κ B-dependent production of inflammatory cytokines, such as TNF- α , IL-6, and IL-1 β , which play a critical role in antibacterial and antiviral immunity. In contrast, the non-canonical pathway is triggered by TNF receptor superfamily members, including receptor activator of NF- κ B (RANK), lymphotoxin β receptor (LT β R), and B-cell activating factor receptor (BAFFR). Unlike the canonical pathway, it does not degrade I κ B but instead processes the NF- κ B2 precursor protein (p100) into its active form, p52, which then heterodimerizes with RelB to promote gene transcription [109].

NF- κ B in Macrophage Polarization and Inflammasome Regulation: Beyond its role in inflammation, NF- κ B is a central regulator of macrophage polarization, determining the differentiation of pro-inflammatory (M1) and anti-inflammatory (M2) macrophages. M1 macrophages, activated through LPS-TLR4 signalling, secrete pro-inflammatory cytokines such as IL-1 β , IL-6, TNF- α , and IL-12, enhancing pathogen clearance. Conversely, M2 macrophages, stimulated by IL-4 and IL-13, secrete IL-10 and TGF- β , contributing to tissue repair and immunosuppression [110, 111]. Additionally, NF- κ B is crucial for inflammasome activation, specifically regulating the NLRP3 inflammasome, which requires two signals: a priming signal via PRR activation (e.g., LPS-TLR4) that upregulates NLRP3 and pro-IL-1 β transcription, and an activation signal triggered by adenosine triphosphate (ATP), pore-forming toxins, or reactive oxygen species (ROS), leading to inflammasome assembly and caspase-1 activation, which processes pro-IL-1 β into its active inflammatory form [112, 113].

Viral Exploitation of NF- κ B signalling: Given its central role in host immunity, NF- κ B is frequently targeted by viruses to evade immune detection and promote viral replication. IAV NS1 protein plays a crucial role in immune evasion by interfering with NF- κ B signalling, with NS1 from avian influenza viruses differentially inhibiting NF- κ B promoter activation to facilitate viral persistence [114]. Additionally, NS1 represses the antiviral immune response by hijacking NF- κ B to regulate type III IFN gene transcription, further dampening immune activation and enabling efficient viral replication [115]. HCV NS3/4A protease cleaves MAVS, inhibiting PRR-induced NF- κ B activation and blocking type I IFN responses [96]. HIV-1 exploits NF- κ B through Vpr and Transactivator of transcription (Tat) proteins, which modulate the pathway to either suppress antiviral immunity or enhance viral transcription depending on the infection stage [116, 117]. SARS-CoV-2 ORF9b protein further disrupts NF- κ B and IFN signalling by targeting mitochondrial TOM70, contributing to immune evasion and delayed IFN responses [117].

1.4.16 The mitogen-activated protein kinase (MAPK) signalling

Besides the activation of NF- κ B signalling, PRRs are also well known for their activation of MAPK signalling [118]. As with NF- κ B signalling, TAK1, which is downstream of PRR activation, is also involved in MAPK pathway activation [119]. Following PRR engagement, TRAF6 recruits the TAK1-binding proteins TAB2/3, leading to the phosphorylation of TAK1 [120]. Subsequently, TAK1 mediates activation of the ERK1/2, p38 and JNK MAPK pathways [121]. Efficient MAPK signalling requires a three-tiered

phosphorylation cascade, where MAPKs are activated by a MAPK kinase (MAP2K), which itself is activated by an upstream MAPK kinase kinase (MAP3K) via phosphorylation. TAK1 acts as a MAP3K and phosphorylates MAP2Ks such as MKK3/6 and MKK4/7, leading to the activation of p38 and JNK, respectively [122]. In parallel, TAK1 modulates ERK1/2 activation through IKK-mediated regulation of TPL2. Under homeostatic conditions, TPL2 remains bound to NF- κ B1 p105, but upon stimulation, it is released and phosphorylated, facilitating ERK1/2 activation. These MAPK cascades regulate the transcription of several cytokines, including TNF- α , IL-8, and IL-10, playing a dual role in inflammation and immune modulation [123, 124]. MAPK signalling is also implicated in the immune response against viral infections, with multiple pathways contributing to antiviral defence mechanisms. For example, RSV infection has been shown to activate multiple protein kinase C (PKC) isoforms, which subsequently drive ERK1/2 phosphorylation and the production of pro-inflammatory cytokines [125]. Similarly, RIG-I-mediated activation of p38 MAPK is critical for viral induction of IFN and the activation of DCs [126]. IAV has been extensively studied in this context, where viral recognition via TLR3, TLR7, and RIG-I leads to MAPK activation, promoting the expression of antiviral genes and cytokine production. However, many viruses have evolved mechanisms to manipulate these pathways to their advantage. For instance, IAV proteins such as NS1 interfere with MAPK activation to dampen the immune response and enhance viral replication [127]. Similarly, EBOV GP has been shown to exploit ERK MAPK signalling to induce cytotoxicity, thereby contributing to viral dissemination and host immune evasion [128]. Moreover, Studies indicate that pharmacological inhibition of MAPKs, particularly p38 (e.g. with SB203580) and JNK, can reduce viral replication and inflammation in infections by HCV, severe fever with thrombocytopenia syndrome virus (SFTSV), HSV-1, and SARS-CoV-2, as well as in more general settings of viral activation seen in influenza, EV71, HIV, and dengue virus [129].

1.4.17 Interferon Signalling in Antiviral Defence

IFNs are pleiotropic cytokines characterized by their ability to inhibit viral replication and are divided into three families: Type I, Type II, and Type III. The Type I IFN family consists of several IFN- α subtypes, IFN- β , and other less-characterized members (IFN- ϵ , IFN- κ , IFN- ω , and IFN- τ). These IFNs are produced mainly by innate immune cells and signal through the IFN- α/β receptor (IFNAR), a two-subunit protein (IFNAR1 and IFNAR2) expressed in most cell types [130]. Type I IFN is produced by pDCs and virus-infected cells after stimulation of PRRs [131]. Upon recognition by IFNAR, signalling occurs via the JAK-

STAT pathway to induce the expression of Interferon stimulation genes (ISGs), which establish an antiviral state by suppressing viral replication at various steps. Different viral infections engage distinct pathways to induce Type I IFN production. For example, in RSV infection, pDCs utilize the TLR7-MyD88-dependent pathway to recognize viral RNA and produce IFN- β , while epithelial cells recognize viral RNA through RIG-I and MDA5 receptors to produce Type I IFN in porcine epidemic diarrhea virus (PEDV) infection [132]. Interferon-gamma (IFN- γ) is the only member of Type II IFN and is produced mainly by T cells and NK cells. It binds to the IFN- γ receptor (IFNGR) and activates macrophages, enhances antigen presentation, and drives adaptive immunity. Type I IFN is a more general antiviral inducer than IFN- γ , which is more specifically involved in cell-mediated immunity and Th1 differentiation [133]. The Type III IFNs, also referred to as IFN- λ s (IFN- λ 1, IFN- λ 2, IFN- λ 3, and IFN- λ 4), signal through a unique receptor complex consisting of IFNLR1/IL28R α and IL10R β , which is restricted to epithelial cells and specific myeloid cells. Type III IFNs signal through pathways similar to those of Type I IFN; however, their restricted receptor expression confines their antiviral actions to mucosal surfaces, including the respiratory and gastrointestinal tracts, thereby providing effective viral control while avoiding systemic inflammation [134].

1.4.18 JAK/STAT signalling

The discovery of Janus Kinases (JAKs) and Signal Transducers and Activators of Transcription (STATs) arose from research into the functioning of IFNs. In 1988, STAT proteins were first identified as crucial regulators of ISG transcription, and JAKs were discovered in 1992 by three separate groups of researchers, culminating in the formal recognition of the JAK-STAT signalling pathway [135]. The JAK-STAT pathway is crucial for orchestrating numerous immune and inflammatory responses, regulating cell proliferation, differentiation, and antiviral defences [136]. Dysfunction of this pathway has been associated with numerous inflammatory diseases and immune disorders [137].

1.4.18.1 Molecular Mechanism of JAK/STAT Activation

Activation of this pathway begins with cytokine binding to its cognate receptor, inducing receptor dimerization and subsequent activation of receptor-associated Janus kinases (JAKs). These kinases undergo trans-autophosphorylation on conserved tyrosine residues, generating docking sites for STAT proteins via their Src homology 2 (SH2) domains. In their inactive state, STATs remain in the cytoplasm. Upon recruitment, they undergo phosphorylation at specific tyrosine residues, inducing conformational changes that

facilitate homo- or heterodimerization. Phosphorylated STAT dimers then dissociate from the receptor complex and translocate into the nucleus through the importin α/β -RanGDP complex, where they bind to gamma-activated sequence (GAS) elements or interferon-stimulated response elements (ISREs) in the promoter regions of target genes, driving transcriptional regulation. A well-characterized example of STAT-dependent transcription is the interferon-stimulated gene factor 3 (ISGF3) complex. ISGF3 is composed of phosphorylated STAT1, STAT2, and interferon regulatory factor 9 (IRF9), and is essential for type I and III IFN-mediated antiviral immunity. This complex orchestrates the induction of ISGs that restrict viral replication and modulate host immune responses [138-140] .

1.4.18.2 JAK-STAT in Cytokine Signalling

The JAK-STAT pathway is not limited to IFN signalling; over 50 cytokines utilise this pathway to mediate immune and inflammatory responses. Among them, the IL-6 family (including IL-6, IL-11, IL-27, LIF, OSM, CNTF, CT-1, and CLC) signals through gp130-containing receptors and primarily activates JAK1. This results in the activation of STAT3, which plays a crucial role in cellular processes such as maturation, proliferation, and survival [141, 142].

1.4.18.3 Structural Components of JAK and STAT Proteins

The human JAK family consists of four members: JAK1, JAK2, JAK3, and TYK2. Each JAK contains multiple functional domains, including the N-terminal FERM (4.1 protein, Ezrin, Radixin, Moesin) domain, which facilitates protein-protein interactions, and the SH2 domain, which binds phosphotyrosine residues to activate STATs. A central pseudokinase domain is present but lacks catalytic function and serves a regulatory role. The C-terminal protein tyrosine kinase (PTK) domain is responsible for phosphorylating specific tyrosine residues on target substrates [143-145].

The human STAT family consists of seven members: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6. These proteins share structural homology in key regions, including the N-terminal domain, which regulates tyrosine dephosphorylation and STAT interactions, and the coiled-coil domain, which mediates nuclear export. The DNA-binding domain recognizes specific promoter sequences (TTCN3-4GAAA) in target genes, while the transactivation domain (TAD) at the C-terminus is essential for transcriptional regulation. In addition to the well-documented tyrosine phosphorylation in JAK-STAT signalling, serine phosphorylation also plays an important role in modulating STAT activity [146, 147].

1.4.18.4 Regulation of JAK-STAT Signalling

The JAK-STAT pathway is subject to multiple layers of regulation to ensure appropriate immune responses. Serine phosphorylation represents an important regulatory mechanism, with studies suggesting that it enhances STAT1 transcriptional potency but negatively regulates STAT3 activation. Several kinases, including p38, ERK, and JNK, contribute to serine phosphorylation, indicating its role in fine-tuning STAT-mediated signalling [148-150]. Additionally, STAT-interacting proteins serve as scaffolds that facilitate STAT phosphorylation and nuclear translocation. Negative regulation is equally important, with Suppressor of Cytokine Signalling (SOCS) proteins acting as key feedback inhibitors that bind to activated JAKs and target them for proteasomal degradation. Protein tyrosine phosphatases (PTPs) also contribute to pathway termination by dephosphorylating activated STAT proteins [151, 152]. While much is known about STAT phosphorylation and activation mechanisms, the precise processes governing STAT dephosphorylation in the nucleus remain poorly understood, representing an important area for future research.

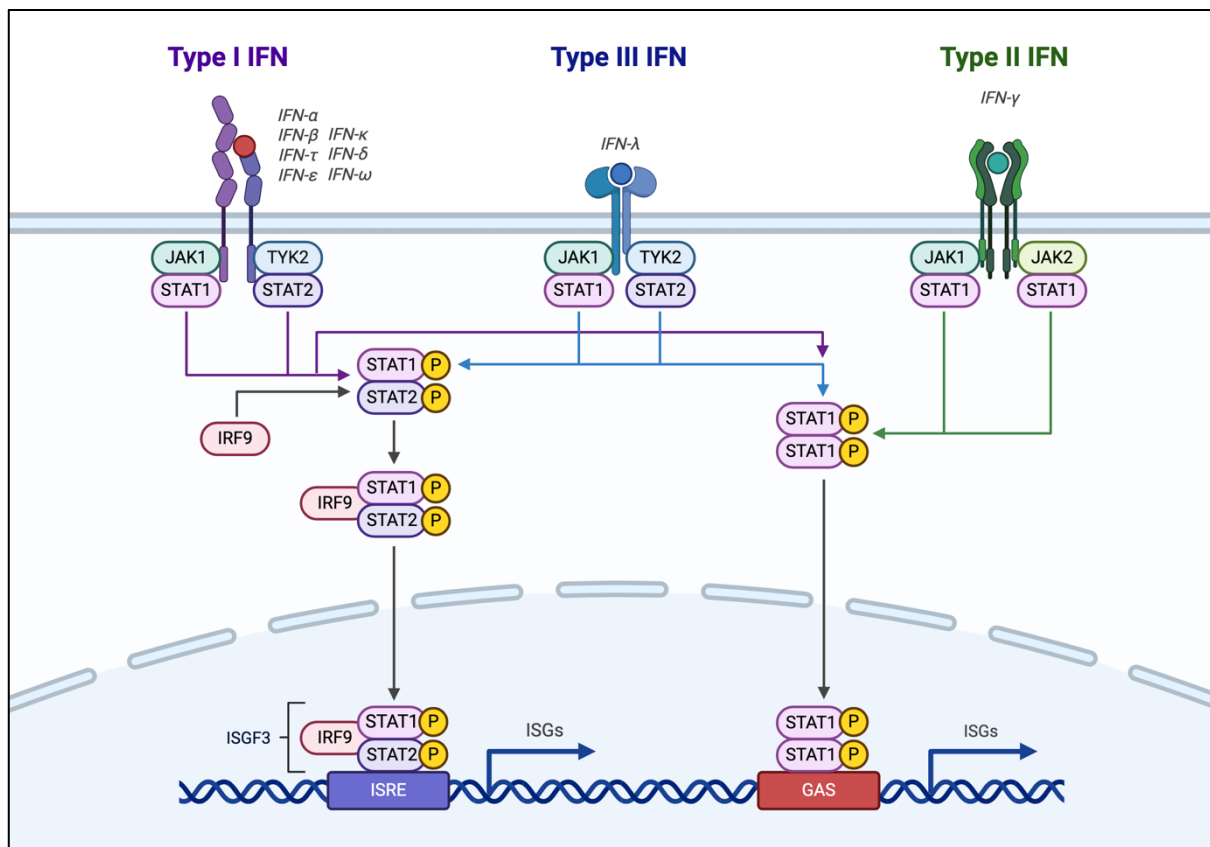


Figure 1.7 JAK-STAT signalling Pathway in Type I, II, and III IFN Responses.

This diagram illustrates the signalling pathways activated by Type I, Type II, and Type III IFNs through the JAK-STAT pathway.

Type I IFNs (IFN-I) comprise IFN- α , IFN- β , and other subtypes including IFN- κ , IFN- τ , IFN- ϵ , and IFN- ω . These IFNs interact with the IFNAR receptor complex, which activates JAK1 and TYK2, leading to the phosphorylation of STAT1 and STAT2. The phosphorylated STAT1-STAT2 complex associates with IRF9 to form the ISGF3 complex, which translocates to the nucleus and binds to ISRE, thereby promoting the transcription of ISGs.

Type III IFNs comprise the IFN- λ family and signal through a unique receptor complex (IFNLR1/IL28R α and IL10R β) that activates JAK1 and TYK2. This pathway shares close similarity with Type I IFN signalling, leading to the phosphorylation of STAT1 and STAT2 and subsequent ISGF3 formation and ISG expression. However, the Type III IFN receptor is expressed mainly on epithelial and some myeloid cells, making the signalling more tissue-restricted than Type I IFNs. Type II IFN (IFN- γ) signals through a distinct receptor complex (IFNGR) that utilizes JAK1 and JAK2 to activate STAT1. This activation leads to STAT1 phosphorylation, which then homodimerizes and translocate to the nucleus where it binds to Gamma-Activated Sequence (GAS) elements, inducing ISG expression and supporting Th1 immune responses.

1.4.19 Viral Evasion and Induction of IFN Responses

IFN Induction by Viral Pathogens: Many viruses, including DENV, EBOV, ZIKV, RSV, and HIV, activate IFN responses through PRRs such as RIG-I and MDA5. Both RIG-I and MDA5 recognize DENV, but RIG-I has the primary responsibility for recognizing viral double-stranded RNA and triggering IFN responses [153]. Similarly, RIG-I is the primary sensor of ZIKV infections and the major inducer of transcriptional changes that lead to the activation of the innate immune response, enabling cells to avoid virus-induced apoptosis and thereby regulating ZIKV infection [154]. Both RIG-I and MDA5 detect RSV, which triggers antiviral IFN production, though the virus possesses mechanisms that inhibit IFN signalling [155]. HIV follows a similar pattern, activating IFN pathways via PRRs while simultaneously developing mechanisms to evade immune responses [156].

HIV Immune Evasion Mechanisms: The role of intrinsic and innate immunity in HIV infection is crucial. Intrinsic immunity is mediated by restriction factors, such as APOBEC3G, SAMHD1, and TRIM5 α , which block viral replication at different stages. Innate immunity depends on PRRs like TLRs and RIG-I to recognize HIV nucleic acids and trigger IFN-I responses and ISGs with antiviral activities. However, while these responses initially help control infection, chronic HIV infection leads to immune exhaustion and systemic inflammation through persistent IFN signalling. HIV subverts these defences using viral proteins like Vif,

Vpu, and Nef to avoid restriction factors and immune recognition [157]. HIV-1 specifically targets IRFs to evade the immune response and facilitate viral replication [158]. Moreover, HIV-1 blocks IRF3 and IRF7 activation, preventing the induction of IFN-I through many mechanisms [159]. The HIV Vpr protein prevents IRF3 phosphorylation and its subsequent activation and nuclear translocation [160]. The HIV Vpr protein inhibits the function of IRF7, leading to a decrease in IFN- α production in pDCs [161]. Additionally, HIV Tat protein modulates host transcription by recruiting E3 ligase HDM2 to degrade IRF1 [162], and HIV-1 Vif protein targets crucial JAK/STAT signalling molecules STAT1 and STAT3 for degradation, thereby disabling ISGs and undermining their capacity to mount an antiviral response [163].

Viral Evasion Strategies Across Different Pathogens: Other viruses employ similar evasion strategies targeting key IFN pathway components. The NS5 protein from DENV reduces STAT2 levels through proteasomal degradation, thus disabling IFN activity and enabling immune evasion. Similarly, EBOV encodes VP35, which prevents IRF3 activation and suppresses IFN production [164]. ZIKV's NS5 protein also degrades STAT2 to ensure persistent infection and contribute to neurological complications [165]. In SARS-CoV-2 infection, MDA5 acts as the main receptor triggering the immune response to viral ssRNA; however, this response is significantly delayed.

SARS-CoV-2 IFN Evasion: Although SARS-CoV-2 can induce the secretion of proinflammatory cytokines such as IL-6, IL-8, and TNF- α , it has a limited capacity to induce Type I and III IFNs. In severe COVID-19 cases, IFN-I responses are markedly suppressed. Children, especially infants, exhibit strong antibody responses but produce lower levels of IFN- γ . In infected Calu3 lung epithelial cells and iPSC-derived airway epithelial cells, transcripts of IFN- β and IFN- λ are upregulated, and ISGs are expressed in both infected and neighbouring bystander cells, underscoring the role of autocrine and paracrine signalling. Despite these immune responses, SARS-CoV-2 actively evades IFN activation in respiratory epithelial cells, which delays the immune response in bystander cells. This evasion strategy contributes to the virus's ability to persist and exacerbate disease severity [166-168]. SARS-CoV-2 employs multiple mechanisms to inhibit IFN responses, which are detailed in the accompanying table.

Table 1. 2 SARS-CoV-2 IFN Inhibition Mechanisms.

SARS-CoV-2 Mechanism	Targeted IFN Type	Description

Inhibiting type I and III IFN production	Type I and Type III	SARS-CoV-2 membrane (M) protein inhibits type I and III IFN production by targeting RIG-I/MDA-5 signalling [169].
Preventing nuclear translocation of phosphorylated IRF3	Type I	SARS-CoV-2 main protease suppresses type I IFN production by preventing nuclear translocation of phosphorylated IRF3 [170].
Upregulating suppressor of cytokine signalling 1 (SOCS1)	Type I	ORF3a, an accessory protein of SARS-CoV-2, inhibited IFN-activated JAK)/STAT signalling via upregulating SOCS1 [171].
NSP5 cleavage of RIG-I	Type I	NSP5 cleaves the RIG-I N-terminal domain, impairing IFN signalling [172].
ORF6 inhibition of IRF3 nuclear translocation	Type I	ORF6 binds to importin KPNA2, preventing IRF3 nuclear translocation and subsequent IFN induction [173].
ORF9b Antagonizes Multiple Components of RIG-I/MDA-5-MAVS, TLR3-TRIF, and cGAS-STING signalling Pathways	Type I and Type III	SARS-CoV-2 ORF9b Antagonizes Type I and III IFN by Targeting Multiple Components of RIG-I/MDA-5-MAVS, TLR3-

		TRIF, and cGAS-STING Signalling Pathways [101].
SARS-CoV-2 viral proteins NSP1 and NSP13 inhibit IFN activation through distinct mechanisms	Type I	NSP1 antagonizes IFN signalling by suppressing host mRNA translation, while NSP13 downregulates IFN and NF- κ B promoter signalling by limiting TBK1 and IRF3 activation [174].

1.4.20 ISGs and Their Functions

Many viruses activate the production of Type I IFN, which induces expression of ISGs. The products of these ISGs perform several antiviral effector actions that target almost every aspect of a virus's life cycle, though their mechanisms remain poorly understood [175]. The first ISGs were defined approximately 25 years ago [176], and microarray analyses now show that 50-1000 ISGs are activated depending on cell type, IFN dose, and treatment time, with 200-500 genes being conserved across many cell types [177-179]. Research has traditionally focused on a relatively small group of well-characterized genes central to the antiviral immune response. Among these classical ISGs, Protein kinase R (PKR) is noted for its role in inhibiting viral replication by arresting protein synthesis, MX1 (Myxovirus resistance protein 1) prevents viral entry and replication, and OAS1 (2'-5' Oligoadenylate Synthetase 1) activates RNase L, which degrades viral RNA and limits viral propagation [180-182]. However, genomic research and high-throughput screening technologies have revealed a more extensive and diverse set of ISGs that act through novel mechanisms to support antiviral immunity. These newly identified ISGs include APOBEC3G, TRIM5, ZAP, ISG15, ADAR, IFITM1/2/3, tetherin, and viperin, each possessing unique mechanisms of viral inhibition. Some, like APOBEC3G, introduce mutations into viral genomes to disable their function, while others, like TRIM5, target viral capsids for degradation. Additional ISGs, such as IFITM1/2/3 and tetherin, physically block virus entry and release from cells, providing another layer of protection against infection [175, 183-

185]. Knockout studies and microarray data demonstrate that IFN-induced effectors constitute a redundant antiviral defence system. Even when some key ISGs or pathways are defective, the immune system can compensate, allowing virus control to persist. This redundancy highlights the critical role of ISGs and explains why viruses find it difficult to completely evade the immune system [186]. Clinical relevance is demonstrated in HCV patients treated with IFN- α , where low pre-treatment ISG levels are observed in responders, while levels rise significantly after treatment. Non-responders exhibit high baseline levels with little change. Additionally, lower HCV viral loads are associated with higher expression of 36 ISGs, though evidence for the direct antiviral effects of most ISGs has only recently begun to emerge [187-189]. Several discovery-based screens have been conducted to identify antiviral ISGs, assessing their effects on virus replication using either fully infectious viruses or replicons that replicate but cannot form infectious particles [190, 191]. Small-scale studies showed that ISGs such as viperin, ISG20, and PKR suppress DENV and West Nile viruses [192]. A large-scale screen of 380 genes against six viruses identified 25 ISGs with antiviral activity, with some having broad-spectrum activity and others being virus-specific. The ISG profiles were quite diverse and showed little overlap between genes acting against replicons versus those acting against infectious viruses. Notably, some ISGs are known to enhance virus replication, adding complexity to the IFN system and highlighting the need for careful analysis of individual ISG functions [193]. The table below highlights key ISGs and their specific antiviral functions.

Table 1. 3 Key ISGs and Their Functions

ISG	Function	Pathogen Targeted
Mx1 (Myxovirus Resistance 1)	Blocks viral nucleocapsid trafficking and inhibits viral replication [194].	Influenza A, La Crosse encephalitis virus (LACV) [194].
OAS/RNase L (2'-5' Oligoadenylate Synthetase/Ribonuclease L)	Detects foreign RNA, activates RNase L to degrade viral RNA [195].	SARS-CoV-2, HCV, [196, 197].
PKR	Activation of PKR by cytosolic PAMPs leads to a dramatic reduction in host cell translation as well as to the degradation of	HIV, HCV [199] [200].

	the inhibitor of κ B (I κ B)—the latter results in activation of the NF- κ B signaling pathway [198].	
IFITM (Interferon-Induced Transmembrane Protein)	Prevents viral entry by blocking membrane fusioninhibit a diverse range of viruses, and this broad antiviral activity strongly suggests that inhibition is not at the level of receptor engagement [201].	DENV, filoviruses, SARS coronavirus, and influenza A, HIV-1 [202-204].
TRIM5 α (Tripartite Motif Protein 5 α)	Binds to retroviral capsids, accelerating uncoating and degradation [205].	HIV, SIV [205, 206]
Viperin (Virus Inhibitory Protein)	Disrupts lipid metabolism to prevent viral budding [207].	HIV, West Nile virus CMV [208-210].
Tetherin (BST-2)	Prevents viral release by tethering virions to the plasma membrane [211].	HIV, Ebola, Kaposi-sarcoma-associated herpes virus (KSHV) [211].
CH25H (Cholesterol-25-Hydroxylase)	inhibits viral entry by production of 25-hydroxycholesterol [212].	Adenovirus [212].
SOCS1/3 (Suppressor of Cytokine Signalling 1 and 3)	Downregulates JAK-STAT signalling to prevent excessive immune activation [213].	Regulates IFN responses in various infections.
ISG15 (Interferon-Stimulated Gene 15)	Functions as an antiviral ubiquitin-like modifier, inhibiting viral replication [214].	Influenza, HCV, HIV [215-217].

1.4.21 Proinflammatory Cytokines

Cytokines are small soluble proteins, usually 6-70 kDa, produced by lymphocytes, macrophages, NK cells, mast cells, and stromal cells. These molecules are important in regulating the body's response to infections, immune challenges, inflammation, and injuries [218]. Some cytokines are proinflammatory, worsening disease by promoting inflammation, while others are anti-inflammatory and support the healing process [219]. Proinflammatory

cytokines include IL-6, IL-8, TNF- α , and IL-1 β , which drive inflammatory responses and recruit immune cells to sites of infection or injury.

1.4.21.1 IL-6

IL-6 is a multifunctional cytokine that acts in both innate and adaptive immunity and affects physiological processes like inflammation, immune cell recruitment, and acute phase reactions [220]. IL-6, first identified in 1973 as a T lymphocyte-secreted protein that helps B cell differentiation, was later found to be identical to several other proteins, suggesting pleiotropic action [221]. IL-6 is a small polypeptide produced by immune and non-immune cells, including macrophages, fibroblasts, and endothelial cells. IL-1 β and TNF- α primarily induce its expression, but it can also be triggered through TLRs, prostaglandins, and stress responses [222]. IL-6 signals through three pathways: classical signalling, trans-signalling, and trans-presentation. Classical signalling occurs when IL-6 signals via its membrane-bound receptor (mIL-6R) on immune cells, resulting in the coupling of the receptor to gp130 and the subsequent activation of the JAK-STAT signalling cascade to regulate immune responses and anti-inflammatory functions. Trans-signalling takes place when IL-6 signals via soluble IL-6R (sIL-6R) to activate gp130 in cells that lack the mIL-6R and is believed to control chronic inflammation and autoimmune diseases. In trans-presentation, DCs present the IL-6/mIL-6R complex to T cells, leading to the pro-inflammatory differentiation of Th17 cells. These pathways identify the role of IL-6 in immunity, inflammation, and disease and illustrate its diverse mechanisms of action [223, 224]. IL-6 plays a crucial role in inflammation and is secreted during tissue injury or infection, inducing acute phase reactants like C-reactive protein, serum amyloid A, and fibrinogen from the liver [225]. It also stimulates the production of hepcidin, which suppresses iron absorption and transport, causing anaemia of chronic disease. Additionally, IL-6 increases ZIP14 expression, which can result in hypozincaemia and impaired wound healing [226]. In the bone marrow, IL-6 is involved in the maturation of megakaryocytes, increasing platelet numbers and contributing to thrombocytosis during the inflammatory process [227]. It also has a role in the pyrogenic response by assisting with the trafficking of lymphocytes through high endothelial venous sinuses (HEVs) to increase immune surveillance [228]. IL-6 is involved in the differentiation of monocytes into macrophages, which participate in phagocytosis, antigen presentation, and the clearance of damaged cells [229]. IL-6 also affects adaptive immunity by stimulating a Th2 response and suppressing a Th1 response through reduced secretion of IFN- γ . In association with TGF- β , it induces Th17 differentiation, which is important for mucosal

immunity but is also involved in autoimmune diseases [230]. Additionally, IL-6 cooperates with IL-7 and IL-15 to induce T cell differentiation and cytolytic activity [231]. It is also involved in B cell maturation into plasma cells, prolonging survival and enhancing immunoglobulin G (IgG) production through regulation of IL-21 [232]. During COVID-19, higher levels of IL-6 are observed in severe cases and are associated with the cytokine storm that leads to respiratory failure and multi-organ damage [233]. Studies also link higher levels of IL-6 with long COVID-19, a state of chronic inflammation following acute COVID-19 characterized by persistent symptoms like fatigue, brain fog, and respiratory issues [234].

1.4.21.2 IL-8

IL-8 is a strongly pro-inflammatory chemokine that is involved in immune responses, neutrophil attraction, and inflammation during normal and pathological conditions [235]. It is secreted by neutrophils, fibroblasts, epithelial cells, hepatocytes, alveolar macrophages, and endothelial cells during infection or in response to tissue injury [236]. IL-8 signals by binding to the CXC receptors, CXCR1 and CXCR2, initiating phosphorylation cascades that induce neutrophil chemotaxis, degranulation, and the production of ROS. However, IL-8/CXCR1/2 signalling has been associated with immunopathology, and an activated, prothrombotic neutrophil phenotype characterized by excessive degranulation and the formation of neutrophil NETs has been implicated in inflammatory and thrombotic disorders [237]. In pathological conditions, IL-8 contributes to disease progression across multiple systems. In the tumour microenvironment, IL-8 promotes angiogenesis, immune evasion, and epithelial-to-mesenchymal transition (EMT), leading to cancer growth and metastasis. Furthermore, IL-8 exacerbates chronic inflammatory diseases like chronic rhinosinusitis by attracting more neutrophils to the affected tissue and increasing their inflammatory response [238]. In respiratory diseases, for example, acute respiratory distress syndrome (ARDS) and COVID-19, IL-8 serves as a biomarker of hyperinflammation and is responsible for lung injury and disease progression. IL-8 levels are elevated in severe cases of COVID-19, which is associated with excessive neutrophil recruitment and activation, resulting in lung tissue damage and worse clinical outcomes [239, 240]. Additionally, IL-8 is involved in neuroinflammatory diseases like Alzheimer's, where it stimulates microglial activation, sustains neuroinflammation, and damages neurons [241]. Beyond its pathological functions, IL-8 serves as an important clinical biomarker, particularly for neonatal sepsis, with reported mean sensitivity of 73% and mean specificity of 81% [242].

1.4.21.3 TNF- α

TNF- α is a potent pro-inflammatory agent involved in the regulation of the immune system, inflammation, and cell signalling. It was first identified due to its ability to induce tumour necrosis in cells [243]. Since then it has been further recognised to be involved in various autoimmune and inflammatory diseases as well as in infectious diseases, including COVID-19 [244, 245]. TNF- α is secreted mainly by activated macrophages, T-lymphocytes and natural killer cells in response to infections, tissue injury or immune activation [246]. It exists in two variations: transmembrane TNF- α (tmTNF- α), which aids in localised cell-to-cell communication, and soluble TNF- α (sTNF- α), which is released into circulation after being cleaved by TNF- α converting enzyme (TACE) [247, 248]. The biological activity of TNF- α is mediated through its interaction with two primary receptors: TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2) [249]. TNFR1 is present in almost all human tissues and is activated by sTNF- α to initiate inflammatory, apoptotic and necroptotic signalling cascades. Upon activation, TNFR1 interacts with intracellular adaptor proteins to activate NF- κ B and MAPKs, which control immune responses. In contrast, TNFR2 is found predominantly on immune cells and endothelium and is preferentially activated by transmembrane TNF- α (tmTNF- α) [250]. While TNFR1 is involved in apoptosis and cell death, TNFR2 does not have a death domain and is involved in cell survival, tissue regeneration and immune modulation. The balance between TNFR1 and TNFR2 signalling is crucial for immune homeostasis, but their dysregulation is implicated in the pathogenesis of chronic inflammation and autoimmune diseases [251]. The role of TNF- α in the pathogenesis of COVID-19 has been recently highlighted. Most severe hyperinflammatory cases of COVID-19 are associated with increased levels of proinflammatory cytokines, such as TNF- α , IL-6, IL-2, IL-7 and IL-10. This type of excessive immune response, representing dysregulated TNF- α signalling, results in the development of ARDS, multi-organ failure and increased mortality in COVID-19 patients [252, 253]. In severe COVID-19, prolonged TNF- α activation leads to lung fibrosis, pneumonia, pulmonary edema, and ARDS. Elevated TNF- α levels are seen in hospitalized COVID-19 patients, and higher levels are associated with worse disease outcomes and mortality. Furthermore, TNF- α has been implicated in cardiovascular complications associated with COVID-19 as it contributes to endothelial dysfunction, thrombosis and atherosclerosis [254, 255].

1.4.21.4 IL-1 β

IL-1 β is a highly active cytokine that regulates the inflammatory process and is involved in the pathogenesis of various diseases. Unlike several other cytokines that are secreted through the standard ER-Golgi secretory pathway, IL-1 β is secreted through non-classical pathways. It is produced as an inactive precursor, pro-IL-1 β , which is activated to the mature IL-1 β by caspase-1 following inflammasome activation. The NLRP3 inflammasome activates caspase-1 and thus promotes IL-1 β maturation in response to PAMPs or DAMPs [256]. IL-1 β can be secreted through different mechanisms, including vesicular release (exosomes, macrovesicles), pore formation mediated by gasdermin D, or passive diffusion during pyroptotic cell death [257-259]. In addition, autophagy has been proposed as a regulatory pathway for IL-1 β , which can be either targeted for degradation or release [260]. IL-1 β triggers immune responses by enhancing the production of other cytokines, upregulating MHC class II molecules and initiating leukocyte migration. The IL-1 β signalling cascade is initiated by the binding of IL-1 β to the receptor IL-1R1. This binding recruits the IL-1 receptor accessory protein (IL-1RAP) to form a receptor complex, which activates intracellular pathways such as NF- κ B, MAPKs (p38, JNK, and ERK), and AP-1 [261]. Additionally, IL-1 β controls the differentiation and activation of Th cell subsets, particularly Th17 cells, by promoting production of IL-6 and IL-23, which are critical for autoimmune and chronic inflammatory conditions [262]. IL-1 β plays a crucial role in the immune response to SARS-CoV-2 by regulating the inflammation that is characteristic of severe COVID-19. The virus activates the inflammasome, particularly the NLRP3 inflammasome, which leads to the activation of caspase-1 and the processing of pro-IL-1 β [263]. Subsequently, IL-1 β is secreted and initiates an inflammatory cascade that may become uncontrolled, leading to lung injury through excessive cytokine release. This hyperinflammation or cytokine storm exacerbates tissue damage and contributes to ARDS development. IL-1 β and its downstream effectors, including IL-18 and IL-6, have been found to be elevated in COVID-19 patients and are associated with disease severity [264]. Recent studies have shown that inflammasome activation is primarily restricted to the lungs and is not systemic in nature, which may explain why IL-1 β levels in the blood are rarely detectable despite the pronounced pulmonary inflammation [263].

1.4.22 TGF- β

TGF- β is a multifunctional cytokine secreted by most tissues and cells, playing crucial roles in embryonic development, wound healing, immune regulation, and tissue homeostasis [265]. The human TGF- β family consists of 33 genes encoding secreted homodimeric or

heterodimeric cytokines, including activins, bone morphogenetic proteins (BMPs), growth differentiation factors (GDFs), and three TGF- β isoforms: TGF- β 1, TGF- β 2, and TGF- β 3 [266]. The family members have been called by various names depending on the historical basis of their molecular identification, and include the activins, bone morphogenetic proteins (BMPs), growth differentiation factors (GDFs), müllerian inhibiting substance (MIS), nodal and the TGF- β s [267]. There are three known TGF- β isoforms: TGF- β 1, TGF- β 2 and TGF- β 3 [268]. These proteins are synthesized as precursors and processed during secretion to produce mature, dimeric ligands linked by disulfide bonds.

TGF- β Processing and Secretion: TGF- β isoforms are initially produced as pro-proteins (pro-TGF- β s) containing large N-terminal pro-domains called latency-associated peptides (LAPs), which facilitate proper folding and dimerization of the C-terminal growth factor domain. After processing in the trans-Golgi apparatus by furin-type enzymes, TGF- β remains non-covalently associated with its pro-peptide to form the small latent complex (SLC). Most cells secrete TGF- β as the large latent complex (LLC), which includes latent TGF- β binding protein (LTBP), a 120-240-kDa glycoprotein that directs TGF- β to the extracellular matrix (ECM) and regulates its bioavailability [269, 270]. TGF- β remains inactive in the ECM, and its activation represents a key regulatory step determining biological activity.

TGF- β Activation Mechanisms:

TGF- β activation occurs through multiple pathways depending on cell type and stimuli. Proteases such as plasmin, matrix metalloproteases (MMP-2 and MMP-9), and neutrophil elastase can activate latent TGF- β [271, 272]. Neutrophil elastase promotes TGF- β 1 production in airway smooth muscle cells via NF- κ B signalling, implicating it in inflammatory responses and tissue repair. Additional activators include thrombospondin-1, ROS, and acidic conditions (pH 4.5) [273, 274]. Integrins α v β 6 and α v β 8 represent critical activation mechanisms through their ability to bind the RGD (Arg-Gly-Asp) sequence in LAPs of TGF- β 1 and TGF- β 3 [280-283]. The α v β 6 integrin, highly expressed in injured epithelial cells, binds and activates latent TGF- β , while α v β 8, found in neuroepithelial and immune cells, enables TGF- β 1 activation through similar RGD-dependent mechanisms. Studies in knockout mice demonstrate that *Itgb6*-deficient mice show severe inflammation but reduced fibrosis, while *Itgb8*-deficient mice suffer intracerebral haemorrhage due to defective CNS vascular development [275]. TGF- β 2 lacks an RGD sequence but can still bind α v β 6 for activation through alternative mechanisms [276].

Clinical Significance and Therapeutic Targeting

The RGD-dependent integrin binding mechanism has significant therapeutic implications. Blocking $\alpha\text{v}\beta 8$ -mediated TGF- β signalling can reverse immunosuppression by shifting macrophages from M2 (tumour-promoting) to M1 (anti-tumour) phenotypes [276]. Therapeutic antibodies such as CRB-601 and 2MW4991 that inhibit TGF- β activation by targeting $\alpha\text{v}\beta 8$ have shown promise in enhancing anti-tumour immunity and promoting CD8⁺ T cell infiltration [277]. These findings highlight RGD-dependent integrin binding as a crucial mechanism for TGF- β activation, with implications for inflammatory diseases, fibrosis, and cancer therapy.

1.4.22.1 Canonical TGF- β Signalling (SMAD Pathway)

The canonical signalling mechanism activated by TGF- β cytokines is the SMAD pathway. Following ligand binding, TGF- β receptors (T β Rs) form heterotetrameric complexes, and the constitutively active T β RII phosphorylates and activates T β RI, which in turn phosphorylates receptor-regulated SMADs (R-SMADs), specifically SMAD2 and SMAD3. Subsequently, the phosphorylated R-SMADs form a complex with the co-mediator SMAD4, which translocates into the nucleus to bind transcription factors (TFs), thereby regulating TGF- β -dependent gene expression [278]. Several regulatory proteins control SMAD activation and signalling. SARA (SMAD Anchor for Receptor Activation) facilitates the recruitment of non-phosphorylated SMADs to the T β Rs at the cell membrane [279]. In contrast, TMEPAI (transmembrane prostate androgen-induced protein) inhibits SMAD activation by preventing SMAD2/3 recruitment and promoting lysosomal degradation of TGF- β receptor I [279]. Following phosphorylation, SMAD2/3 is released from SARA, forms heteromeric complexes with SMAD4, and translocates to the nucleus. The E3 ubiquitin ligases Smurf1 and Smurf2 regulate SMAD protein levels through targeted degradation, while the inhibitory SMAD7 functions as a negative feedback regulator by promoting T β R degradation and competing with R-SMADs for receptor binding [280]. Within the nucleus, SMAD complexes associate with diverse transcriptional cofactors including FOX, HOX, RUNX, AP1, and CREB/ATF family members to determine target gene specificity and recruit either coactivators or corepressors [281]. Additionally, R-SMADs regulate chromatin modifications by recruiting histone acetyltransferases such as CBP and p300, and by interacting with histone deacetylases to modulate chromatin accessibility [282]. Beyond transcriptional regulation, R-SMADs connect TGF- β signalling with the microRNA processing machinery, including the microprocessor complex

components p68, Drosha, and DGCR8, to control the biogenesis of TGF- β -regulated microRNAs [283]. These interconnected mechanisms mediated by the SMAD pathway are essential for precise gene regulation, cell fate determination, and tissue homeostasis.

1.4.22.2 Non-SMAD TGF- β Signalling

In addition to the canonical SMAD pathway, TGF- β activates several non-SMAD signalling pathways that mediate diverse cellular responses including apoptosis, epithelial-mesenchymal transition (EMT), cell migration, proliferation, and differentiation. TGF- β regulates multiple MAPK pathways, including ERK1/2, JNK, and p38, as well as PI3K/AKT signalling [284]. Notably, JNK and p38 activation can occur through both SMAD-dependent and SMAD-independent mechanisms, as demonstrated in NMuMG mammary epithelial cells expressing mutant T β RI [285]. Beyond MAPK signalling, TGF- β engages additional pathways including AKT/PKB, small GTP-binding proteins (RAS, RHOA, RAC1, CDC42), mTOR signalling, and protein tyrosine kinases such as FAK, SRC, and ABL. These pathways are particularly active in mesenchymal cells and dedifferentiated epithelial cells undergoing EMT [286, 287]. The activation of these non-canonical pathways often occurs rapidly following TGF- β stimulation and can regulate cytoskeletal reorganization, cell motility, and metabolic reprogramming. Furthermore, TGF- β signalling exhibits extensive crosstalk with other major signalling networks, including NF- κ B and the Wnt/ β -catenin pathway, expanding its influence on cell survival, differentiation, and tumour progression. Furthermore, TGF- β signalling is known to interact with NF- κ B and the Wnt/ β -catenin pathway, suggesting a wider role in cell survival, differentiation and tumour progression [288, 289]. These non-SMAD pathways reflect the complexity of the TGF- β signalling network and its context-dependent effects in normal and malignant cells. The integration of SMAD-dependent and SMAD-independent pathways allows TGF- β to elicit context-dependent responses that can be either tumour-suppressive or tumour-promoting, depending on the cellular environment and stage of malignant transformation. These non-SMAD pathways highlight the complexity of the TGF- β signalling network and its ability to coordinate multiple cellular processes simultaneously in both normal physiology and disease states.

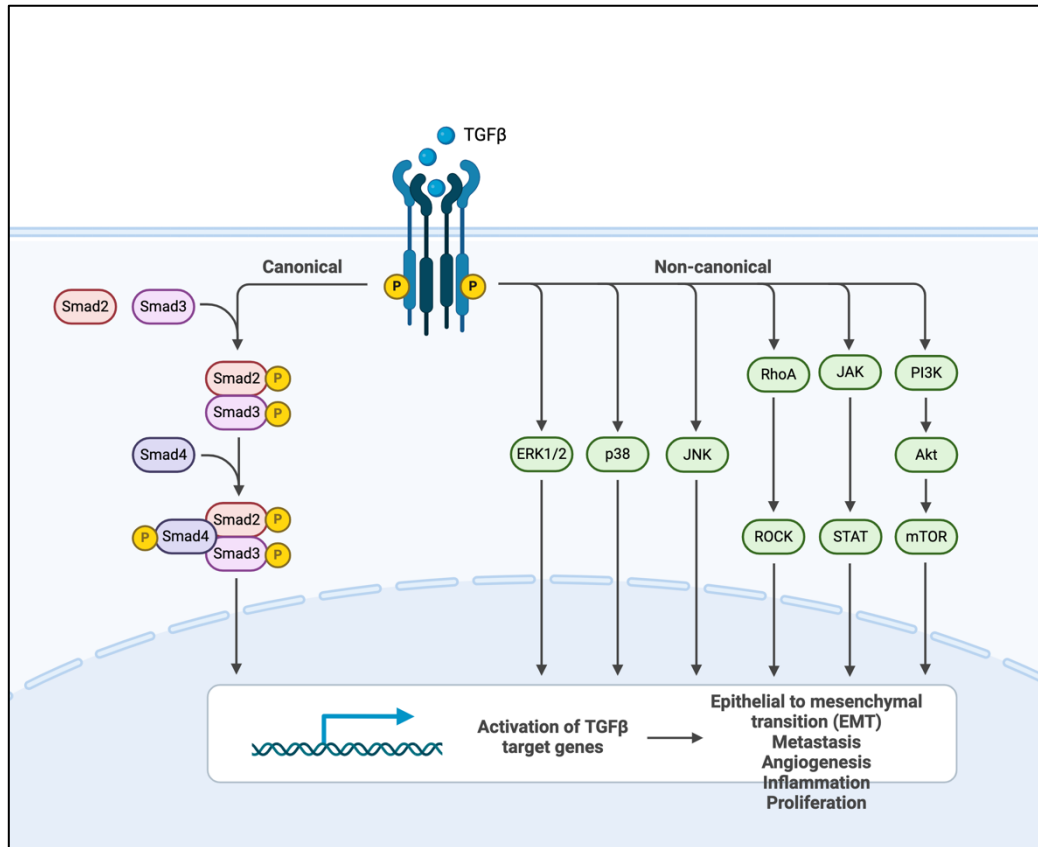


Figure 1.8 TGF-β Signalling Pathway: Canonical and Non-Canonical Pathways.

The TGF-β signalling pathway controls cell growth, differentiation, immune responses, and tumour progression through canonical (SMAD-dependent) and non-canonical (SMAD-independent) pathways. In the canonical pathway, TGF-β binding to its receptors leads to phosphorylation and activation of SMAD2/3, which then form complexes with SMAD4 and translocate to the nucleus to regulate target gene transcription. The non-canonical pathways involve activation of MAPK (ERK1/2, JNK, p38), RhoA/ROCK, JAK/STAT, and PI3K/AKT/mTOR signalling cascades that regulate diverse cellular processes including epithelial-mesenchymal transition (EMT), metastasis, angiogenesis, inflammation, and cell proliferation. Crosstalk between canonical and non-canonical pathways enables context-dependent TGF-β responses in both physiological and pathological conditions.

1.4.22.3 TGF-β1 in COVID-19

Severe COVID-19 is characterized by a TGF-β-dominated immune response that leads to persistent inflammation, immune suppression, fibrosis, and vascular dysfunction without effective SARS-CoV-2 clearance. TGF-β activation plays a central role in microvascular injury, endothelial dysfunction, and coagulation disorders, which exacerbate ARDS and systemic inflammation [290]. In post-COVID-19 survivors, TGF-β1 overexpression is associated with early-phase fibrotic abnormalities and long-term respiratory impairment.

Elevated plasma and exhaled breath condensate TGF- β 1 levels correlate with persistent lung fibrosis and reduced lung function, particularly in patients with COVID-19-associated ARDS [291]. SARS-CoV-2 viral proteins directly contribute to TGF- β pathway dysregulation. Both SP and Nucleocapsid (NP) proteins activate the TGF- β -MRTF (myocardin-related transcription factor) axis, inducing endothelial-mesenchymal transition (EndMT) that promotes vascular fibrosis, endothelial dysfunction, and thrombotic complications [292]. Additionally, the SARS-CoV-2 SP utilizes its RGD motif to bind integrins, particularly α V β 3, leading to vascular barrier disruption, endothelial leakage, and enhanced microvascular thrombosis with subsequent systemic inflammation [293]. The clinical significance of TGF- β 1 in COVID-19 extends beyond pulmonary complications. A prospective case-control study demonstrated that patients with COVID-19 and concurrent non-alcoholic fatty liver disease (NAFLD) exhibit elevated TGF- β 1 levels associated with increased ICU admissions, respiratory complications, and mortality [294]. These findings highlight the systemic impact of TGF- β dysregulation in COVID-19 pathogenesis. Given the multiple pathological roles of TGF- β activation in COVID-19, TGF- β -integrin interactions represent promising therapeutic targets for reducing SARS-CoV-2 susceptibility and disease severity. The integrin/TGF- β 1 inhibitor GLPG-0187 has demonstrated efficacy in preventing SARS-CoV-2 Delta and Omicron pseudovirus infection of airway epithelial cells while suppressing TGF- β 1 activation, suggesting potential for slowing fibrosis progression and improving clinical outcomes [295].

1.5 Neutrophils

Neutrophils have historically been identified as the first leukocytes to respond to inflammatory or infectious etiologies. They are rapidly recruited from the bloodstream to sites of inflammation in response to chemokines, such as IL-8. They are a type of polymorphonuclear (PMN) leukocyte and have a crucial role in innate immune response, which mainly involves containing and eliminating pathogens by multiple mechanisms, e.g., phagocytosis, the formation of ROS, intracellular degradation, and the formation of NETs, a process known as NETosis [296]. Neutrophils are the most abundant circulating leukocyte in humans, but also relatively short-lived, with a circulating half-life of 6-8 hours. The human bone marrow produces more than 0.85×10^9 cells/kg per day, accounting for 50-70% of all leukocytes [297]. Neutrophils are produced progressively in the bone marrow from self-renewing hematopoietic stem cells (HSCs), by a process called granulopoiesis. Granulopoiesis is a part of hematopoiesis, where HSCs develop into highly proliferative

multipotent and lineage-committed granulocytic progenitor cells. These cells differentiate into fully differentiated, functionally active granulocytes [298]. During neutrophil development, first HSCs differentiate into non-self-renewing multipotent progenitor (MPP) cells. These cells have the ability to differentiate into all blood cell lineages. MPPs then transform into lymphoid-primed multipotent progenitors (LMPPs) [299]. LMPPs then differentiate into granulocyte-monocyte progenitor cells. These GMPs, under control of the cytokine granulocyte colony-stimulating factor (G-CSF), differentiate into neutrophils through subsequent differentiation stages: myeloblasts, promyelocytes, myelocytes, metamyelocytes, and band neutrophils [300] (**Fig.1.9**). In tissue, the clearance of apoptotic neutrophils is carried out by resident macrophages and dendritic cells through phagocytosis. Aged neutrophils are cleared from the circulation via the bone marrow, in a CXCR4-dependent manner. Aged neutrophils upregulate the chemokine receptor CXCR4, which allows them to traffic back to stromal cell-derived factor (SDF)-1 α , the ligand for CXCR4. SDF-1 α , also known as C-X-C motif chemokine 12 (CXCL12), is expressed in the bone marrow [301].

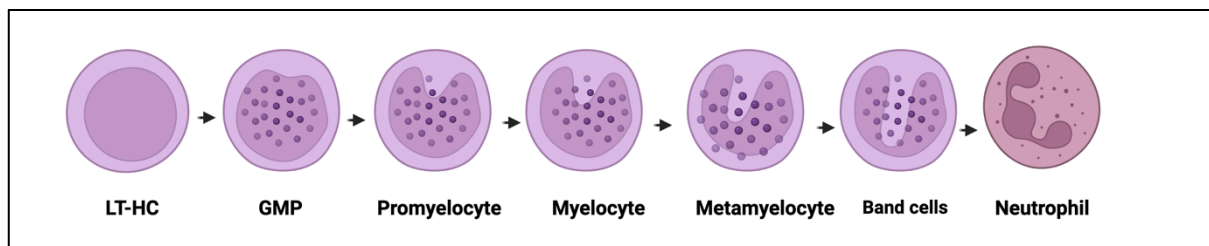


Figure 1.9 Neutrophil Development and Granulopoiesis.

Neutrophils are generated from long-term hematopoietic stem cells (LT-HSCs) through sequential differentiation stages. The granulopoietic pathway progresses from LT-HSCs to granulocyte-monocyte progenitors (GMPs), then through morphologically distinct precursor stages including promyelocytes, myelocytes, metamyelocytes, and band cells before giving rise to mature, functionally competent neutrophils. LT-HSC: long-term hematopoietic stem cell; GMP: granulocyte-monocyte progenitor.

1.5.1 Neutrophils recruitment to the site of infection “Neutrophil release from bone marrow and Neutrophil extravasation into tissue”

Chemokines control the balance between neutrophil release and retention within the bone marrow. Under normal homeostatic conditions, only 1-2% of all bone marrow neutrophils are released into the bloodstream. The CXCR4/SDF-1 and VLA-4/VCAM-1 pathways

regulate neutrophil retention within the bone marrow microenvironment. Osteoblasts and other bone marrow stromal cells produce SDF-1, which interacts with CXCR4, causing neutrophil retention in the bone marrow niche. Both CXCR4 and SDF-1 expression are downregulated by G-CSF during inflammatory conditions. VLA-4/VCAM-1 signalling is essential for neutrophil adherence to marrow endothelial cells and stromal cells. Neutrophil release from the bone marrow is primarily mediated by CXCR2 signalling. CXCR2 ligands, including CXCL1, CXCL2, CXCL5, and CXCL8 (in humans), are expressed by endothelial cells outside the bone marrow when neutrophils need to be mobilized to the systemic circulation [302, 303].

1.5.2 Mechanism of Neutrophil Activation

Upon release into tissues, neutrophils encounter an inflammatory microenvironment containing host-derived mediators, including TNF- α , IL-1 β , IFN- γ , and granulocyte-macrophage colony-stimulating factor (GM-CSF), which signal for neutrophil activation and enhance their recruitment to sites of infection. To execute their antimicrobial functions and support host defence, neutrophils must undergo stepwise activation [304]. While neutrophil recruitment initiates their activation, multiple priming steps are required to achieve full activation, enabling degranulation and oxidative burst [305]. During circulation, neutrophils exist in a quiescent state, remaining unattached and spherical with minimal transcriptional activity. They exhibit limited responsiveness to activating stimuli, which serves as a protective mechanism against unnecessary inflammatory responses and associated tissue damage [306]. Upon exposure to proinflammatory cytokines, chemokines, mitochondrial components, and microbial products, neutrophils become sensitized or 'primed' for enhanced immune activation. Primed neutrophils display phenotypic alterations including augmented ROS production, enhanced granule content release, increased cytokine and chemokine secretion, improved adhesion and transmigration capabilities, enhanced chemotaxis, and delayed apoptosis [306]. Neutrophil activation is a multi-step process involving several key receptor families, including PRRs, opsonic receptors, and G-protein-coupled receptors (GPCRs). Immune activation is initiated through recognition of PAMPs by PRRs or DAMPs released from necrotic cells. TLRs such as TLR2, TLR4, and TLR9 enhance neutrophil phagocytosis, cytokine secretion, and survival [306]. Additionally, Dectin-1 and TREM-1 facilitate bacterial and fungal recognition, with TREM-1 playing a crucial role in sepsis-induced neutrophil activation. Opsonic receptors, including Fc γ receptors (Fc γ RIIA, Fc γ RIIIB, and Fc γ RI) and complement receptors (CR3, CR4, and CR1), enable neutrophils to recognize and eliminate IgG- and complement-opsonized pathogens,

further enhancing phagocytosis and ROS production. GPCRs, which respond to bacterial formyl peptides, leukotrienes, IL-8 (CXCL8), and C5a, mediate neutrophil chemotaxis and activation during infection and inflammation [307]. However, excessive neutrophil activation can result in uncontrolled inflammation, potentially causing tissue damage and contributing to the pathogenesis of inflammatory disorders such as ARDS and sepsis. Therefore, precise regulation of neutrophil activation is essential to maintain the balance between protective immune responses and prevention of collateral tissue damage [308].

1.5.3 Neutrophil Surface Marker Changes During Infection

Changes in the expression of cell surface markers represent hallmarks of neutrophil activation and vary depending on the type of pathogen encountered. For instance, following activation, neutrophils exhibit increased expression of CD64 (high-affinity Fc γ receptor I), CD11b, CD66b, and CD69, while showing decreased expression of CD62L, CD16, and CD182 [309-314]. Understanding these marker changes is crucial for elucidating neutrophil functions across different infectious contexts. Table 1.4 provides a summary of key changes in neutrophil surface markers observed in different viral infections.

Table 1. 4 Summary of Neutrophil Surface Marker Expression Changes Across Different Viral Infections

Marker	Regulation	Infection Type	Notes
CD64 (Fc γ RI)	↑ / ↓	IAV (Human) RSV	For IAV upregulated in one study, downregulated in another [315-317]. For RSV downregulated.
CD11b	↑ / ↓	IAV (Human), Rhinovirus (COPD), RSV SARS-CoV-2 ↓	Upregulated in blood neutrophils during IAV infection [316]; in sputum neutrophils during rhinovirus infection in Chronic obstructive pulmonary disease (COPD) patients[317, 318]. Downregulated in SARS-CoV-2
CD69	↑	IAV (Mouse)	Upregulated in BAL neutrophils during IAV infection but not in RSV [309, 315].
CD62L	↓	ZIKV, RSV SARS-CoV-2	Downregulated after neutrophil activation [317, 319].
CD182	↓	RSV	Downregulated after neutrophil activation [317].

CD63	↑	Rhinovirus (COPD	Upregulated in sputum neutrophils of COPD patients [318]
CD66	↑	Rhinovirus (COPD) and DENV	Upregulated in sputum neutrophils of COPD patients [313, 318].

These receptor modifications directly affect neutrophil functions including adhesion, migration, phagocytosis, and interactions with other immune cells. For example, the upregulation of CD64 enhances phagocytic activity to facilitate pathogen and cellular debris clearance [320]. The specific pattern of marker expression is influenced by the type of infection and the surrounding inflammatory milieu, highlighting the adaptability of neutrophils in mounting appropriate immune responses.

1.5.4 Neutrophils roles in innate immunity

Neutrophils combat infection through phagocytosis, degranulation (releasing proteases, oxidants, antimicrobial peptides, and defensins), NET formation, respiratory burst producing ROS, chemotaxis toward infection sites, cytokine production for immune cell recruitment, and direct antimicrobial activity via lysozyme and membrane-disrupting proteins [321].

Phagocytosis

Phagocytosis is a crucial process whereby neutrophils engulf microbes rapidly through both opsonin-dependent and opsonin-independent mechanisms. In opsonin-dependent phagocytosis, Fcγ receptors (FcγRIIA, FcγRIIb and FcγRI) bind immunoglobulins attached to pathogens, while β2 integrin MAC-1 (CD11b/CD18) interacts with complement fragment iC3b to facilitate pathogen internalization [322]. Neutrophils efficiently engulf IgG-opsonized particles within 20 seconds, significantly faster than macrophages which have a slower, more regulated phagocytic process [323]. For non-opsonized pathogens, neutrophils utilize PRRs including scavenger receptors, dectin-1 (a C-type lectin receptor recognizing fungal β-glucans), mannose receptors that identify mannose-rich glycans on microbial surfaces, and TLRs that enhance phagocytosis while recognizing PAMPs [324, 325]. Following uptake, phagocytic vacuoles fuse with preformed granules containing hydrolytic enzymes and NADPH oxidase subunits that generate ROS for pathogen destruction. Unlike macrophages that rely on gradual lysosomal fusion and acidic phagosome maturation, neutrophils rapidly deploy prestored granules and maintain neutral phagosomal pH due to their intense oxidative burst, enabling faster pathogen elimination [323]. Beyond microbial

clearance, neutrophil phagocytosis contributes to immune regulation by removing apoptotic cells and debris, preventing excessive inflammation [326]. However, dysregulated phagocytosis and uncontrolled granule release can contribute to chronic inflammatory diseases, autoimmune conditions, sepsis, and ARDS [327]. While neutrophils can phagocytose some pathogens like bacteria and opsonized respiratory viruses (RSV, IAV) through surfactant protein D-mediated mechanisms [328, 329], their interaction with certain viruses differs significantly. Studies by Ratcliffe and colleagues demonstrated that neutrophils do not directly engulf free influenza virions but instead bind them through sialic acid sites on viral hemagglutinin, suggesting that against such viral infections, neutrophils primarily rely on alternative mechanisms including degranulation, ROS production, and NET formation rather than classical phagocytosis [330, 331].

Degranulation

Neutrophil degranulation is a crucial controlled release process involving membrane-bound cytoplasmic vesicles containing over 1,200 unique proteins, including proteolytic enzymes, antimicrobial molecules, NADPH oxidase components, and receptors that regulate endothelial adhesion, extracellular matrix remodelling, and inflammatory signalling [332]. Neutrophils contain four types of granules that undergo sequential release: secretory vesicles (released first), followed by tertiary (gelatinase) granules, secondary (specific) granules, and finally primary (azurophil) granules, which require the most potent activation signals [333]. The released granule contents serve multiple effector functions, with gelatinases and collagenases (MMP-8 and MMP-9) from secondary and tertiary granules degrading extracellular matrix to facilitate neutrophil migration while releasing proline-glycine-proline tripeptides that enhance further neutrophil recruitment [334, 335]. Primary granule components including neutrophil elastase (NE) and myeloperoxidase (MPO) provide direct antimicrobial activity through proteolysis and enhanced ROS production [336]. Neutrophil degranulation is particularly significant in viral infections. Research has found that RSV and DENV viruses trigger the release of key granule enzymes such as MPO and lactoferrin from neutrophils at the sites of infection [337, 338], while elevated NE levels are found in serum and airways of RSV-infected hospitalized infants [339, 340]. COVID-19 patients demonstrate increased azurophilic granules containing MPO, elastase, and cathepsin G in nasopharyngeal samples compared to healthy controls [341]. Lactoferrin, a secondary granule protein, inhibits viral infections by preventing microbial adhesion and blocking viral entry, as demonstrated with SARS-CoV-2 where it binds viral receptors and reduces human norovirus infections through indirect IFN response activation [342-344]. Additionally,

neutrophil granules store antimicrobial peptides including cathelicidins (such as LL-37) and α -defensins, with cathelicidins being activated by proteinase 3 (PR3) to exhibit direct antimicrobial effects and modulate chemokine/cytokine production, while α -defensins disrupt pathogens by forming membrane pores or coating them [333, 345-347]. LL-37 specifically inhibits IAV and RSV infections, and neutrophil peptides enhance IAV uptake through viral aggregation, demonstrating the multifaceted antiviral capabilities of degranulated neutrophil components [348, 349] .

ROS

Neutrophils employ nicotinamide adenine dinucleotide phosphate (NADPH) oxidase to produce ROS through respiratory burst as a primary antiviral defence mechanism, generating highly reactive molecules including superoxide ion (O_2^-) and hydrogen peroxide (H_2O_2) that serve multiple antimicrobial functions [350] . During viral infections, ROS generation is heightened due to immune activation and viral-induced oxidative stress, contributing to antiviral defence through induction of apoptosis in infected cells, restriction of viral replication, and activation of immune responses [351]. Superoxide and hydrogen peroxide can induce programmed cell death of virus-infected cells and their progeny, thereby containing viral spread, as demonstrated in infections with influenza A virus (IAV), herpes simplex virus (HSV), and respiratory syncytial virus (RSV), which all stimulate ROS production through the NADPH oxidase system [352-355]. However, this relationship is complex as many viruses including HCV, HIV, and influenza virus exploit ROS pathways to enhance their own replication and pathogenesis, while SARS-CoV-2 infection has been linked to oxidative stress that contributes to coagulation disorders and severe inflammatory responses [351, 356]. Beyond direct antiviral activity, ROS regulate neutrophil responses by inducing autophagy through mTOR kinase inhibition to improve antiviral defences, promoting NET formation, and triggering apoptosis of infected cells [357-359]. The delicate balance between ROS-mediated antiviral defence and potential oxidative damage to host tissues is crucial, as overproduction can result in chronic inflammation, immune dysregulation, and tissue damage, making understanding of this balance essential for developing therapeutic strategies that target viral infections without causing excessive collateral damage[360].

NETs

NETs are web-like structures composed of nuclear chromatin, antimicrobial proteins, and cytoplasmic components including histones, NE, MPO, and α -defensins that, although first discovered for bacterial defence, are now recognized as important antiviral mechanisms

against pathogens including RSV, SARS-CoV-2, influenza virus, DENV, and HIV through encapsulation of viral particles, inhibition of spread, and direct killing via high local concentrations of antimicrobial components [361, 362]. NET formation occurs primarily through NETosis, a specific form of neutrophil death dependent on NADPH oxidase-mediated ROS production that results in neutrophil rupture and chromosomal DNA release, although an alternative non-lytic pathway exists where viable neutrophils release mitochondrial DNA-based NETs following TLR4 or C5a stimulation after GM-CSF priming without cell death [363, 364]. While NETs provide antiviral protection, they are associated with inflammatory damage and are overproduced in COVID-19, H1N1, H7N9, and RSV infections, with elevated NET levels correlating with pulmonary pathology [365-367]. RSV induces NET formation by human neutrophils *in vitro*, capturing viral particles and preventing target cell infection. *In vivo*, extensive NET formation occurs in the airways of infants with severe RSV lower respiratory tract disease and calves with bovine RSV. Lung pathology shows dense NET-containing airway plugs that sometimes harbor viral antigens [368]. Moreover, the RSV fusion (F) protein directly induces NET formation and promotes MPO association with NETs [369]. Similarly, NETs have been detected in lung injury regions during infections with influenza A virus, Sendai virus, and rhinovirus in murine models, though some findings may represent extracellular DNA from dying cells rather than true NETs [370-372]. The role of NETs in COVID-19 demonstrates a complex dual function: protective studies show that NETosis encapsulates SARS-CoV-2 particles and prevents viral spread, with NET inhibition enhancing infection and DNase I degradation of NETs increasing viral load both *in vitro* and in ACE2 transgenic mice, suggesting viricidal activity independent of NE and MPO [373]. Conversely, SARS-CoV-2-induced NETs contribute to COVID-19 pathology through inflammatory tissue damage, with elevated NET concentrations found in plasma, tracheal aspirates, and lung tissues of hospitalized patients, and NET formation depending on ACE2, serine proteases, viral replication, and PAD-4 activity, ultimately causing lung epithelial cell death and disease progression [374]. These findings reveal that NETosis serves dual functions in COVID-19, acting both as a host defence mechanism against viral spread and as a pathogenic factor that exacerbates disease severity through inflammatory tissue destruction.

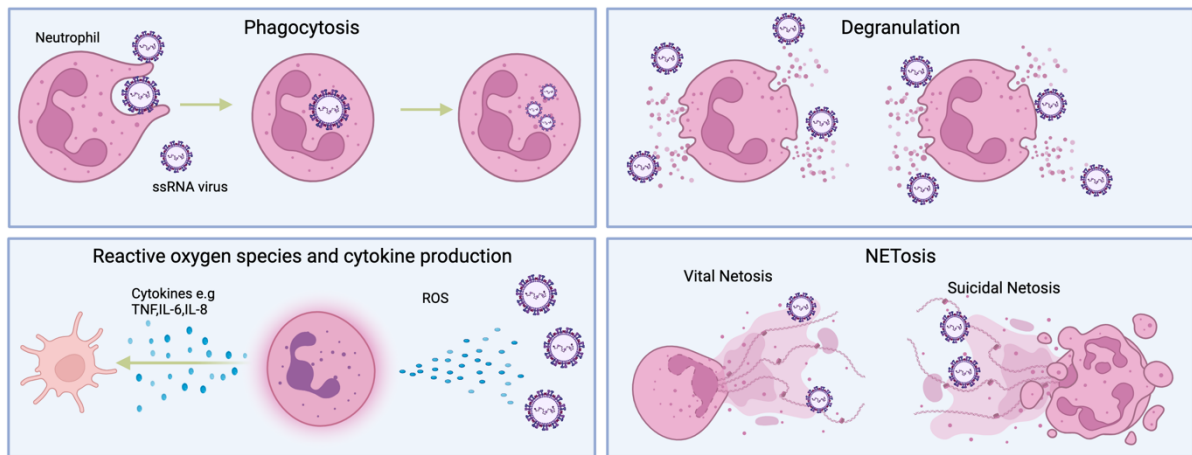


Figure 1.10 Neutrophils in Viral Defence: Mechanisms of Phagocytosis, Degranulation, ROS Production, and NETosis.

Neutrophils combat viral infections through four key mechanisms: phagocytosis (engulfing ssRNA viruses into phagosomes for degradation), degranulation (releasing antimicrobial proteins like MPO, NE, and defensins), ROS production (generating superoxide and hydrogen peroxide that damage viral components and trigger cytokine release including TNF- α , IL-6, and IL-8), and NETosis (expelling chromatin and antimicrobial proteins to trap viruses through either vital or suicidal pathways). While these responses effectively control infections, excessive activation can cause tissue damage and inflammation in diseases like COVID-19, influenza, and RSV.

1.5.5 Neutrophil Heterogeneity

Neutrophils, which have been traditionally classified as a relatively uniform cell population, are now recognized to display significant phenotypic and functional heterogeneity. Similar to the M1/M2 polarization model described for macrophages, neutrophils have been suggested to polarize into functionally distinct subtypes designated as N1 and N2, although the criteria for distinguishing these subsets are still emerging and can vary by experimental context. N1 neutrophils are typically characterized as pro-inflammatory cells that produce high levels of ROS and pro-inflammatory cytokines, thereby enhancing pathogen clearance and, in some instances, anti-tumour immunity, while N2 neutrophils exhibit immunosuppressive properties and secrete anti-inflammatory mediators that promote tissue repair and may facilitate tumour progression in cancer [375]. Another important dimension of neutrophil heterogeneity involves the presence of low-density neutrophils (LDNs), which are distinguished from conventional normal-density neutrophils (NDNs) through density gradient centrifugation of blood, with NDNs sedimenting in the granulocyte fraction and LDNs co-localizing with the mononuclear cell layer due to their altered density properties.

LDNs have been identified in various disease conditions and possess both pro-inflammatory and immunosuppressive properties, suggesting additional complexity within neutrophil populations. In systemic lupus erythematosus (SLE), LDNs demonstrate elevated levels of activation markers CD11b and CD66b compared to NDNs, while analysis of healthy donors reveals that LDNs and NDNs can be differentiated by surface marker expression, with NDNs showing reduced levels of CD15 and CD16b compared to LDNs. These findings demonstrate the functional and phenotypic differences between neutrophil subsets and reveal the increasing complexity of neutrophil biology beyond traditional concepts of cellular uniformity [376].

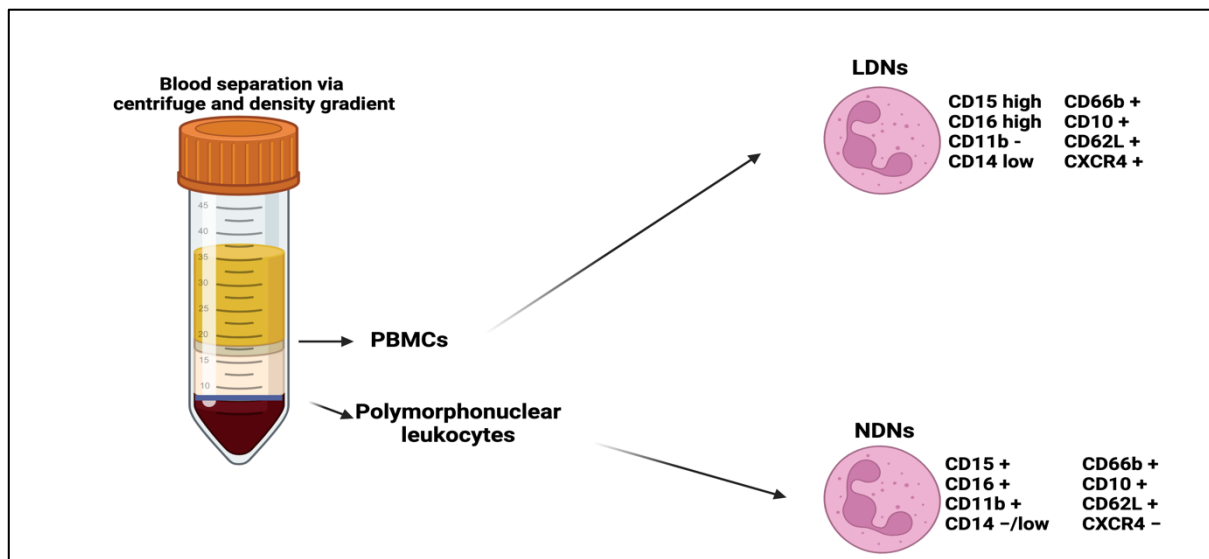


Figure 1.11 Morphologically distinct subsets of neutrophils are observed in circulation.

Density gradient centrifugation of blood yields two distinct neutrophil populations: NDNs found in the polymorphonuclear cell layer and LDNs co-localizing with peripheral blood mononuclear cells (PBMCs) in the upper layer. These populations exhibit distinct phenotypic surface marker profiles, with LDNs characterized by high CD15 and CD16 expression but low CD14 levels, while NDNs show positive but lower CD15 and CD16 expression with higher CD11b and CD14 levels. Both populations express CD66b, CD10, CD62L, and CXCR4 but at different intensities, and demonstrate functional differences in ROS production and phagocytic capacity. In COVID-19 patients, neutrophil heterogeneity is further revealed through characterization as CD15+CD66b+CD193-CD16bright/dim populations, with their proportion increasing with disease severity and involving both mature (CD16+) and immature (CD16-/dim) neutrophils. Patients with severe COVID-19 exhibit more immature neutrophils with reduced CD66b and CD11b surface expression, while moderate cases are characterized by mature neutrophils with enhanced CD11b,

CD66b, and CD177 expression that produce molecules supporting type 1 responses (CXCL9, CXCL10, IL-15) and IFN-related adaptive immune responses (IL-2, B-cell Activating Factor (BAFF)). Immature neutrophil counts normalize to healthy control levels upon patient recovery, demonstrating the dynamic nature of neutrophil heterogeneity in disease states [377].

1.6 SARS-CoV-2

The virus that causes COVID-19 is an enveloped, positive-sense, single-stranded RNA virus from the genus Betacoronavirus. It has a genome of approximately 30kb in length, encoding structural proteins including S glycoprotein, Membrane (M) protein, Envelope (E) protein, and Nucleocapsid (N) protein, along with non-structural proteins involved in viral replication. The viral E protein is acquired through budding from the host cell membrane. The SP are glycoproteins with three subunits attached to the envelope. The SP is divided into two functional subunits: S1, which contains the receptor-binding domain (RBD) responsible for recognizing the ACE2 receptor, and S2, which facilitates membrane fusion. ACE2 is expressed on type II alveolar epithelial cells, endothelial cells, and enterocytes, contributing to the virus's broad tissue tropism and the systemic nature of COVID-19 [376]. Upon ACE2 binding, the host protease TMPRSS2, found in respiratory epithelial cells, triggers SARS-CoV-2 SP cleavage, inducing conformational changes in the S2 subunit that culminate in fusion peptide exposure, allowing viral envelope fusion with the host membrane. In certain cases, alternative proteases such as cathepsins found within endosomes can also mediate this process if TMPRSS2 is not available. After fusion, the viral nucleocapsid is released into the cytoplasm, and the positive-sense RNA genome undergoes direct translation and replication via the viral RNA-dependent RNA polymerase (RdRp). These processes enable rapid viral propagation and result in widespread infection and severe complications such as ARDS, hyperinflammation, and immunothrombosis, which are characteristic features that define severe COVID-19 [378-381].

1.6.1 COVID-19

COVID-19 primarily affects the lungs and can also have complications that affect other organs, including the cardiovascular system, the nervous system, and the kidneys. The disease has a variety of symptoms, from mild flu-like illness to severe respiratory failure that requires intensive care. The severity of COVID-19 differs between people and is classified into four main categories: mild, moderate, severe and critical, according to the clinical features and the degree of respiratory failure [382].

1.6.2 Classification of COVID-19 Severity

The table below outlines the classification criteria for mild, moderate, severe, and critical COVID-19 cases:

Table 1. 5 Clinical Criteria for Classification of COVID-19 Severity Levels

Severity Level	Clinical Features	Respiratory Status	Complications
Mild	Fever, cough, sore throat, headache, muscle pain, and fatigue	No shortness of breath, $\text{SpO}_2 \geq 95\%$ on room air	No pneumonia or organ dysfunction
Moderate	Pneumonia with fever, cough, and mild respiratory distress	SpO_2 90-94% on room air, respiratory rate ≤ 30 breaths per minute	No signs of severe pneumonia or ARDS
Severe	Severe pneumonia with significant respiratory distress	$\text{SpO}_2 < 90\%$ on room air, respiratory rate > 30 breaths per minute	High risk of ARDS, cytokine storm, or organ failure
Critical	Respiratory failure, septic shock, or multi-organ dysfunction	Requires mechanical ventilation or high-flow oxygen support	ARDS, sepsis, multiple organ failure, high mortality risk

This classification is important for informing treatment decisions, from supportive care management at home for mild cases to intensive care and mechanical ventilation for critical cases. Identifying severe and critical forms of COVID-19 at an early stage allows for early intervention, resulting in better patient outcomes and reduced mortality.

1.6.3 Neutrophil Responses to SARS-CoV-2

Neutrophils play a crucial role in the immune response to SARS-CoV-2, with their activation and dysfunction significantly contributing to COVID-19 pathogenesis across different severity levels. Both *in vivo* and *in vitro* studies have been conducted to explore their involvement in disease progression, providing complementary insights into neutrophil biology during infection. While *in vivo* studies provide a more accurate understanding of how neutrophils function during infections within the complex host environment, *in vitro* experiments are valuable for examining specific interactions and mechanisms associated

with the disease under controlled conditions. A comparison of these two approaches highlights essential aspects of neutrophil activation, cytokine production, neutrophil NET formation, tissue damage, and interactions with other immune cells. The table below summarizes the key findings from studies conducted *in vivo* and *in vitro* on the role of neutrophils in SARS-CoV-2 infection.

Table 1. 6 Comparison of Neutrophil Responses in COVID-19: In Vivo Versus In Vitro Findings

Aspect	In Vivo Findings	In Vitro Findings
Neutrophil Activation	Elevated neutrophil counts observed in severe COVID-19 patients; hyperactivation correlates with disease severity [383, 384].	SARS-CoV-2 directly activates neutrophils via TLR7/8 and STING pathways, leading to ROS and cytokine release [385].
Cytokine Production	Increased levels of IL-6, IL-8, IL-1 β , and TNF- α detected in patient plasma and bronchoalveolar lavage fluid [386, 387].	Neutrophils release IL-8, IL-6, and TNF- α in response to virus exposure; cytokine production varies by viral variant[385, 388].
NET Formation	NETs detected in lung tissues, blood, and sputum of COVID-19 patients; associated with thrombosis and ARDS [365, 366, 374].	SARS-CoV-2 induces robust NETosis in isolated neutrophils; NETs can inhibit the virus in culture [373].
Tissue Damage	Lung damage due to neutrophil-induced inflammation, protease release, and NET toxicity [365, 374].	Neutrophil-derived NETs and proteases damage epithelial layers in lung co-culture models [373].
Interaction with Other Cells	Neutrophils interact with epithelial cells, monocytes, and lymphocytes, contributing to cytokine	Co-culture with epithelial cells enhances inflammatory response; neutrophils reduce lymphocyte survival in mixed immune cell cultures Also

	storm and immune suppression [389-391].	SARS-CoV-2 induces dysregulated degranulation in neutrophils and affects lymphocyte numbers[392] [374, 388].
Neutrophil-Lymphocyte Ratio	High neutrophil-to-lymphocyte ratio (N-LR) linked with poor prognosis and mortality in COVID-19 patients [393].	Neutrophil activation in culture models mirrors clinical findings; enhanced activation correlates with increased inflammation [388].

1.6.4 Neutrophils as Mediators of Immunopathology in COVID-19

While neutrophils are important for host defence, there is growing evidence that they are also key players in the immunopathogenesis of severe COVID-19, specifically in lung injury and the development of ARDS. ARDS is characterized by diffuse alveolar damage with exudative edema, hyaline membrane formation, and inflammatory infiltrates. Neutrophils are evident in ARDS histopathology, and COVID-19 is no different. However, excessive neutrophil-driven inflammatory responses are key drivers of tissue injury in severe COVID-19. The following are key mechanisms by which neutrophils contribute to pathological outcomes.

1.6.4.1 Damage to Alveolar-Capillary Barrier

Neutrophils release proteases such as neutrophil elastase, matrix metalloproteinases, and ROS in the lung, which degrade alveolar epithelium and capillary endothelium, thereby increasing vascular permeability [394]. This leads to pulmonary edema and hyaline membrane formation, impairing gas exchange [395]. Moreover, NET-associated enzymes further break down lung tissue, worsening the injury [396].

1.6.4.2 Cytokine Storm Amplification

Neutrophil production of pro-inflammatory cytokines (IL-1 β , IL-6, TNF- α) and chemokines (IL-8, CXCL2) sustains excessive inflammatory responses by maintaining a pro-inflammatory environment [397]. Furthermore, IL-8 is strongly expressed by activated neutrophils in the lung, creating an autocrine loop that perpetuates neutrophil activation and tissue recruitment [398].

1.6.4.3 NETs and Immunothrombosis:

NET components (histones, NE, MPO) are cytotoxic to endothelial cells and thus worsen lung injury. NETs act as scaffolds for platelet aggregation and fibrin deposition, resulting in widespread microthrombosis in pulmonary capillaries. High circulating NET markers correlate with severe disease, suggesting systemic thromboembolic complications [399, 400].

1.6.4.4 Neutrophil Heterogeneity and Dysfunction:

LDNs and other atypical neutrophil populations are observed in severe COVID-19 and possess enhanced inflammatory phenotypes. These LDNs are often immature stages (promyelocytes, myelocytes) released from the bone marrow prematurely through emergency myelopoiesis [401]. Morisey *et al.*, identified a population of LDGs characterized as

CD45⁺CD66b⁺CD16^{int}CD44^{low}CD11b^{int} that are associated with disease severity and hypercoagulable states in COVID-19 patients. These cells demonstrate increased phagocytic capacity, spontaneous NET formation, and higher cytokine production [400]. They express lower levels of the chemokine receptor CXCR2 and adhesion molecule CD62L compared to conventional neutrophils. These neutrophils express S100A8/A9 (alarmins) and ISGs, promoting inflammation and NET formation. Some neutrophils upregulate PD-L1 and Arginase-1, suppressing T-cell responses and potentially impairing viral clearance. Metabolic reprogramming, including increased glycolysis and succinate accumulation, sustains neutrophil hyperactivation and contributes to persistent inflammatory responses. Neutrophils from children with COVID-19 exhibit different phenotypic profiles compared to adult patients and healthy controls. They show reduced expression of adhesion molecules including CD11b, CD66b, and L-selectin that are required for neutrophil accumulation at inflammation sites. However, they display increased activation markers such as HLA-DR, CD64, and PECAM-1, indicating maintained functional capacity. Additionally, elevated levels of inhibitory receptors LAIR-1 and PD-L1 suggest possible regulatory mechanisms that may limit inflammation and tissue infiltration, potentially contributing to the generally milder disease course observed in COVID-19 patients [402].

1.6.4.5 Multi-Organ Damage:

Disseminated NETs and systemic neutrophil activation contribute to multi-organ complications including acute kidney injury, cardiac dysfunction, and cerebrovascular events. Neutrophil-rich microthrombi in glomeruli, brain, and cardiac vessels indicate

systemic immunothrombosis extending beyond pulmonary involvement [401]. High circulating NET levels correlate with markers of tissue damage (LDH, AST), hypercoagulability parameters, and poor clinical outcomes, highlighting the systemic nature of neutrophil-mediated pathology in severe COVID-19 [403].

1.7 Overall Rationale Underpinning this Thesis

Severe COVID-19 cases are characterized by distinctive immune dysregulation, with patients exhibiting lymphopenia alongside elevated neutrophil counts, resulting in an increased neutrophil-to-lymphocyte ratio that strongly correlates with disease severity and mortality [400]. These findings implicate neutrophils, the most abundant circulating innate immune cells, as key contributors to COVID-19 pathogenesis. While neutrophils serve as critical frontline defenders that typically facilitate infection clearance, their excessive activation has been directly linked to the hyperinflammation and multi-organ damage characteristic of severe COVID-19 [404]. Neutrophil-mediated pathological processes, including degranulation, ROS generation, and NETosis, can cause extensive tissue damage during acute SARS-CoV-2 infection [400]. This presents a paradox: neutrophils are essential for antiviral defence, yet their dysregulated activation may drive disease progression through inflammatory injury. Understanding the precise mechanisms by which SARS-CoV-2 influences neutrophil behaviour is therefore critical for developing targeted therapeutic interventions that preserve beneficial immune functions while mitigating harmful inflammatory responses.

Despite growing recognition of neutrophil involvement in COVID-19, significant mechanistic gaps remain. The molecular pathways through which SARS-CoV-2 triggers neutrophil activation are incompletely understood, particularly whether activation occurs through direct virus-neutrophil interactions or predominantly via indirect mechanisms involving infected cell-derived mediators. Additionally, the specific viral components responsible for neutrophil activation and the downstream signalling cascades involved require elucidation. Addressing these knowledge gaps forms the foundation of this thesis.

1.8 Importance of this Investigation

The strong association between neutrophil activation and COVID-19 severity makes mechanistic investigation of neutrophil-SARS-CoV-2 interactions both scientifically compelling and clinically urgent. By elucidating how SARS-CoV-2 and its components trigger neutrophil activation and inflammatory mediator release, this research will advance our understanding of COVID-19 immunopathogenesis and identify potential therapeutic

targets. Understanding the molecular basis of neutrophil activation could inform precision therapeutic strategies. If specific viral components (such as the SP or ssRNA) are identified as primary neutrophil activators, targeted inhibitors could be developed to block these interactions or their downstream signalling cascades. Conversely, characterizing the beneficial antiviral functions of activated neutrophils will help optimize the timing and selection of immunomodulatory treatments to prevent interference with necessary host defence mechanisms. Several neutrophil-targeted therapies are already under clinical investigation for COVID-19, including DNase enzymes to degrade NET-associated extracellular DNA in ARDS [401], neutrophil elastase inhibitors, and chemokine receptor antagonists targeting IL-8-mediated neutrophil recruitment. By identifying the critical steps in SARS-CoV-2-induced neutrophil activation, this research will provide evidence-based guidance for prioritizing and optimizing these therapeutic approaches. Ultimately, these findings may contribute to improved COVID-19 management by reducing immunopathology while preserving essential antimicrobial functions.

1.9 Hypothesis

We hypothesize that SARS-CoV-2 activates PMNs through both direct and indirect mechanisms, with distinct activation profiles depending on the mode of interaction.

Direct activation hypothesis: SARS-CoV-2 viral particles, purified SP, and viral ss RNA will directly activate PMNs through PRR engagement, including TLRs and other innate immune sensors. This activation will manifest as:

- Increases in PMN activation markers (CD11b upregulation, CD62L shedding).
- Enhanced NET formation.
- Increased ROS production and pro-inflammatory cytokine release (IL-6, IL-8, TNF- α , IL-1 β).

Indirect activation hypothesis: Factors secreted from SARS-CoV-2-infected epithelial cells activate PMNs through paracrine signalling mechanisms, potentially producing more sustained activation compared to direct viral exposure.

1.10 Overall Objective

The primary aim of this thesis is to elucidate the mechanisms by which SARS-CoV-2 induces PMN activation and to characterize the functional consequences of this activation in the context of COVID-19 immunopathogenesis. Through systematic investigation of direct and indirect activation pathways, we seek to identify key molecular interactions that

could serve as targets for therapeutic intervention to reduce excessive inflammation while preserving beneficial immune responses.

1.11 Specific Aims

Aim 1: Characterize PMN Activation Markers

Quantify changes in neutrophil surface activation markers (CD11b, CD16, CD66b, CD62L) following exposure to SARS-CoV-2 components, live virus, or conditioned media from infected cells.

Aim 2: Assess NETosis and ROS Production

Investigate SARS-CoV-2-induced NET formation following exposure to SARS-CoV-2 components, live virus, or conditioned media from infected cells using SYTOX Orange by flow cytometry. Simultaneously measure intracellular and extracellular ROS production using CellROX by flow cytometry. This aim will establish whether SARS-CoV-2 triggers the pathological NET formation associated with COVID-19 tissue damage.

Aim 3: Profile Cytokine and Chemokine Production

Quantify neutrophil production of key inflammatory mediators (IL-6, IL-8, TNF- α , IL-1 β) following exposure to SARS-CoV-2 components, live virus, or conditioned media from infected cells using ELISA. This aim will characterize how activated neutrophils contribute to the cytokine storm and determine whether specific viral components elicit distinct inflammatory profiles.

Each aim incorporates appropriate controls, including unstimulated neutrophils, non-SARS-CoV-2 viral controls, and validated positive controls to ensure SARS-CoV-2-specific effects are distinguished from general activation responses.

Chapter 2:

Materials and Methods

2. Materials and Methods:

2.1 Ethics

Written informed consent was obtained from each healthy individual volunteer and the study received ethical approval from the local Research and Ethics Committee in the Trinity Biomedical Sciences Institute, in accordance with the guidelines of the 1975 Declaration of Helsinki. Ethics #: BI-JH-100924

2.2 Cell culture

Human embryonic kidney 293T (HEK293T), Vero, and ACE2 Stably expressing HEK293 (hACE2) cells were cultured in T75 flasks with Dulbecco's Modified Eagle Medium (DMEM) (Sigma Aldrich) supplemented with 10% Fetal bovine serum (FBS) and 1% penicillin and at 37°C in a humidified atmosphere containing 5% CO₂. Cells were passaged every 2-3 days to maintain a high level of confluency (70-90%). When the cells were ~80% confluent, the DMEM was removed and the cells were washed with 5 mL of Dulbecco's Phosphate buffered saline (PBS) (Sigma Aldrich). In order to remove the cells from the flask, 1ml of trypsin: Ethylenediaminetetraacetic acid (EDTA) (Sigma Aldrich) was added to the HEK293T cells while 2ml of trypsin. The trypsinised cells then recovered by adding DMEM up to a volume of 10ml which was transferred into a 15ml falcon tube and centrifuged for 5 minutes at 1200rpm. The DMEM was poured off carefully and the cell pellet was re-suspended in 1ml of DMEM. 9ml of DMEM was added and the cells were re-suspended by pipetting. 10µl of the cell suspension was removed and placed on a haemocytometer. Cells were counted in the 16-square area of the haemocytometer. This value was multiplied by 10,000 to give the cell count per ml. The volume of cells needed to obtain a concentration of 2x10⁵ was then calculated and the appropriate volume of cells was then added to a new T75 flask with fresh DMEM to a total volume of 15ml. Alternatively, cells were seeded at the same concentration in 6 well plates. Human ACE2 & TMPRSS2 expressing A549 Cells were cultured in RPMI-1640 (Sigma-Aldrich; Merck Millipore) supplemented with 10% FBS (Sigma Aldrich), 100 U/ml penicillin, and 100 µg/ml streptomycin, and incubated at 37°C under 5% CO₂.

2.3 PMN isolation

Polymorphonuclear neutrophils (PMNs) were isolated from heparinized peripheral blood, collected by venepuncture from healthy volunteers. Ethics was granted from the Trinity Biomedical Sciences Institute (TBSI) ethics board – Ethics #: BI-JH-100924. The isolation of PMN was done through the removal of red blood cells using dextran (Sigma) promoted

rosette formation, followed by Ficoll-Hy-paque density gradient (GE healthcare). This involved mixing freshly isolated heparinised blood with 3% dextran in a 2:1 ratio in a 50 ml falcon tube and leaving for 30 minutes to allow red blood cells to sediment out. The top 'straw' coloured layer was carefully added onto 10 mL of lymphoprep. Cells were spun down at 1200 rpm for 20 minutes at room temp (20°C) with the brake off, resulting in a cell pellet containing the granulocytes and remaining red blood cells. Red blood cells were removed by hypotonic lysis using Ammonium-Chloride-Potassium lysis (ACK) lysing buffer (Gibco).

2.4 Cell counting

Cells were counted using a haemocytometer, under a light microscope to ensure the cells were seeded at the same density per experiment.

2.5 Propagation of SARS-CoV-2

Vero cells were infected with full-length SARS-CoV-2, including the Alpha, Delta, Omicron, and IC-19 variants. Vero cells were cultured as previously described above before being seeded into a T175 flask at 1×10^7 cells per flask in 25ml complete DMEM and left to incubate for 24h to reach a confluency of 70-80%. To infect the Vero cells with SARS-CoV-2 variants, all complete DMEM was aspirated from the flasks and the cells washed with 10ml warmed PBS. The viral stock was diluted to 0.1pfu/cell in 3 mL serum-free (SF) media, assuming that the number of cells had doubled overnight and dispersed over the cellular monolayer at 37°C for 2 hours. After 2 hours, 22 mL of complete DMEM was added and allowed to incubate for a further 24 hours. The following day the serum concentration was reduced to 2% and allowed to incubate until about 80% cell death. In order to harvest virus cells were scraped from the flask and sonicated for 2 minutes in an ice bath before being aliquoted into cryovials and snap freezing in liquid nitrogen.

2.6 Titration of SARS-CoV-2

To calculate the viral titer, serial dilutions of the viral stock were made and used to infect permissive cells (Vero cells for SARS-CoV-2). Vero cells were used as the permissive cell line for SARS-CoV-2 titration. Cells were seeded into 96-well plates at 2×10^4 cells per well in 200 µL of complete DMEM, cells are incubated for 24h until confluent. Media is removed from the cells and gently washed with warmed PBS. Serial dilutions of viral stocks are added to the wells in triplicate, alongside stocks from the control flask. The plate is then returned to the incubator for 2h to allow the virus to infect the cells. The inoculum is then removed from the wells and replaced with 200µl complete 63 DMEM. The plate is returned

to the incubator for 5 days. The media is discarded and the plate washed with PBS. Cells are fixed by adding 100µl of 2% hydrogen peroxide in methanol to each well, taking care not to disturb the cell monolayer. The monolayer is washed with 1% Bovine Serum Albumin (BSA) in PBS and stained with biotinylated anti-SARS-CoV-2 SP diluted in 1% BSA for 1h at room temperature. The plate is washed twice with 1% BSA, followed by 100ul of a 1 in 500 dilution of Extravidin Peroxidase (Merck, USA) added to each well for 1 hour at room temperature, and focus forming units (FFU) were counted. To calculate the multiplicity of infection (MOI): $MOI = FFU / \text{number of cells infected}$.

2.7 SARS-CoV-2 Infection of PMNs

Human primary PMNs were seeded at a density of 1×10^6 cells/well in a 6-well plate and infected with SARS-CoV-2 Delta at MOI of 0.1, 0.5 and 2 MOI. The plates were centrifuged for collection of supernatants and cells. Supernatant media were used for Enzyme Linked Immunosorbent Assay (ELISA) and while cells were used for flow cytometry analysis.

2.8 Stimulations and treatments

After seeding, the PMNs were incubated at 37°C for 30 mins. Cells were stimulated for 30 minutes with a range of inhibitors before subsequently being stimulated with a range of agonists.

Inhibitor	Concentration
CU-CPT-9a (TLR8 inhibitor)	5nM
BAY11-7082 (NF-κB inhibitor)	5nM
SB 203580 (P38 inhibitor)	20nM
PD 98059 (ERK inhibitor)	20nM
ATN-161 (integrin inhibitor)	1 µM
CLI-095 (TLR4 inhibitor)	1 µg/mL
TL2-C29 (TLR2 inhibitor)	10 µM

Table 2. 1 List of intracellular signalling inhibitors

Agonist	Concentration
CL075 (TLR8 agonist)	1µg/mL
LPS (TLR4 agonist)	100ng/mL
TGF-β1	10-11 ng/ml
IL-10	100ng/mL

SARS-CoV-2 SP	10-100 ug/mL
SARS-CoV-2 ssRNA	1-10 ug/mL
tert-Butyl hydroperoxide tBuOOH	10 nM
Phorbol Myristate Acetate (PMA)	20 nM

Table 2. 2 List of cell culture agonists and viruses

2.9 RNA extraction using Norgen Biotek corp

RNA was isolated as per the manufactures protocol. Cell suspension was transferred to an RNase free tube and centrifuged for 200xg for 10 minutes to pellet the cells. 350µL of buffer RL (guanidinium salts) and 200µl of 100% ethanol was added, vortexed and added to the RNA column. The column was spun at 3500xg to allow the RNA to bind. The RNA was subsequently washed 3 times with a wash buffer before being eluted in an RNase free tube.

2.10 Reverse transcription (cDNA synthesis)

250ng of total RNA from each sample was reverse transcribed into cDNA using the SensiFAST reverse transcription cDNA kit (Bioline, MSC, Dublin), using the following reaction: 0.25µl of reverse transcriptase and 2.25µl of 5X Transamp buffer was added to 250ng RNA and made up to 17.5µl in RNase free DNase free H2O (Sigma). Following this, the reaction was placed on the thermocycler at 25°C for 10 minutes (annealing), 42°C for 15 minutes (reverse transcription), 85°C for 5 minutes (inactivation of the enzyme) and 4°C hold. cDNA was quantified using the NanoDrop ND-1000 spectrophotometer.

2.11 Quantitative Real-time-Polymerase Chain Reaction (qRT-PCR)

Each qRT-PCR reaction was carried out in duplicate in a 96-well PCR plate. Total volume per well was 10 µl: 1µl of a 1 in 10 dilution of cDNA, 4µl of SYBR green PCR master mix (Biorad), 0.5 µl of forward and reverse primer and 4 µl of H2O. qRT-PCR was performed using the Biorad quantitative PCR system using the following cycling parameters: 95°C for 15 minutes, 92°C for 30 seconds, 65°C for 1min, 72°C for 30secs. The cycle was repeated 40 times. All gene amplifications were normalised to Beta-actin (β-Actin) gene.

Steps	Temperature	Time	Cycle n2mber
Initial Denaturation	95°C	15 minutes	One cycle
Denaturation	95°C	30 seconds	30 cycles
Annealing	65°C	60 seconds	
Elongation	72°C	30 seconds	

Final Elongation	72°C	30 seconds	One cycle

Table 2. 3 PCR cycle

2.12 qRT-PCR data analysis

Data analysis was carried out using the 2- $\Delta\Delta C_t$ method. A ratio of gene of interest relative to housekeeping gene (β -Actin) was generated. β -Actin was selected as it was found to be the most stably expressed gene, compared to other normalisers (including glyceraldehyde 3-phosphate 64 dehydrogenase [GAPDH] in peripheral blood cells by previous lab members.

Gene Name	Forward primer sequence (5' – 3')	Reverse primer sequence (3' - 5')	Primer supplier
PKR	GAAGTGGACCTCTACGCTTTGG	TGATGCCATCCCGTAGGTCTGT	Sigma
MXA	GGTGGTGGTCCCCAGTAA TG	ACCACGTCCACAACCTT GT	Sigma
OAS	GAAGCCCTACGAAGAATGTCAGA	TCGGAGTTGCCTCTTAAGACTGT	Sigma
Viperin	CGTGGAAGAGGACATGACGGAAC	CCGCTCTACCAATCCAGCTTC	Sigma
ISG15	TCCTGCTGGTGGTGGACA	TTGTTATTCCTCACCAGG	Sigma
IFN α 2	TGGGCTGTGATCTGCCTCAAA C	CAGCCTTTTGGAAGTGGTTGCC	Sigma
IFN- β	CTTGGAATCCTACAAAGAAGCAGC	TCCTCCTTCTGGAAGTGGTGC A	Sigma
IFN- λ	CGCCTTGGAAGAGTCACTCA	GAAGCCTCAGGTCCCAATTC	Sigma

ACE2	TAACCACGAAGCCGAAGACC	TTTGGGCAAGTGTGGACTGT	Sigma
TGF- β	TACCTGAACCCGTGTTGCTCT C	GTTGCTGAGGTATCGCCAGG AA	Sigma

Table 2. 4 Primer sequences

2.13 Protein extraction

Cells were stimulated as described above. Then, the media was removed and the cells were washed with 1ml of PBS. Another 1ml of PBS was then added and cells were scraped and removed into 1.5ml tubes which were centrifuged at 5,000rpm for 5 minutes. The PBS was then removed and cells were re-suspended in 100 μ l of lysis buffer consisting of 1 $\times$ RIPA buffer (20mM Tris-HCl pH7.4, 150mM NaCl, 1mM EDTA pH 8, 1% TRITON-X, and 0.5% SDS), containing the following proteases and phosphatase inhibitors; Leupeptin (5 μ g/ml), Na₃VO₄ (1mM), PMSF (1mM), and DTT (1mM). The cells were lysed on ice for 30 minutes, and re-suspended every 10 mins to ensure complete lysis. The lysates were then centrifuged at 12,000 rpm at 4°C for 10 minutes and the lysate was transferred to new tubes while the pellets were discarded. Lysates were stored at -20°C until required for immunoblot analysis.

2.14 Western Immunoblotting

2.14.1 SDS-PAGE

Discontinuous Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the BioRad Gel Electrophoresis system. Polyacrylamide gels were made by preparing a 12.5% resolving gel (Table 2.5) and a 5% stacking gel (Table 2.6), using 1mm plates and combs. 5X loading buffer (Table 2.9) was added to each sample to dilute the loading buffer to 1X. Samples were then boiled at 100°C for 10 minutes to denature the proteins, and briefly centrifuged to capture any evaporation. Upon setting of the gels, the BioRad apparatus was then set up and 1 $\times$ SDS running buffer (250mM Tris, 1.9M glycine, 1% SDS) was added to the trough. Running buffer was added until it reached the same level as the guideline level etched on the trough's side. 1 μ l of the protein ladder was added to the first well of each gel. 10 μ l of the protein sample was then loaded. The gels were run at 80V until the samples had migrated through the stacking gel at which stage the voltage was increased to 110V and the gels were run until the sample had reached the end of the gel.

Resolving gel

Gel Percentage	12.5%	15%
Acrylamide	6.25 ml	7.5 ml
dH2O	3.55 ml	4.2ml
1.5M Tris-HCL pH 8.8	5.6ml	5.6ml
10%SDS	150µl	150µl
10% APS	150µl	150µl
TEMED	15µl	15µl

Table 2. 5 Composition of Resolving Gel

Stacking gel

Acrylamide	1ml
dH2O	4.61
1M Tris-HCL PH 6.6	750µl
10% SDS	60µl
10% APS	60µl
TEMED	12µl

Table 2. 6 Composition of Stacking Gel

Running Buffer

250 mM Tris	30g
1.8 M glycine	144g
1% w/v SDS	10g
dH2O	Make up to 1L

Table 2. 7 Composition of running buffer

Sample Buffer

0.5M Tris pH 6.8	10 mL
SDS	3 g
Bromophenol Blue	(pinch)

Glycerol	10 mL
Distilled water	5 mL

Table 2. 8 Composition of 5x Sample Buffer

2.14.2 Transfer of proteins to the PVDF membrane

Protein was transferred to a polyvinylidene fluoride (PVDF) membrane in 1 X transfer buffer (250mM Tris, 1.9M glycine, 20% methanol) at 100v for 90 minutes. After transfer, membranes were blocked using blocking buffer (5% dried skimmed milk in Tris-buffered saline with 0.05% Tween 20 [TBST]) for 1 hour at room temperature. Primary antibody was diluted appropriately in blocking buffer and membranes were incubated with gentle agitation at 4°C overnight. Antibodies used for Western blotting are listed in Table 3. After incubation, membranes were washed 3 times for 10 minutes each in TBST. Horseradish peroxidase-conjugated secondary antibody was diluted 1:2000 in blocking buffer, and membranes were incubated for 1 hour at room temperature with gentle agitation. Secondary antibody was removed and membranes were washed 3 times for 10 minutes in TBST. Membranes were incubated in enhanced chemiluminescent (ECL) substrate before being visualised using the Biorad ChemiDoc system.

10X Transfer Buffer

1.8M glycine	30g
250mM Tris	144g
dH2O	Make up to 1L

Table 2. 9 Composition of 10X transfer buffer

Antibody	Concentration	Source
ACE2	1:1000	Abcam
IκBα	1:1000	University of Edinburgh gift
p-STAT1	1:1000	Cell Signalling
SARS-CoV-2 NP	1:1000	Cell Signalling
β-Actin	1:2000	Sigma-Aldrich

Table 2. 10 Antibodies used in Western immunoblotting.

Table showing antibodies used in Western blot analysis of proteins. All antibodies were diluted in 5% skimmed milk in TBST.

2.15 Flow cytometry staining

Extracellular antibodies were added at a 1:100 dilution and incubated at 4°C for 20 minutes. The cells were spun at 300g for 5 minutes at room temperature and washed once with PBS. 100ul of 1X BD Cytofix/Cytoperm was added to each well, resuspended thoroughly to ensure no clumping and incubated at room temperature in the dark for 20 minutes. 100ul of BD 1x Permwash (1 part Permwash, 9 parts deionized water) was added. Cells were spun at 300g for 5 minutes. Cells were washed once more in 1X Permwash prior to intracellular staining. Cells were resuspended in 50µl of 1X Permwash that contained the intracellular antibody Mastermix (1:50 dilution) and placed in the dark for 20 minutes. Cells were washed once in 1X Permwash and then resuspended in PBS before acquisition.

Primary antibody	Company	Cat:	Fluorochrome
Live/Dead	Biolegend	558537	BV510
CD11b	Biolegend	612597	PE/Cyanine7
CD66b+	Biolegend	305118	Allophycocyanin (APC)
CD16	Biolegend	30203 7	BV421
TLR8	Biolegend	39550 3	PE
CD62L	Biolegend	304806	PE
CD64	Biolegend	305026	APC/Cyanine7
CD35	Biolegend	BL- 332406	Fluorescein isothiocyanate (FITC)
CD63	Biolegend	353024	Alexa Fluor® 700
TLR8	Biolegend	395503	PE

Table 2. 11 Fluorescently conjugated antibodies and dyes

2.16 Identified PMNs population by flow cytometry:

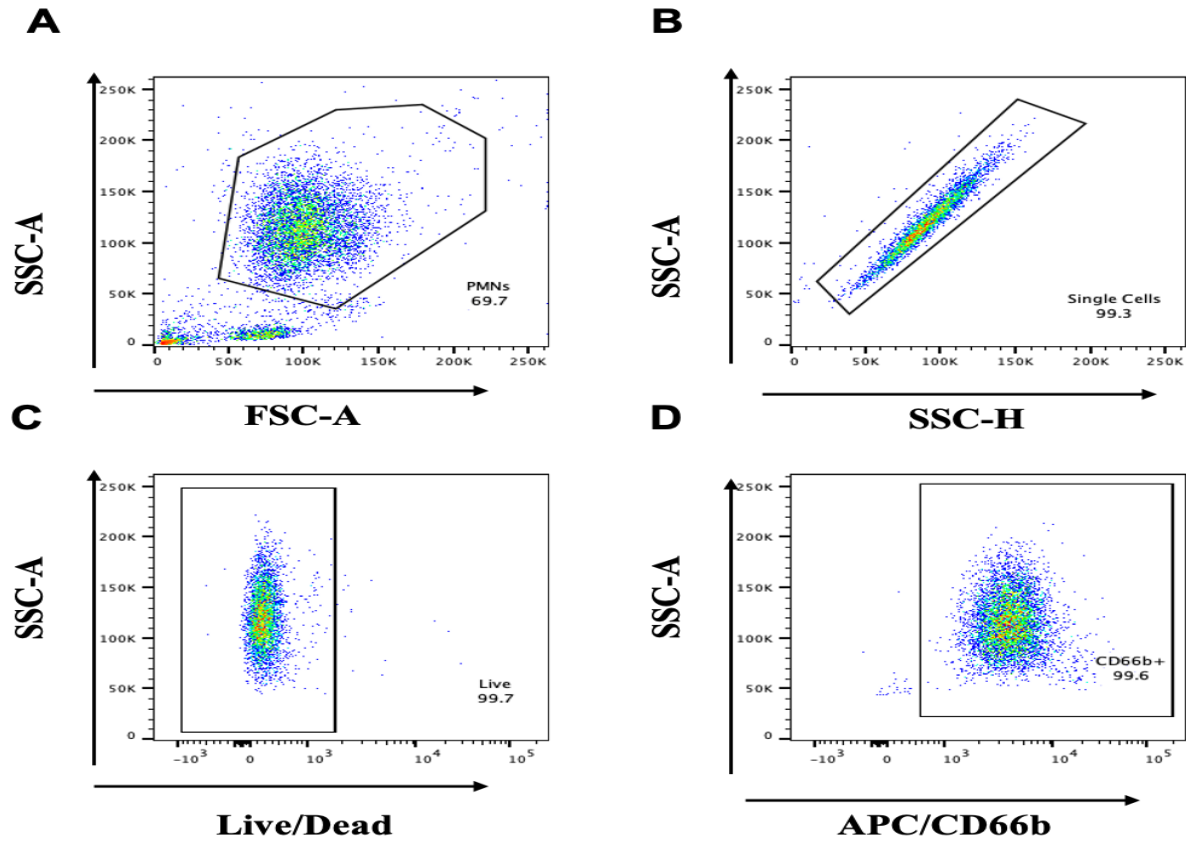


Figure 2.1 Gating strategy for identification of PMNs

Flow cytometric analysis showing sequential gating for PMN identification. (A) Initial cell population gated based on forward scatter (FSC-A) and side scatter (SSC-A) to identify PMNs based on size and granularity characteristics (69.7% of total events). (B) Singlet discrimination using SSC-A versus SSC-H to exclude doublets and multiplets (99.3% single cells). (C) Viability assessment by live/dead dye exclusion to identify viable cells (99.7% live cells). (D) Final PMN identification using APC-conjugated anti-CD66b antibody, a neutrophil-specific surface marker (99.6% CD66b-positive cells). Numbers indicate the percentage of cells within each gate relative to the parent population.

2.17 Measurement of the ROS levels

For the ROS measurement, the CellROX™ (ThermoFisher scientific) Deep Red fluorogenic probe was used. ROS production was measured using Flow cytometric analysis as previously described. Cells were incubated with 4 μ M CellROX for 30 min at 37°C in the dark. The ROS levels were measured using a BD FACSCanto II flow cytometer; the

fluorescence was captured at 644/665 nm of excitation/emission light and the values reported as percentage of positive cells.

2.18 ELISA Proinflammatory Cytokines

To measure the IL-6, IL-1, IL-8 and TNF- α protein production of neutrophils primary human PMNs sandwich ELISA kits were used. 96-well polystyrene microplates were coated with 100 μ l/well of specific antibody, diluted 1:200 (according to biolegend) in 1x coating buffer, and incubated at 4°C overnight. The following morning the plates were washed with 1% (v/v) PBS-Tween 20 by immersing the plate into wash buffer and then completely removing the wash buffer by inverting and tapping the plate on tissue paper. This was done 3-4 times. The plate was then blocked for 2 hours with 200 μ l of 1x reagent diluent (FBS in PBS) in order to inhibit non-specific binding. The plates were washed as described above and in accordance with the manufacturer's protocol the Standards were added in triplicate. Samples were diluted 1/4 for IL-6 and IL-8 and 1/2 for TNF- α and IL-1 β . Plates were allowed to incubate at 4°C overnight. The following day the plate was aspirated and washed 4 times. 100 μ l of secondary biotin labelled antibody in accordance with the manufacturer's recommended concentration were added to the plate and allowed to incubate at room temperature for 2 hours. The plate was washed 4 times before 100 μ l of HRP-Streptavidin conjugate (SABC) working solution was added to each well for 30 minutes at 37°C. The plate was washed 5 times. 100 μ l of the 3,3',5,5'-Tetramethylbenzidine (TMB) substrate was added and incubated for 15-30 minutes at 37°C for colour development and ELISA assays were stopped with 50 μ l/well of "stop solution" (Phosphoric acid). Colour development was read at 450 on a microplate reader

2.19 ELISA for TGF- β 1

To measure the TGF- β 1 protein production of neutrophils primary human PMNs sandwich ELISA kits were used. 96-well polystyrene microplates were coated with 100 μ l/well of specific antibody, diluted 1:180 (according to R&D Systems, DuoSet ELISA) in PBS overnight at 4°C. The next day, wells are washed with wash buffer (0.05% Tween-20 in PBS) and blocked with 1% BSA in PBS for at least 1 hour at room temperature. Meanwhile, samples and standards undergo acid activation using HCl, followed by neutralization with NaOH to activate latent TGF- β 1. After blocking, the plate is washed and activated samples, standards, and controls are added in duplicate, incubated for 2 hours at room temperature. Following incubation, wells are washed, and a biotinylated detection antibody specific for TGF- β 1 is added (1:180), incubating for another 2 hours. After washing, streptavidin-HRP

conjugate is added for 20–30 minutes in the dark. Wells are then washed thoroughly before adding the TMB substrate, allowing colour development for 20 minutes, stopped with (Phosphoric acid), and read at 450 nm with wavelength correction at 540 nm. Sample concentrations are calculated based on a standard curve generated from recombinant TGF- β 1.

2.20 ELISA for MPO and NE

To measure the MPO and NE production of neutrophils primary human PMNs sandwich ELISA kits were used. 96-well polystyrene microplates were coated with 100 μ L/well of specific antibody, diluted 1:180 (according to R&D Systems, DuoSet ELISA) in PBS overnight at 4°C. The next day, wells are washed with wash buffer (0.05% Tween-20 in PBS) and blocked with 1% BSA in PBS for at least 1 hour at room temperature. Meanwhile, MPO standards and samples were prepared according to the kit instructions. After blocking, the plate was washed, and 50 μ L/well of standards, controls, and diluted samples were added in duplicate, incubating for 2 hours at room temperature on a horizontal orbital shaker. Following incubation, wells are washed, and a biotinylated detection antibody specific for TGF- β 1 is added (1:180), incubating for another 2 hours. After washing, streptavidin-HRP conjugate is added for 20–30 minutes in the dark. Wells are then washed thoroughly before adding the TMB substrate, allowing colour development for 20 minutes, stopped with (Phosphoric acid), and read at 450 nm with wavelength correction at 540 nm. Sample concentrations are calculated based on a standard curve generated from recombinant TGF- β 1.

2.21 NETosis Assay

Freshly isolated PMNs were first washed with 1 $\times$ PBS to remove residual media and unwanted debris. Cells were then stained with a live/dead viability dye (1:1000 dilution in PBS) and incubated at 4°C for 20 minutes in the dark. After incubation, neutrophils were washed again with PBS and then labelled with CD66b (1:100 dilution in PBS, APC channel), a granulocyte marker, along with SYTOX Orange (1 μ M final concentration, PE channel), a membrane-impermeable DNA dye that stains extracellular DNA indicative of NETosis. The cells were incubated for 10–15 minutes in the dark at 4°C. After incubation, cells were washed with FACS buffer (1% BSA in PBS) to remove unbound antibodies and dyes. Cells were then fixed by adding 100 μ L of 1 $\times$ BD Cytofix/Cytoperm to each well, resuspended thoroughly to prevent clumping, and incubated at room temperature in the dark for 20 minutes. After fixation, cells were resuspended in FACS buffer and immediately analysed

using flow cytometry. SYTOX Orange fluorescence was measured in the PE channel (excitation: 547 nm, emission: 570 nm), while CD66b staining was detected in the APC channel (excitation: 650 nm, emission: 660 nm). The percentage of SYTOX Orange-positive CD66b⁺ neutrophils was used to quantify NET formation.

2.22 Phagocytosis Assay

Phagocytosis assay was performed using GFP-expressing *Staphylococcus aureus* (SA-GFP). Primary human PMNs were isolated from healthy donor blood using density gradient centrifugation and resuspended in RPMI 1640 medium supplemented with 10% FBS. PMNs were seeded at a concentration of 5×10^6 cells per well in 96-well plates and incubated at 37°C in a humidified 5% CO₂ incubator. SA-GFP was cultured overnight in LB broth, washed, and resuspended in PBS before being added to PMNs at MOI of 1:100. The co-culture was incubated for 1 hour at 37°C to allow phagocytosis, after which extracellular bacteria were removed by washing three times with PBS. PMNs were then fixed with 4% paraformaldehyde (PFA) for 15 minutes at room temperature, and phagocytosis was quantified as the mean fluorescence intensity (MFI) of GFP-positive PMNs using flow cytometry (FACS analysis). Data were analysed using FlowJo software, and the Mean fluorescence intensity (MFI) of GFP-positive PMNs was used to quantify bacterial uptake.

2.23 Apoptosis Assay Using Annexin V-FITC/PI Staining

To assess apoptosis in PMNs, an Annexin V-FITC/PI staining assay was performed following the manufacturer's protocol (Miltenyi Biotec). First, cell numbers were determined, and 1×10^6 cells were washed in 1 mL of 1× Binding Buffer and centrifuged at 300×g for 10 minutes. The supernatant was completely aspirated, and the cell pellet was resuspended in 100 µL of 1× Binding Buffer per 10^6 cells. Annexin V-FITC (10 µL per 10^6 cells) was added, mixed thoroughly, and incubated for 15 minutes in the dark at room temperature. Following incubation, cells were washed by adding 1 mL of 1× Binding Buffer per 10^6 cells, centrifuged at 300×g for 10 minutes, and the supernatant was aspirated. The washing step was optionally repeated for increased purity. The final cell pellet was resuspended in 500 µL of 1× Binding Buffer per 10^6 cells, and 5 µL of propidium iodide (PI) solution was added immediately before analysis. Flow cytometry was then performed to assess apoptotic (Annexin V-positive), necrotic (PI-positive), and viable (Annexin V/PI-negative) cell populations.

2.24 Identification of potential TLR7/8-triggering ssRNA PAMPs

Salvi, Valentina, et al. identified ssRNA fragment from the SARS-CoV-2 genome as direct activators of endosomal TLR7/8 and MyD88 pathway. They scanned the reference SARS-CoV-2 genome (NC_045512, positive strand) for GU-rich ssRNA fragments with the SequenceSearcher tool in the Fuzzy mode. In their work, they defined “GU-enriched sequences” as short strings with a maximal length of 20 bp that were composed of GU and/or UG pairs for more than 40% of their length. They identified 491 GU-rich sequences in the SARS-CoV-2 genome. From these 491 GU-rich sequences we selected this sequence 5'-GUGUGUGUGUUCUGUUAUU*G-3' (genome position: 20456-20475; *indicates a phosphorothioate linkage). We checked this sequence for uniqueness with BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) selecting RNA viruses (taxid: 2559587) as organism in the “search set” window”. additional sequence was synthesized in which U was substituted with A in order to impair TLR7/8 stimulation. These sequences were synthesized by Integrated DNA Technologies (IDT).

2.25 Statistics

All graphing was carried out using Graphpad Prism 5. A student's T-test was used for comparisons in Microsoft Excel. A p value of < 0.05 was considered as significant.

Chapter 3:

The Effect of SARS-CoV-2 SP on Activation and Inflammation in Primary Human PMNs

3.1 Introduction

The emergence of SARS-CoV-2 has raised numerous questions about how viral components interact and evade the human immune system [405]. The outbreak of SARS-CoV-2 is one of the worst global catastrophes of all time, resulting in the loss of millions of lives globally [406]. The unique pathogenic characteristics of the virus have stimulated an intense wave of studies exploring the molecular and cellular basis of its infectivity and the evolution of the disease it produces [407]. At the core of SARS-CoV-2 pathogenesis is the SP, a surface glycoprotein that is crucial for the virus's entry into host cells through binding to the ACE2 receptor. S1 and S2 subunits form the SP that enables the virus to attach to host cells and subsequently fuse its membrane with the host cell membrane [408]. Recent evidence shows that SARS-CoV-2 SP plays a vital role in controlling how the host's immune system response to the virus. For example, some studies have found that SARS-CoV-2 SP is capable of stimulating immune cells, such as macrophages, which can then result in the secretion of cytokines [409]. Additionally, these effects include, but are not limited to, the stimulation of cytokine storms and the disruption of immune cells, which can lead to overwhelming inflammation in severe cases of COVID-19 [408]. Neutrophils are an essential component of the innate immune system. As the most prevalent category of peripheral blood leukocytes, neutrophils serve as the primary barrier against invading microorganisms. They are heavily involved in ARDS, and their presence in the lungs is often tied to how severe the disease gets, whether viral infections, trauma, or autoimmune conditions cause it [410]. High neutrophil levels and an increased neutrophil-to-lymphocyte ratio are key indicators of severe COVID-19. The more severe the disease, the higher the neutrophil levels, with the highest seen in the most critical cases [411]. These cells are indeed highly efficient in fighting infections through several different mechanisms, including, but not limited to, phagocytosis, the secretion of antimicrobial peptides through degranulation, and the generation of NETs [412]. NETs are web-like structures formed by DNA and granular proteins, playing a role in trapping and neutralising pathogens. However, their excessive or uncontrolled activation has consequences on neutrophils as well as the surrounding healthy tissues, resulting in inflammatory responses [413]. This paradoxical role of neutrophils highlights their significance during both the protective and pathological events.

This chapter seeks to explain the molecular mechanisms through which SARS-CoV-2 SP modulates neutrophil cell functions, with special emphasis on their protective and pathological balance. These interrelationships may explain the immune dysregulation

characteristics of severe cases of COVID-19 and assist in the subsequent development of therapies.

3.1.1 Viral Proteins and Neutrophils

Already established in literature is the fact that interaction between viral proteins and the host immune system is of utmost importance in viral pathogenesis. Numerous studies on various infections have indicated that these proteins are involved in altering the activity of neutrophils. For example, the viral hemagglutinin and neuraminidase proteins, at the time of influenza virus infection, are capable of provoking neutrophil activation and elicit the release of NETs [414]. Additionally, in severe influenza, the excessive formation of NETs may lead to enhanced lung inflammation and tissue injury [415]. Likewise, exposure to RSV has been shown to provoke the influx and activation of neutrophils, at least partly through the action of the RSV F protein, which elicits the secretion of chemokines and the migration of neutrophils. These effects emphasise the paradox of neutrophils, as both antiviral and immunopathological agents [416]. Moreover, it has been suggested that the glycoproteins of HSV can influence the recruitment of neutrophils and inhibit their apoptosis, allowing the virus to infect and survive in the host's tissues [417]. CMV takes a subtle approach by suppressing neutrophil activity and lowering the inflammatory response, which could otherwise increase the risk of the virus spreading. CMV uses various methods to manipulate the immune system, including its impact on neutrophils. It extends the lifespan of neutrophils by blocking their natural cell death through specific survival pathways. These survival pathways operate independently of viral gene expression and are mediated by the ERK/MAPK and NF- κ B pathways of activation, with further enhancement by CMV-induced cytokine secretion. Since prolonged neutrophil survival can induce tissue damage, this mechanism may also be relevant to HCMV immune pathology. Furthermore, CMV-exposed neutrophils release factors that promote monocyte recruitment and maturation into a virus-permissive phenotype through the mediation of IL-6, thereby facilitating the propagation of the virus [418]. This interplay between viral proteins and neutrophils is not limited to the aforementioned viruses. Other pathogens, such as HIV and DENV, also leverage similar mechanisms to influence neutrophil behaviour and drive disease progression. The HIV-1 transactivator protein, Tat, can activate neutrophils, leading to their migration and release of pro-inflammatory cytokines [419]. The non-structural protein NS1 of DENV can directly interact with neutrophils, inducing the release of NETs and inflammatory cytokines. This response contributes to vasculopathy and the severe clinical

picture which encompasses dengue hemorrhagic fever [313]. Collectively, these examples illustrate how diverse viral proteins modulate neutrophil responses, either aiding viral dissemination or intensifying immunopathology. This pattern becomes particularly evident in the context of SARS-CoV-2, where the SP serves as a key trigger for innate immune recognition and response.

3.1.2 Immune Recognition and Responses to the SARS-CoV-2 SP

As a foreign antigen, SARS-CoV-2 is recognized by the immune system, initiating a cascade of innate and adaptive immune responses aimed at neutralizing the virus and preventing infection.

3.1.3 Innate Immune Recognition

The body's first line of defence, the innate immune system, uses PRRs, such as TLRs, RLRs, and NLRs, to sense the conserved elements of viral structures, including the SP. The involvement of TLR4 and TLR2 in recognising SP and subsequently activating specific signalling pathways is a case in point (**Fig. 3.1**). This cascade leads to the activation of signals, resulting in the secretion of IFNs and pro-inflammatory cytokines, including IL-6 and TNF- α . One study showed that the SARS-CoV-2 SP stimulates an inflammatory reaction in macrophages and lung epithelial cells, producing IL-6, IL-1 β , TNF- α and chemokines through the activation of NF- κ B. This response is TLR2-dependent, since it is not observed in TLR2-deficient cells and mice. The other viral proteins (M, E, N) do not trigger inflammation. Another study demonstrated that the SARS-CoV-2 SP directly binds to and activates TLR4, triggering the production of IL-1 β and IL-6 in THP-1 monocytes, HL-60-derived neutrophils, Raw 264.7 macrophages, and primary macrophages. Using surface plasmon resonance (SPR), researchers confirmed that SP trimer binds TLR4 (~300 nM affinity). Inhibition studies with Resatorvid (a TLR4 inhibitor) and JSH-23 (an NF- κ B inhibitor) demonstrated that SP-induced inflammation occurs via the TLR4/MyD88/NF- κ B pathway. This activation is independent of ACE2 and TMPRSS2. Other coronaviruses, including SARS-CoV and HCoV-229E, also trigger TLR4-mediated responses, suggesting a conserved mechanism. However, although such responses are necessary for bringing immune cells to the area of infection, an extreme release of cytokines can lead to a condition referred to as a cytokine storm, which is counterproductive as it leads to further tissue injury and contributes to severe disease symptoms [409, 420].

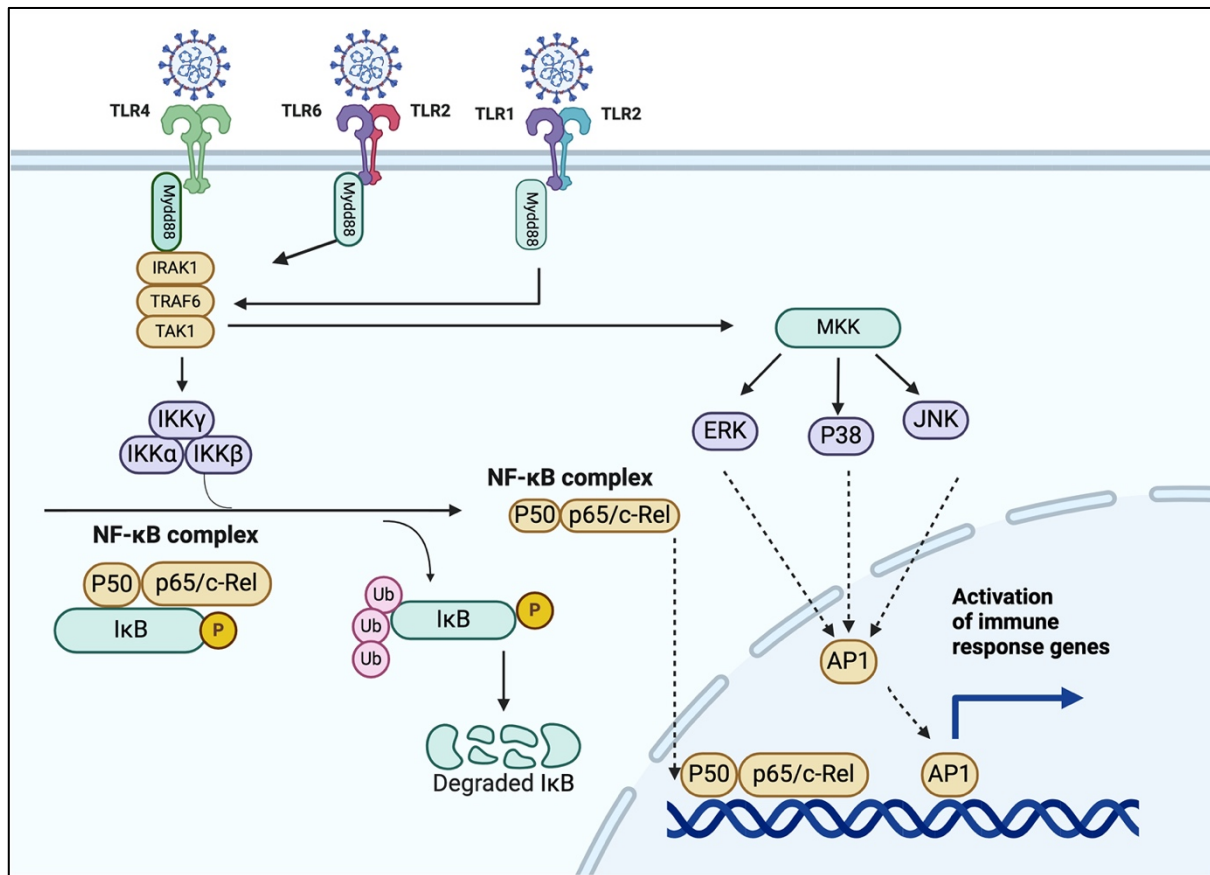


Figure 3.1 TLR-Mediated NF-κB and MAPK Signalling Pathways in Response to SARS-CoV-2 SP.

This diagram shows how the SARS-CoV-2 SP triggers immune responses through TLR signalling pathways. The SARS-CoV-2 SP binds to TLR dimers, such as TLR4 or TLR2/1 and TLR2/6, which activate a MyD88-dependent signalling cascade. In the NF-κB pathway, MyD88 recruits IRAK1, TRAF6, and TAK1, leading to activation of the IKK complex. This results in the degradation of IκB, freeing NF-κB (p50, p65/c-Rel) to enter the nucleus and activate inflammatory genes. Simultaneously, TAK1 activates MAPKs (ERK, P38, JNK) through MKK, leading to the activation of AP-1, another transcription factor involved in immune gene expression. Together, NF-κB and AP-1 drive the production of pro-inflammatory cytokines, such as IL-6 and TNF-α, and chemokines. Although these pathways are critical for antiviral defence, overactivation by the SP can cause excessive inflammation, cytokine storms, and severe COVID-19 symptoms.

3.1.4 Adaptive Immune Responses

Considered crucial when it comes to providing long-term protective immunity against infections involving the SP, the adaptive immune system has B cells that respond to SP epitopes, such as those located in the RBD, by generating neutralising antibodies. These antibodies neutralise the ability of the SP to bind to ACE2 receptors, therefore, blocking the entry of the virus into the host cells. Furthermore, memory B cells and long-lived plasma cells are responsible for the continuity of the antibody response and the protection against reinfections [420]. T cells also have a significant role in the adaptive response. CD4⁺ Th cells do this by providing adequate signals to B cells, thus facilitating antibody production, while Th cells kill. CD8 CTLs destroy SP peptide-presenting cells through MHC-restricted recognition. This combination of responses is crucial for eradicating the infection and reducing the viral load [421].

3.1.5 Immune Evasion and Challenges

The outcome of an infection depends on the virus's ability to manipulate the infected cell to evade the immune system, as well as the immune response's capacity to counteract this evasion. The SARS-CoV-2 SP is central to the virus's ability to evade immune defences and establish infection. One of its strategies involves masking the RBD, making it less visible to neutralising antibodies while preserving its ability to bind the ACE2 receptor. Mutations such as N439K, E484K, and N501Y alter the surface of the SP, strengthening its interaction with ACE2 and diminishing the effectiveness of antibody neutralisation. These mutations enable the virus to evade immune detection and increase its ability to spread [422]. Additionally, the SP not only directly facilitates viral entry but also indirectly contributes to suppressing IFN responses. The SP disrupts IFN-I pathways, which are critical for initiating antiviral defences. The SARS-CoV-2 SP inhibits IFN-I by disrupting RIG-I signalling, thus reducing IFN β activation and ISGs expression. This results in a higher viral load in infected cells. Mechanistically, SP interacts with IRF3, downregulates its expression, and promotes its degradation through the proteasome. However, SP does not affect NF- κ B and STAT1 levels [423]. However, SARS-CoV-2 SP binds to STAT1, STAT2, and IRF9, blocking STAT1/STAT2 phosphorylation and ISGF3 complex formation, thereby preventing ISG expression. This inhibitory mechanism, primarily driven by the conserved S2 domain, is shared across multiple human coronaviruses, including SARS-CoV, MERS-CoV, and others

[424]. The SP also interferes with adaptive immunity by impairing the function of CTLs. Specifically, it disrupts the formation of immune synapses, which are essential for CTLs to identify and kill infected cells. This interference occurs through interactions between the SP and ACE2 on CD8⁺ T cells [425]. The SP mutations allow SARS-CoV-2 to evade neutralising antibodies. Variants like Beta and Delta have mutations in RBD and NTD that reduce antibody binding and neutralisation. Some mutations, such as E484K and L452R, significantly decrease the effectiveness of monoclonal antibodies and convalescent plasma [426]. Additionally, the SP downregulates activating ligands on infected cells, thereby impairing NK cell activation and facilitating viral immune escape [427]. Together, these mechanisms, including antibody evasion, suppression of IFN responses, and CTL disruption, highlight the virus's sophisticated immune evasion tactics and underscore the need for broadly protective vaccines and therapies that can target these challenges [428].

3.1.6 SARS-CoV-2 and TGF- β Signalling

SARS-CoV-2 SP disrupts vascular integrity by activating TGF- β signalling through its RGD integrin-binding motif, independent of ACE2. *In vitro* studies showed that SP increased TGF- β secretion and upregulated TGF- β -related genes (TGFB1, LTBP2, ITGB5). Blocking TGF- β receptor 1 (TGFB1) with inhibitors (SB431542) or gene knockout prevented endothelial barrier dysfunction. *In vivo*, SP induced vascular leakage in mice, similar to that observed in SARS-CoV-2 infection. The mouse-adapted SARS-CoV-2 model confirmed that viral-induced leak was reduced by blocking TGF- β activation via integrins. Treatment with ATN-161, an integrin inhibitor, significantly reduced SP-mediated vascular leak, confirming the role of the RGD integrin-binding motif in triggering TGF- β activation. These findings highlight TGF- β and integrins as key therapeutic targets for COVID-19-associated vascular damage [429].

3.1.7 TGF- β Regulation of Neutrophil Function

TGF- β is involved in the regulation of neutrophil functions in both normal and pathological conditions. Table 3.1 highlights the multifaceted role of TGF- β in modulating neutrophil function. TGF- β 1 has been shown to inhibit neutrophil degranulation without affecting chemotaxis, thereby limiting excessive inflammation [430]. In the tumour microenvironment, TGF- β drives neutrophils toward a pro-tumour N2 phenotype, while its blockade can promote anti-tumour activity [431]. More recently, TGF- β 1 was found to activate SMAD3 and ERK1/2 pathways and upregulate pro-tumour genes in neutrophils without inducing their migration, suggesting a regulatory role in cancer progression [432].

Table 3. 1 TGF- β and Neutrophil Interaction.

Study	Focus	Key Findings	Functional Impact
Shen et al. (2007) [430].	TGF- β 1 and neutrophil degranulation in innate immunity	TGF- β 1 inhibits neutrophil degranulation by reducing lactoferrin secretion but does not affect chemotaxis.	Prevents excessive inflammation in the female reproductive tract.
Fridlender et al. (2009) [431].	TGF- β and tumour-associated neutrophils (TANs).	TGF- β polarizes TANs into a pro-tumour N2 phenotype; blocking TGF- β promotes an anti-tumour N1 phenotype.	Promotes tumour growth via N2 neutrophils but can be targeted for anti-tumour therapy.
Hein et al. (2023) [433].	TGF- β 1 activation of neutrophil signalling and gene expression	TGF- β 1 activates SMAD3 and ERK1/2 signalling and upregulates genes like OSM and VEGFA but does not induce migration.	Modulates neutrophil function in cancer without directly affecting movement.

3.2 Hypothesis

Since the SARS-CoV-2 SP is known to activate PMNs directly by binding to PRRs, including TLR2 and TLR4, we hypothesize that this interaction activates MyD88-dependent NF- κ B and p38 MAPK signalling pathways, leading to the up-regulation of PMN activation markers (CD11b, CD66b), increased ROS production, NET formation, and degranulation. These events collectively contribute to tissue damage and cytokine storm (IL-6, IL-8, and TNF- α), exacerbating the severity of COVID-19. We also hypothesize that the SARS-CoV-2 SP directly induces TGF- β production in PMNs, which contributes to immune dysregulation, downregulation of ACE2 expression, and promotion of sustained inflammation and fibrosis. This integrated mechanism may underlie neutrophil-driven immunopathology in severe COVID-19 and supports the rationale for targeting these pathways therapeutically.

3.3 Specific Aims

Aim 1: Analyse PMN activation markers in COVID-19 patients with varying disease severity.

Hypothesis: CD11b and CD66b mRNA transcripts of PMNs correlate with the degree of illness in the patients suffering from COVID-19 and indicate that in severe cases there is an increase in the activity of PMNs.

Approach: Nanostring gene expression data derived from whole blood samples of patients with COVID-19 of varying disease severity were analysed. A comparative analysis of the mRNA levels of genes encoding PMN activation markers (CD11b, CD66b) was performed to assess their association with disease progression and severity.

Aim 2: Explore the effects of the SARS-CoV-2 SP on PMN activation markers (CD11b, CD66b, CD16, and CD62L) and functional responses.

Hypothesis: Directly interacting with PMN surface receptors, the SARS-CoV-2 SP interacts with the target cell receptor, influencing the expression of PMNs activation markers (CD11b, CD66b, CD16, and CD62L), thus leading to greater activation and functional modulation.

Approach: Primary human PMNs were isolated and then treated with recombinant SARS-CoV-2 SP. The quantification of changes in the expression of selected activation markers, including CD11b, CD66b, CD16, and CD62L, was performed using flow cytometry. Notably, dose-response studies were conducted to establish concentration-related effects, and the treated samples were compared to the untreated ones to determine their significance.

Aim 3: Examine the role of the SARS-CoV-2 SP in inducing ROS production in PMNs at varying concentrations.

Hypothesis: The SARS-CoV-2 SP causes a dose-dependent rise in ROS within PMNs, explaining the oxidative stress or inflammatory pathology.

Approach: Human PMNs were cultured with different concentrations of the SARS-CoV-2 SP, before assessing the production of ROS by flow cytometry. The concentration dependent effects of treatment were analysed by measuring the level of ROS in the tested samples vs control samples.

Aim 4: Investigate the role of the SARS-CoV-2 SP in inducing NET formation measured by flow cytometry.

Hypothesis: The SARS-CoV-2 SP leads to the induction of NET formation in PMNs which can be quantitated by flow cytometry and contributes to inflammatory pathogenic processes.

Approach: PMNs were treated with the SARS-CoV-2 SP and measure NET formation using flow cytometry with SYTOX™ Orange dye to detect extracellular DNA. Treated samples were compared to untreated controls to evaluate NET formation.

Aim 5: Investigate whether the SARS-CoV-2 SP induces degranulation in PMNs.

Hypothesis: The SARS-CoV-2 SP enhances PMN degranulation leading to the release of antimicrobial peptides and other inflammatory mediators.

Approach: PMNs were exposed to the SARS-CoV-2 SP and their degranulation by release of specific granule contents such as MPO and NE was measured using ELISA.

Aim 6: Investigate the effect of the SARS-CoV-2 SP on PMN phagocytosis.

Hypothesis: The SARS-CoV-2 SP influences the phagocytic action of PMNs, which may increase or decrease a PMNs capacity to phagocytise.

Approach: PMNs were treated with the SARS-CoV-2 SP and assessed phagocytosis using fluorescently labelled bacteria (e.g., GFP-labeled strains). Phagocytic uptake was quantified via flow cytometry, comparing treated and untreated samples to evaluate the SP's effect on PMN phagocytosis.

Aim 7: Examine whether the SARS-CoV-2 SP induces apoptosis in PMNs.

Hypothesis: SARS-CoV-2 SP induce apoptosis of PMNs through the activation of specific intracellular pathways, thereby contributing to immune dysregulation and tissue damage.

Approach: PMNs were treated with the SARS-CoV-2 SP and neutrophil apoptosis was assessed using Annexin V/PI staining and flow cytometry.

Aim 8: Investigate whether the SARS-CoV-2 SP induces proinflammatory cytokine production in PMNs.

Hypothesis: The SARS-CoV-2 SP encourages the production of proinflammatory cytokines by PMNs, thus adding to the hyper-inflammatory condition experienced in severe cases of COVID-19.

Approach: PMNs were exposed to the SARS-CoV-2 SP and proinflammatory cytokines levels produced were measured (IL-6, IL-8, and TNF- α) by ELISA. Cytokine levels in treated versus untreated cells were evaluated to assess the ability of the SP to induce cytokines.

Aim 9: Determine whether TLR2 or TLR4 mediates proinflammatory cytokine production in response to the SARS-CoV-2 SP and investigate the role of NF- κ B, p38, and PERK signalling pathways.

Hypothesis: The SARS-CoV-2 SP induces proinflammatory cytokine production through TLR2 or TLR4 activation, mediated by NF- κ B, p38, and PERK signalling pathways.

Approach: SARS-CoV-2 SP was used to treat PMNs under the specific conditions of TLR2 and TLR4 inhibition, then cytokine production (IL-6, IL-8, TNF- α) was assessed using ELISA. Pharmacological antagonists of NF- κ B, p38 and PERK were used to determine the effects on the production of cytokines.

Aim 10: Human PMNs were exposed to SARS-CoV-2 SP, before measuring the production of TGF- β by ELISA.

Hypothesis: We hypothesize that the SARS-CoV-2 SP initiates TGF-beta synthesis in PMNs.

Approach: Exposed human PMNs to SARS-CoV-2 SP. We measured the production of TGF- β by ELISA.

Aim 11: Investigate the mechanism of TGF- β induction by the SARS-CoV-2 SP and its impact on ACE2 expression.

Hypothesis: The SARS-CoV-2 SP transactivates TGF- β synthesis of PMNs via either ACE2 or integrin receptors, and TGF- β or IL-10 increases the levels of ACE2 expression.

Approach: PMN expression of ACE2 was assessed by employing quantitative polymerase chain reaction (qPCR) and western blot To assess the role of integrin signaling, integrin receptors were selectively blocked prior to TGF- β secretion by ELISA. Furthermore, the impacts of TGF- β and IL-10 on PMNs expression of ACE2 was determined using qPCR.

Aim 12: Investigate the effect of TGF- β on PMN function (activation, ROS production, degranulation, phagocytosis, and NETosis).

Hypothesis: TGF- β modulates PMN functions, including activation, ROS production, degranulation, phagocytosis, and NETosis, in a concentration-dependent manner.

Approach: PMNs were treated with varying concentrations of TGF- β and their activation state was evaluated by measuring surface marker expression (CD11b, CD66b). ROS production was assessed using fluorescence-based ROS detection assays, degranulation through granule content release assays (ELISA for MPO and NE), phagocytosis using labelled bacteria and flow cytometry, and NETosis using SYTOX™ Orange dye and microscopy. Responses across treated and untreated samples were compared, to establish concentration-dependent effects.

3.4 Experiments & Discoveries:

3.4.1 CD66b and CD11b mRNA gene expressions in whole blood is associated with the severity of COVID-19

We analysed Nanostring data from Hadjadj et al. [434] who studied gene induction in COVID-19 blood samples from patients who experienced moderate, severe and critical disease. In order to investigate if neutrophil markers of activation were affected in COVID-19 patients, we analysed the Nanostring dataset for CD66b and CD11b gene expression in patients with differing severities of COVID-19 and healthy controls (**Fig. 3.2**). We saw that the expression of CD66b was not significantly increased in COVID-19 patients with moderate or severe disease, when compared with healthy controls. However, CD66b was increased in critically ill patients (**Fig. 3.2A**). Additionally, the CD11b gene expression was elevated in moderate, severe and critically ill COVID-19 patients, compared to healthy controls (**Fig. 3.2B**). These data suggest a correlation between the expression of CD66b and CD11b and disease progression in patients with COVID-19.

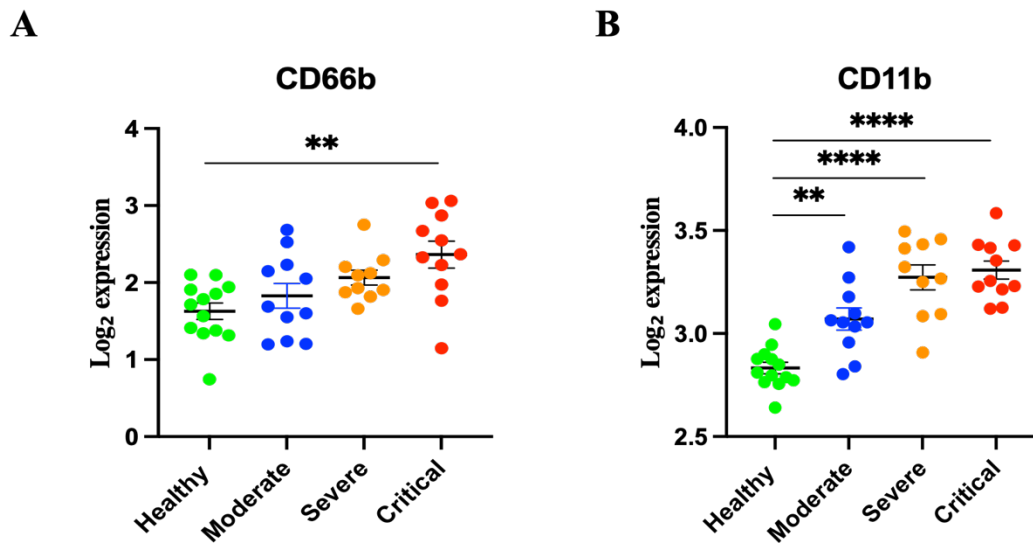


Figure 3.2 CD66b and CD11b mRNA expression in COVID-19 patients with moderate, severe and critical disease and healthy individuals.

Normalized log₂ expression of CD66b and CD11b mRNA in healthy controls (n = 13) and patients with mild-to-moderate (n = 11), severe (n = 10), and critical (n = 11) COVID-19. Data are shown as mean $\pm$ SEM. Statistical analysis was performed using one-way ANOVA. Significant differences compared with the healthy group are indicated as: **P < 0.01 and ****P < 0.0001.

3.4.2 CD11b (and not CD66b) is increased in PMNs from COVID-19 patients

To further investigate the effect of COVID-19 upon levels of neutrophil activation markers, we measured the cell surface protein expression of CD11b and CD66b by flow cytometry from PMNs isolated from COVID-19 patients and healthy controls. No significant change was observed in CD66b expression (Fig. 3.3A). However, CD11b expression was significantly increased in PMNs from COVID-19 patients, when compared with healthy controls (Fig. 3.3B).

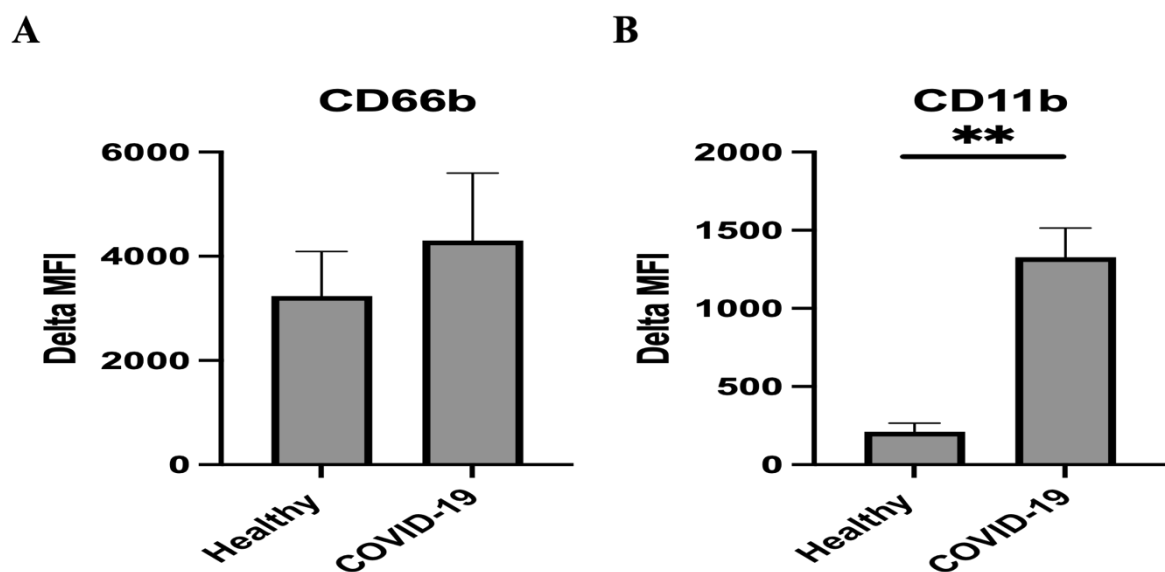


Figure 3.3 Neutrophil activation marker expression in PMNs from COVID-19 and healthy individuals.

PMNs were obtained by density gradient isolation and analysed by flow cytometry (delta MFI) for (A) CD66b and (B) CD11b in healthy donors (n=3) and COVID-19 (n=3). Data represent mean $\pm$ SEM from, with statistical analysis performed using one-way ANOVA. Significant differences compared with the healthy group are denoted as: **P < 0.01.

3.4.3 SARS-CoV-2 SP does not induce the expression of CD66b in human primary PMNs

Based on our findings that primary human PMNs are activated in COVID-19 patients (Fig 3.2), we hypothesized that the SARS-CoV-2 SP might directly activates these cells. CD66b, a glycoprotein highly expressed on the surface of activated neutrophils, plays a crucial role in adhesion and migration to sites of inflammation, making it an important marker of neutrophil activation [435]. Also during rhinovirus infections in COPD patients, sputum

neutrophils exhibit increased expression of CD66b [318] . Therefore, to explore the effect of SP upon CD66b levels of PMNs, we treated neutrophils with SARS-CoV-2 SP at various concentrations (0, 10, 50, and 100 $\mu\text{g/mL}$) for 2 hours and measured CD66b expression by flow cytometry. No significant changes in CD66b expression were observed following treatment with SARS-CoV-2 SP, as shown in (Fig. 3.4).

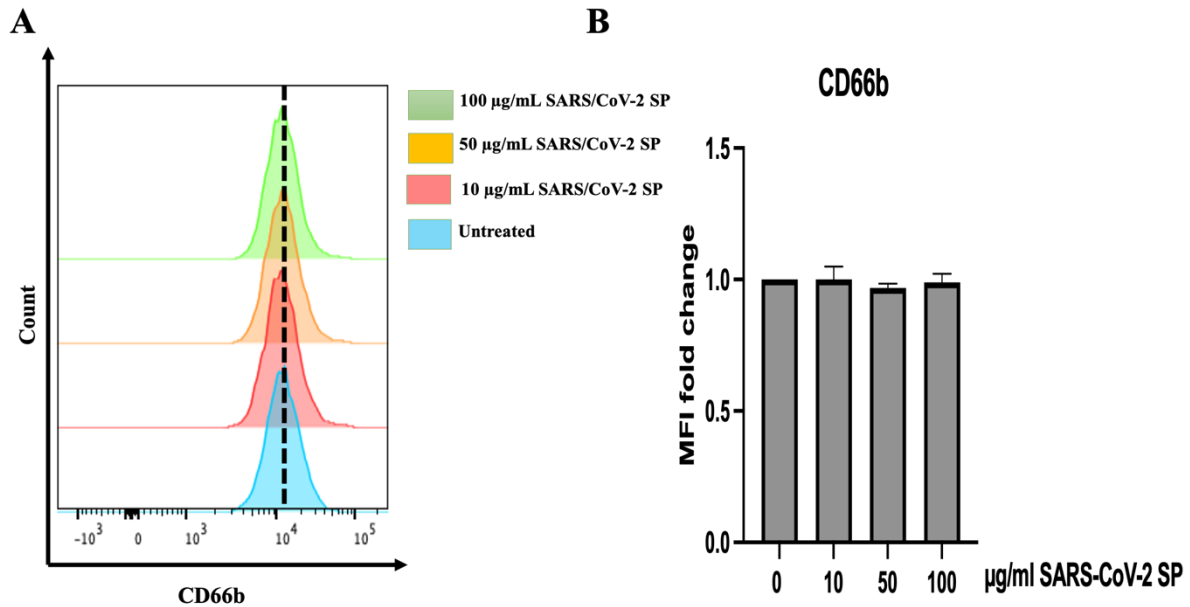


Figure 3.4 CD66b expression in treated PMN with SARS-CoV-2 SP.

Human PMNs were obtained by density gradient isolation and analysed by flow cytometry for CD66b in healthy donors ($n=4$). PMNs (1×10^6) were treated with SARS-CoV-2 SP at various concentrations (0, 10, 50, and 100 $\mu\text{g/mL}$) for 2 hours. Flow cytometric analysis was performed using a standard gating strategy: cells were first gated based on FSC and SSC properties to exclude debris and select the PMNs population, followed by singlet discrimination using SSC-Area versus SSC-Height, and finally gated for live cells using live/dead stain exclusion. (A) Representative flow cytometry histograms showing CD66b fluorescence intensity distribution. (B) Quantitative analysis showing mean fluorescence intensity (MFI) fold change relative to untreated control. Data represent mean $\pm$ SEM, with statistical analysis performed using one-way ANOVA.

3.4.4 SARS-CoV-2 SP Does Not Induce the Expression of CD11b in human primary PMNs

Since we found that CD11b mRNA levels were upregulated in the whole blood samples of COVID-19 patients in our NanoString analysis (Fig 3.2A), and the isolated neutrophils from COVID-19 patients had enhanced CD11b surface expression (Fig 3.3A), we hypothesised

that the SARS-CoV-2 SP may have a direct responsibility for CD11b expression. CD11b, an integrin expressed on neutrophils, plays a critical role in adhesion, migration, and immune responses during inflammation. Increased surface expression of CD11b is a key marker of neutrophil activation [436]. CD11b upregulation in neutrophils is a critical response during infections, including viral pneumonia [437]. To investigate whether the SARS-CoV-2 SP induces CD11b expression, we treated isolated neutrophils with SARS-CoV-2 SP at various concentrations (0, 10, 50, and 100 $\mu\text{g/mL}$) for 2 hours and measured CD11b expression by flow cytometry. No significant changes in CD11b expression were observed following treatment with SARS-CoV-2 SP, as shown in (Fig. 3.5).

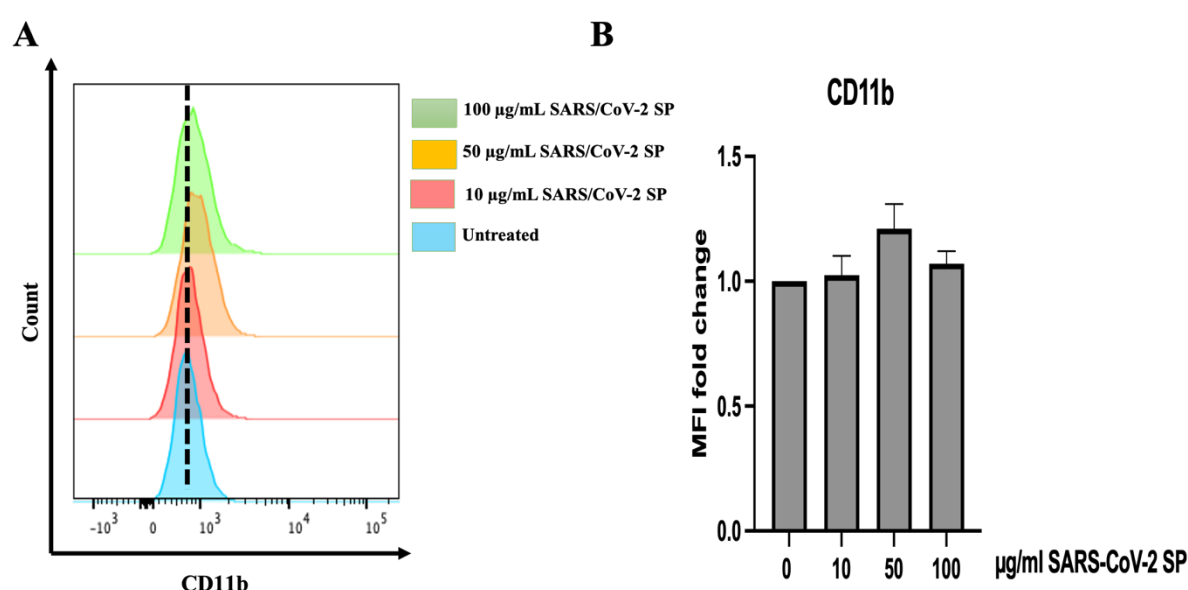


Figure 3.5 CD11b expression in treated PMNs treated with SARS-CoV-2 SP.

Human PMNs were obtained by density gradient isolation and analysed by flow cytometry for CD11b in healthy donors ($n=4$). PMNs (1×10^6) were treated with SARS-CoV-2 SP at various concentrations (0, 10, 50, and 100 $\mu\text{g/mL}$) for 2 hours. Flow cytometric analysis was performed using a standard gating strategy: cells were first gated based on FSC and SSC properties to exclude debris and select the PMNs population, followed by singlet discrimination using SSC-Area versus SSC-Height, and finally gated for live cells using live/dead stain exclusion. (A) Representative flow cytometry histograms showing CD11b fluorescence intensity distribution. (B) Quantitative analysis showing MFI fold change relative to untreated control. Data represent mean $\pm$ SEM, with statistical analysis performed using one-way ANOVA.

3.4.5 SARS-CoV-2 SP Does Not Induce Shedding of CD16 in human primary PMNs

CD16, a low-affinity Fc receptor expressed on neutrophils, plays a critical role in antibody-dependent immune responses, including phagocytosis and cytotoxicity [438]. Shedding of CD16b from the neutrophil surface is a key marker of neutrophil activation [438]. The Ebola virus' secretory glycoprotein (sGP) binds to Fc γ RIIB (CD16b) and inhibits L-selectin shedding [439]. Ebola sGP impacts the interaction between CD16b and CR3 (complement receptor 3) on neutrophils. By modifying the spatial relationship between these molecules, sGP affects signalling pathways within neutrophil, potentially hindering their normal function and assisting the virus in evading the host's immune defences [440]. To investigate whether the SARS-CoV-2 SP induces CD16 shedding, we treated isolated PMNs with SARS-CoV-2 SP at various concentrations (0, 10, 50, and 100 μ g/mL) for 2 hours and measured CD16 expression by flow cytometry. No significant CD16 shedding was observed following treatment with SARS-CoV-2 SP, as shown in (Fig. 3.6).

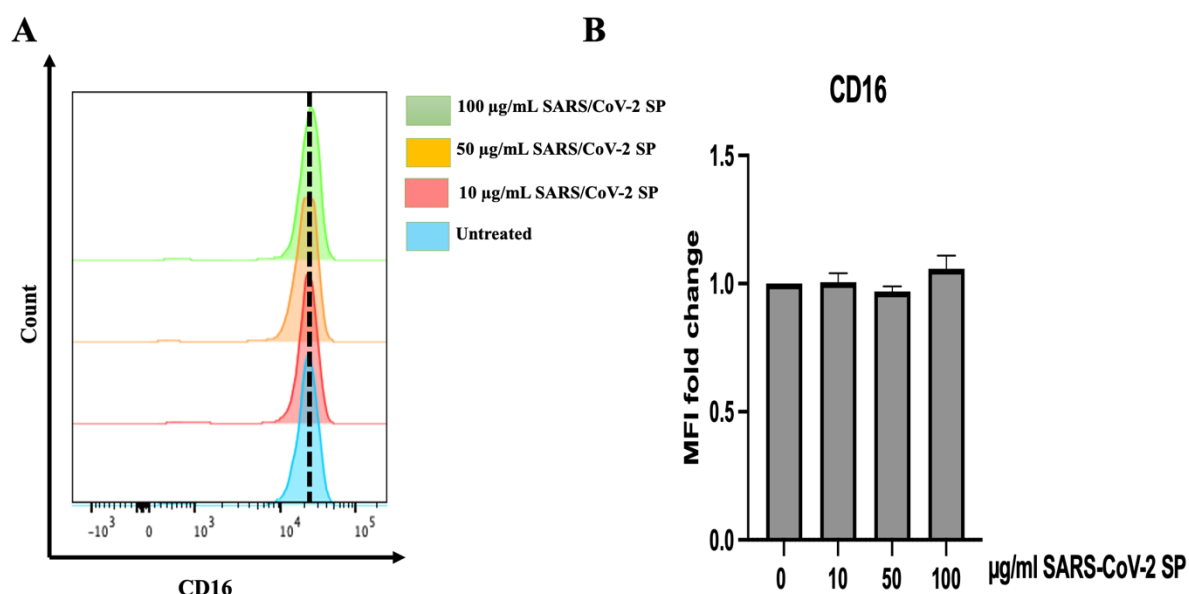


Figure 3.6 CD16 expression in treated PMNs with SARS-CoV-2 SP.

Human PMNs were obtained by density gradient isolation and analysed by flow cytometry for CD16 in healthy donors (n=4). PMNs (1×10^6) were treated with SARS-CoV-2 SP at various concentrations (0, 10, 50, and 100 μ g/mL) for 2 hours. Flow cytometric analysis was performed using a standard gating strategy: cells were first gated based on FSC and SSC properties to exclude debris and select the PMNs population, followed by singlet discrimination using SSC-Area versus SSC-Height, and finally gated for live cells using live/dead stain exclusion. (A) Representative flow cytometry histograms showing CD16 fluorescence intensity distribution. (B) Quantitative analysis showing MFI fold change

relative to untreated control. Data represent mean $\pm$ SEM, with statistical analysis performed using one-way ANOVA.

3.4.6 SARS-CoV-2 SP Does Not Induce Shedding of CD62L in human primary PMNs

CD62L, also known as L-selectin, is an adhesion molecule expressed on neutrophils that plays a critical role in leukocyte rolling and migration to inflamed tissues. Shedding of CD62L from the neutrophil surface is a key marker of neutrophil activation [441]. In a cohort of COVID-19 patients treated in the ICU, Loyer et al. reported that neutrophils showed an increase in CD11b and ROS production, along with a decrease in CD62L expression, compared to neutrophils from patients with community-acquired pneumonia (CAP) and healthy controls [442]. Also in children affected by HIV-1, PMNs show changes in CD62L expression. This includes a reduction in surface CD62L accompanied by an increase in its soluble form, which could interfere with normal cell migration and heighten vulnerability to secondary infections [443]. Based on these findings, we sought to investigate whether SARS-CoV-2 SP can decrease CD62L expression. We treated isolated PMNs with SARS-CoV-2 SP at various concentrations (0, 10, 50, and 100 $\mu\text{g/mL}$) for 2 hours and measured CD62L expression by flow cytometry. No significant CD62L shedding was observed following treatment with SARS-CoV-2 SP, as shown in (Fig. 3.7).

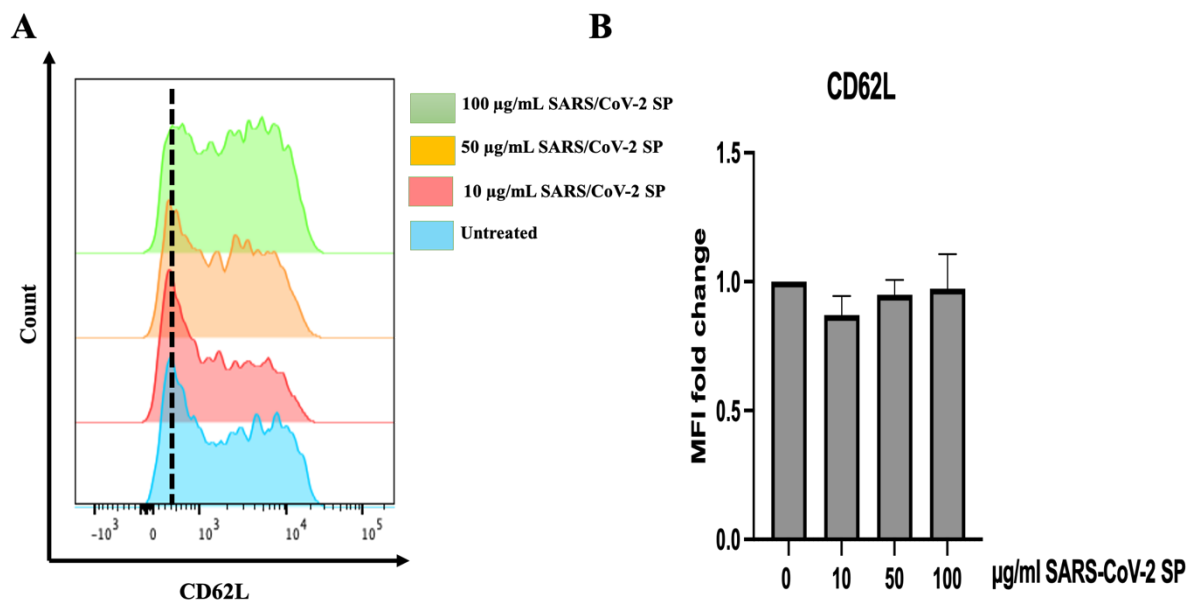


Figure 3.7: CD62L expression in PMNs treated with SARS-CoV-2 SP.

Human PMNs were obtained by density gradient isolation and analysed by flow cytometry for CD62L in healthy donors (n=4). PMNs (1×10^6) were treated with SARS-CoV-2 SP at various concentrations (0, 10, 50, and 100 $\mu\text{g/mL}$) for 2 hours. Flow cytometric analysis was

performed using a standard gating strategy: cells were first gated based on FSC and SSC properties to exclude debris and select the PMNs population, followed by singlet discrimination using SSC-Area versus SSC-Height, and finally gated for live cells using live/dead stain exclusion. (A) Representative flow cytometry histograms showing CD62L fluorescence intensity distribution. (B) Quantitative analysis showing MFI fold change relative to untreated control. Data represent mean $\pm$ SEM, with statistical analysis performed using one-way ANOVA.

3.4.7 SARS-CoV-2 SP Does Not Induce ROS Production in human primary PMNs

Next, we sought to determine whether SARS-CoV-2 SP stimulates ROS production in PMNs. ROS production plays a vital role in the antimicrobial and inflammatory responses of neutrophils. Elevated ROS production is a hallmark of neutrophil activation and is commonly used as a marker to assess functional responses [444]. It has been recognized that some viral proteins result in the production of ROS in neutrophils. For instance, neutrophils are activated by Influenza hemagglutinin HA and RSV fusion protein which in turn induces oxidative bursts in a TLR dependent pathways [445, 446]. Dengue Virus serotype-2 (DV2) triggers the generation of ROS in neutrophils, playing a role in the formation of NETs and altering neutrophil functionality in a manner that evades typical immune detection mechanisms [447]. To investigate whether the SARS-CoV-2 SP stimulates ROS production in PMNs, we treated isolated PMNs with SARS-CoV-2 SP at various concentrations (0, 10, 50, and 100 μ g/mL) for 2 hours and measured ROS production by flow cytometry. As a positive control, we treated cells with tBuOOH, which significantly induced ROS production. In contrast, treatment with SARS-CoV-2 SP did not result in significant ROS production, as shown in (Fig. 3.8).

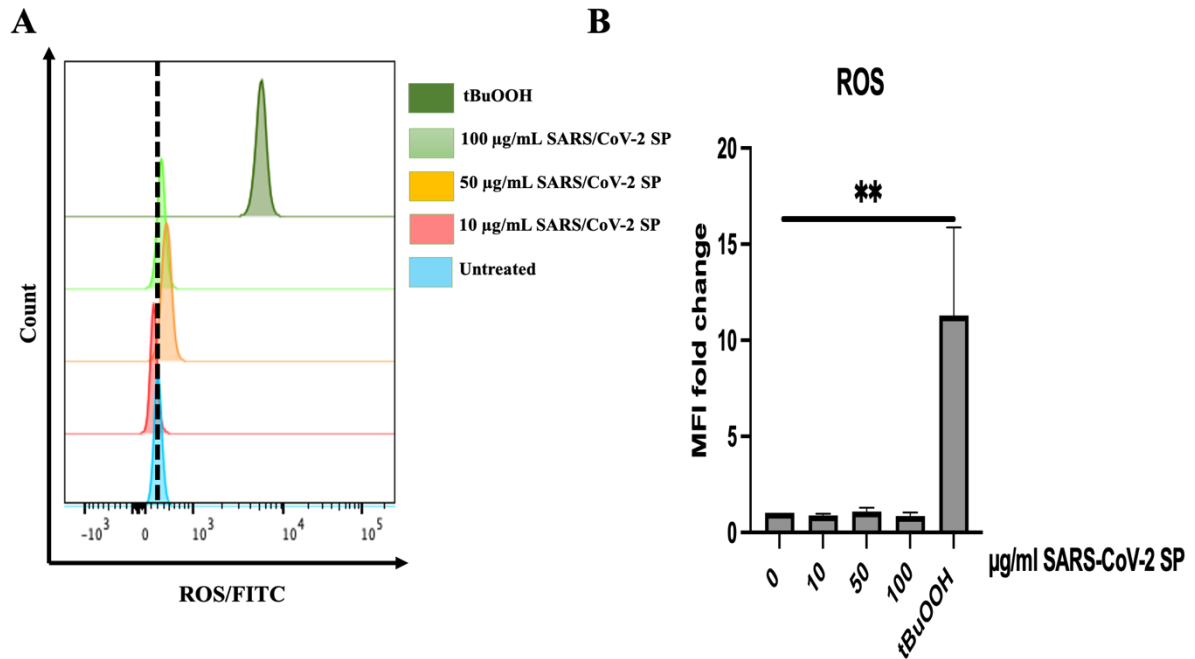


Figure 3.8 ROS production in PMNs treated with SARS-CoV-2 SP

Human PMNs were obtained by density gradient isolation and analysed by flow cytometry for ROS production in healthy donors ($n=3$). PMNs (1×10^6) were treated with SARS-CoV-2 SP at various concentrations (0, 10, 50, and 100 µg/mL) for 2 hours. Additionally, 10 nM tBuOOH was included as a positive control. Flow cytometric analysis was performed using a standard gating strategy: cells were first gated based on FSC and SSC properties to exclude debris and select the PMNs population, followed by singlet discrimination using SSC-Area versus SSC-Height, and finally gated for live cells using live/dead stain exclusion. (A) Representative flow cytometry histograms showing ROS fluorescence intensity distribution. (B) Quantitative analysis MFI fold change relative to untreated control. Data represent mean $\pm$ SEM, with statistical analysis performed using one-way ANOVA. * $P < 0.05$ compared to untreated control.

3.4.8 SARS-CoV-2 SP Decreases NETosis Production in human primary PMNs

The formation of NETs or NETosis is integral to the immune defence mediated by neutrophils and is a common indicator of neutrophil activation. SARS-CoV-2 induces significant NET formation, which has been linked to severe COVID-19 outcomes. Also excessive NETs can lead to vascular injury, clot formation, and heightened immune responses, worsening patient conditions[366]. Also one study showed that of SARS-CoV-2 SP and N proteins induced NET formation from neutrophils [448]. Based on this finding, to assess whether the SARS-CoV-2 SP is capable of inducing NETs, we placed isolated PMNs

in the presence of 10 $\mu\text{g/mL}$ of SARS-CoV-2 SP for a duration of 6 hours. We included PMA (20 nM) as a positive control as it is well known to effectively cause NETosis. PMA significantly increased the amount of NETs. On the other hand, while not statistically significant, treatment with SARS-CoV-2 SP showed a reduction in the mean fluorescence intensity of NETosis, compared to the untreated baseline control; with the mean value decreasing by 3682.25 units (from 6716.5 to 3034.25), representing approximately a 55% reduction in NETs upon SP treatment (**Fig. 3.9**).

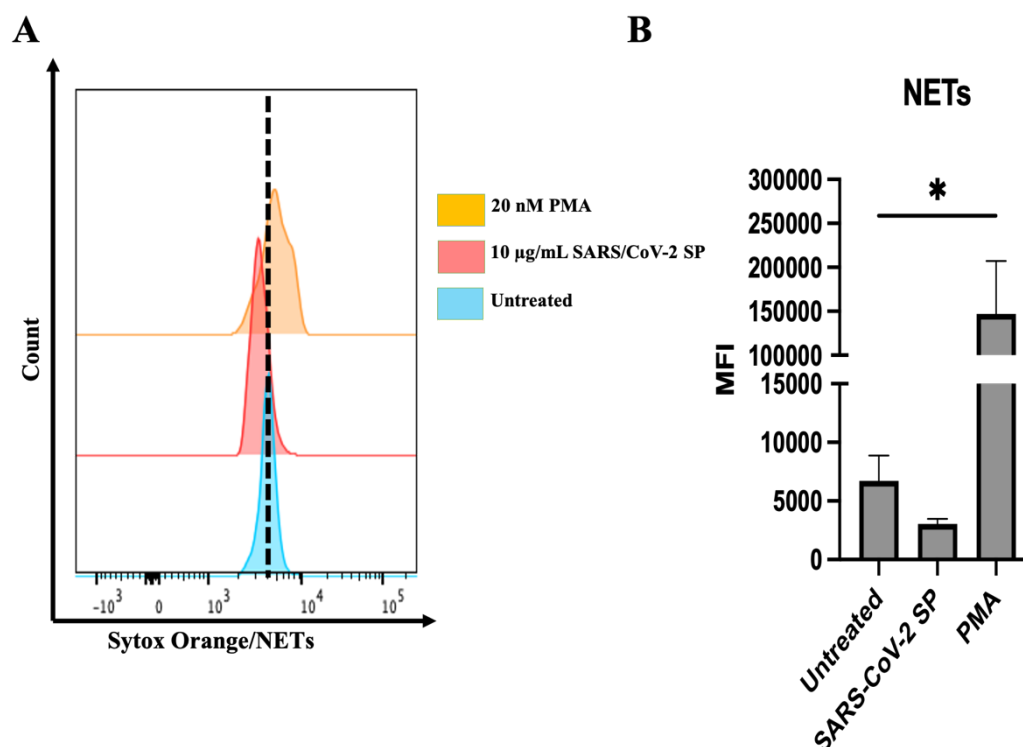


Figure 3.9 NETosis formation in treated PMNs with SARS-CoV-2 SP.

Human PMNs were obtained by density gradient isolation and analysed for NETosis formation in healthy donors ($n=4$). PMNs (1×10^6) were treated with 10 $\mu\text{g/mL}$ SARS-CoV-2 SP for 6 hours. Additionally, 20 nM PMA was included as a positive control. NETosis formation was measured using Sytox Orange, a fluorescent dye that binds to extracellular DNA. Flow cytometric analysis was performed using a standard gating strategy: cells were first gated based on FSC and SSC properties to exclude debris and select the PMNs population, followed by singlet discrimination using SSC-Area versus SSC-Height, and finally gated for live cells using live/dead stain exclusion. (A) Representative flow cytometry histograms showing Sytox Orange fluorescence intensity distribution. (B) Quantitative analysis showing MFI for each treatment condition. Data represent mean $\pm$

SEM from, with statistical analysis performed using one-way ANOVA. * $P < 0.05$ compared to untreated control.

3.4.9 SARS-CoV-2 SP Does Not Significantly Induce Degranulation in human primary PMNs

Degranulation, the release of granule-stored proteins such as MPO and neutrophil elastase, is a critical process in neutrophil-mediated immune responses and serves as a marker of neutrophil activation [449]. In severe cases of COVID-19, SARS-CoV-2 activates neutrophils excessively, leading to MPO and elastase through NET formation. These substances cause oxidative damage, harm blood vessel linings, and increase the risk of blood clots, including conditions like pulmonary embolism. MPO intensifies inflammation, while elastase breaks down structural proteins, worsening tissue injury. High levels of MPO-related markers have been linked to worse outcomes [366]. A proteomic analysis of nasopharyngeal swab samples also supports the role of neutrophil degranulation in COVID-19 pathology. A study of samples from 15 PCR positive and 15 PCR negative cases identified 207 proteins, 17 of which were differentially regulated. The key pathways affected included neutrophil degranulation, the innate immune system and antimicrobial peptide activity. 17 proteins were significantly dysregulated in SARS-CoV-2 positive patients, including NE, Azurocidin (AZU1), MPO, Myeloblastin (PRTN3), Cathepsin G (CTSG) and Transcobalamin-1 (TCN1) [341]. To investigate whether the SARS-CoV-2 SP induces degranulation, we treated isolated PMNs with SARS-CoV-2 SP at a concentration of 10 $\mu\text{g/mL}$ for 6 hours. PMA (20 nM) was used as a positive control due to its well-established ability to induce robust degranulation. Levels of MPO and neutrophil elastase were measured by ELISA. While treatment with SARS-CoV-2 SP did not result in a significant increase in the release of MPO (**Fig. 3.10A**) and NE (**Fig. 3.10B**), PMA treatment resulted in a notable, though not statistically significant, increase in their release.

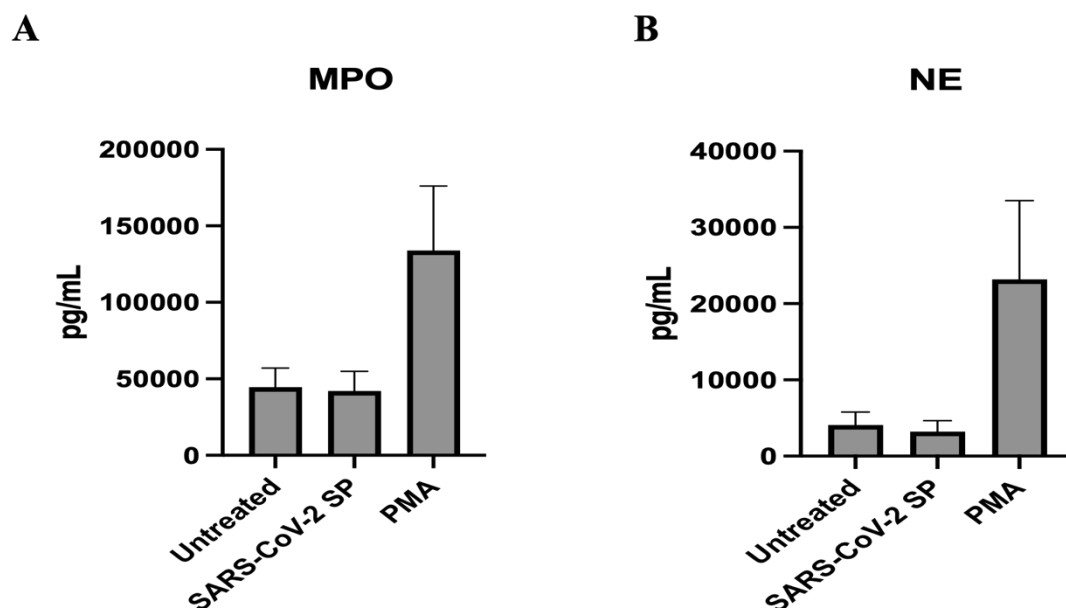


Figure 3.10 MPO and NE release in PMNs treated with SARS-CoV-2 SP.

Human PMNs were obtained by density gradient isolation and analysed for MPO (n=3) and neutrophil elastase release (n=4) in healthy donors. PMNs (1×10^6) were treated with $10 \mu\text{g/mL}$ SARS-CoV-2 SP for 6 hours. Additionally, 20 nM PMA was included as a positive control. MPO and NE release were quantified using ELISA. data expressed as concentration (pg/mL). Data represent mean $\pm$ SEM from, with statistical analysis performed using one-way ANOVA.

3.4.10 SARS-CoV-2 SP does not Induce matrix metalloproteinase 9 (MMP-9) in human primary PMNs

MMP9 is an antimicrobial enzyme produced by activated neutrophils, playing a key role in tissue remodelling, immune cell migration, and the defence against pathogens, including viruses. In the context of viral infections, MMP-9 has been shown to contribute to the immune response by facilitating neutrophil recruitment and extracellular matrix degradation, processes that help eliminate infected cells and resolve inflammation [450, 451]. Excessive activity of MMP-9 can result in tissue damage, as observed in conditions like influenza and hepatitis B, where it plays a role in driving inflammation, fibrosis, and disruptions in immune function. High levels of MMP-9 are linked to the severity of these diseases [451, 452]. Also, SARS-CoV-2 infection in K18-hACE2 mice is associated with increased MMP8, MMP9, and MMP14 that degrade extracellular matrix components [453]. A study also showed that SARS-CoV-2 SP can be activated by TMPRSS13 and MMPs, especially MMP-2 and MMP-9, which allows protease-independent viral entry, particularly

in high-MMP expressing cells. MMP-dependent entry required S1/S2 cleavage, with the Delta variant favouring this route, while Omicron could not utilize MMPs. As MMP-2/9 are released during inflammation, they may impact viral spread, cytopathic effects, and disease severity [454].

To evaluate whether SARS-CoV-2 SP stimulates MMP-9 production, PMNs were treated with 1 µg/mL of SARS-CoV-2 SP (**Fig. 3.11A**) or 100 ng/mL of LPS (a positive control) for 0, 2, 4, and 6 hours (**Fig. 3.11B**). There was no significant increase in MMP-9 expression with LPS treatment, but mean concentrations showed a modest elevation from baseline levels to approximately 250 ng/mL at 2 hours, 280 ng/mL at 4 hours, and 260 ng/mL at 6 hours. However, treatment with SARS-CoV-2 SP did not induce significant changes in MMP-9 protein expression at any time point, suggesting that the SARS-CoV-2 SP does not activate this response in human neutrophils (**Fig. 3.11**).

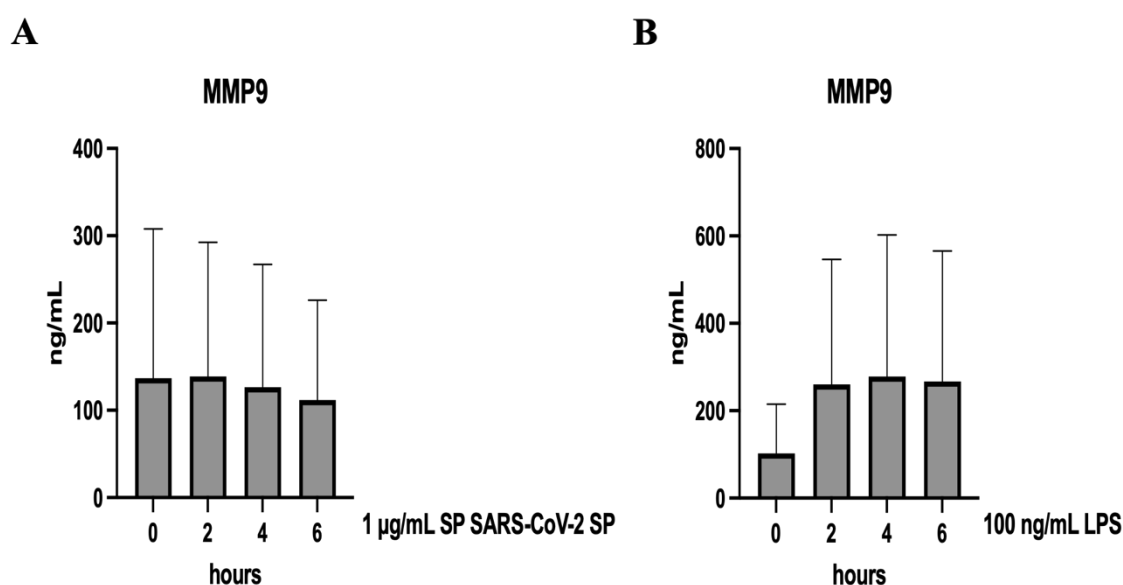


Figure 3.11 MMP9 expression in PMNs treated with SARS-CoV-2 SP.

Human PMNs were obtained by density gradient isolation. PMNs (1×10^6) were treated with 1 µg/mL of SARS-CoV-2 SP or 100 ng/mL of LPS for the indicated time points ($n=3$). MMP-9 production was analysed using ELISA. Data expressed as concentration (ng/mL). Data represent mean $\pm$ SEM from, with statistical analysis performed using one-way ANOVA.

3.4.11 SARS-CoV-2 SP Does Not Alter Phagocytosis of human primary PMNs

Next, we examined the impact of SARS-CoV-2 on neutrophil phagocytic function. Phagocytosis is one of the essential parts of the pathogenic immune response. Perera, S et

al., investigated whether the rates of phagocytosis correlated with COVID-19 severity. They obtained neutrophils from 20 COVID-19 patients and incubated them with *Staphylococcus aureus* bioparticles, before quantifying phagocytosis over 2 hours. They found that neutrophils from critically ill COVID-19 patients had significantly increased phagocytosis rates, when compared to healthy controls [455].

Another study reported the opposite finding, showing that neutrophils from COVID-19 patients exhibit a significant reduction in their ability to engulf bacteria, compared to those from healthy individuals. This diminished capacity may be linked to disruptions in receptor activity or alterations in the cytoskeletal structures necessary for the phagocytosis process [456]. Also, in monocytes, SP and anti-SP antibodies may either enhance or reduce phagocytosis, depending on their concentration [457]. Therefore, to evaluate whether the SARS-CoV-2 SP affects PMN phagocytosis, we conducted experiments comparing PMNs treated with SARS-CoV-2 SP to untreated controls. In our experiment, PMNs were pre-treated with 10 µg/mL of SARS-CoV-2 SP or kept untreated (control group) for 4 hours before being exposed to GFP-labelled *Staphylococcus aureus* (*S. aureus*) (at a 1:100 MOI) for 1 hour. Phagocytosis of the GFP *S. aureus* was subsequently analysed by flow cytometry. The results showed no significant difference in phagocytosis between neutrophils pre-treated with SARS-CoV-2 SP and the untreated control group. Both groups demonstrated similar levels of bacterial uptake, indicating that the SARS-CoV-2 SP does not significantly influence the phagocytic capacity of neutrophils under these experimental conditions (**Fig. 3.12**)

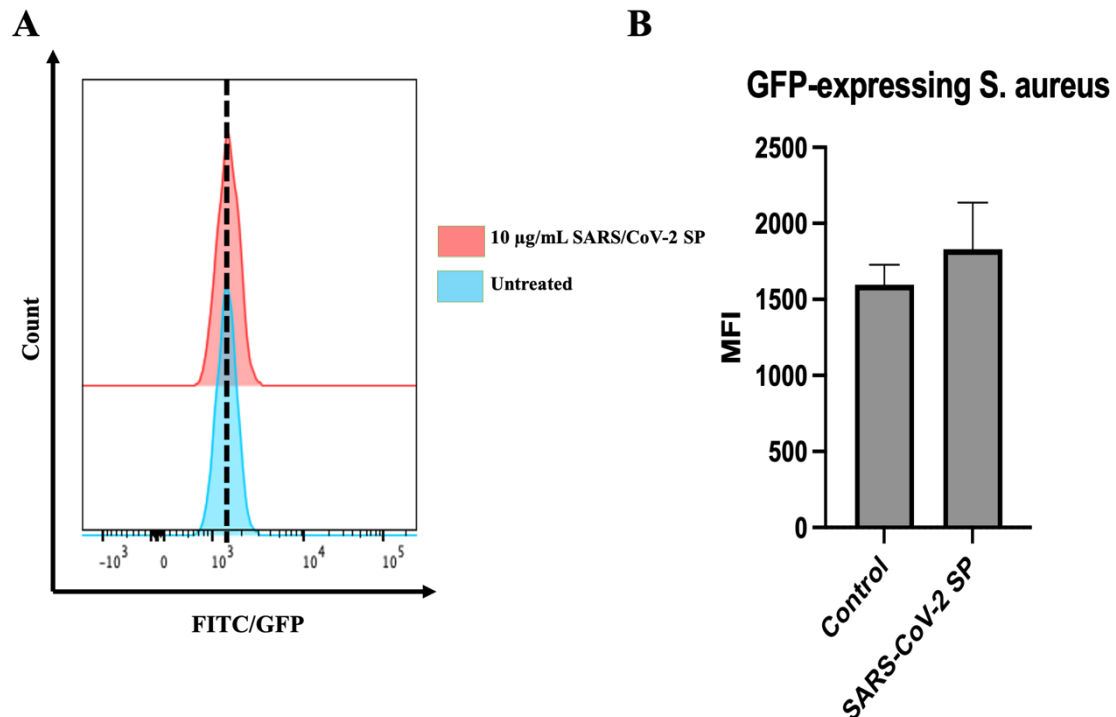


Figure 3.12 Effect of SARS-CoV-2 SP on PMNs phagocytosis.

Human PMNs were obtained by density gradient isolation. PMNs (1×10^6) pre-treated with 10 µg/mL of SARS-CoV-2 SP for 4 hours or left untreated (control) were then exposed to GFP-labelled *S. aureus* (at a 1:100 MOI) for 1 hour ($n=3$). Phagocytosis was quantified by flow cytometry measuring the uptake of GFP-labelled bacteria. Flow cytometric analysis was performed using a standard gating strategy: cells were first gated based on FSC and SSC properties to exclude debris and select the PMNs population, followed by singlet discrimination using SSC-Area versus SSC-Height, and finally gated for live cells using live/dead stain exclusion. (A) Representative flow cytometry histograms showing GFP fluorescence intensity distribution. (B) Quantitative analysis showing mean MFI of GFP uptake for each treatment condition. Data represent mean $\pm$ SEM from, with statistical analysis performed using one-way ANOVA.

3.4.12 SARS-CoV-2 SP Does Not Induce Type I or Type III IFN mRNA Expression in human primary PMNs

Given that certain viral proteins, such as the Influenza NS1 and RSV NS2 protein [458, 459], are capable of regulating IFNs through RIG-I and TLR signalling pathways, we next investigated whether SARS-CoV-2 SP induces IFNs production.. One study shows that the SARS-CoV-2 SP induces cell fusion, which in turn results in DNA damage and the activation of the cGAS-STING pathway, leading to the production of IFN-I. In this study,

using HEK293T, A549-ACE2, HeLa-ACE2 and Calu-3 cells, the researchers observed that fused cells gave rise to micronuclei, that in turn activated cGAS and boosted IFN production. This process turned out to require protease cleavage at the S1/S2 and S2' sites. Similar effects were seen in cells infected with VSV engineered to express the SARS-CoV-2 SP [460]. However, to determine if similar effects occur in neutrophils, we examined the mRNA levels of type I IFNs (IFN- α and IFN- β) and type III IFNs (IFN- λ). Neutrophils were incubated with 10 μ g/mL of SARS-CoV-2 SP or 10 μ g/mL of Polyinosinic acid poly I:C (a synthetic double-stranded RNA mimic that activates PRRs, induces type I and III IFN responses, and serves as a positive control for viral RNA recognition and innate immune activation) for 0, 2, and 4 hours. The mRNA expression levels of IFN- λ (**Fig. 3.13.A**), IFN- α (**Fig. 3.13.B**), and IFN- β (**Fig. 3.13.C**) were assessed using qRT-PCR. Following poly I:C treatment while there was no significant increase in mRNA expression, at 4 hours IFN- λ showed a 6-fold increase, IFN- α a 2.7-fold increase, and IFN- β a 4.7-fold increase, compared to baseline levels. However, SP treatment did not enhance the mRNA levels of IFN- λ , IFN- α , or IFN- β .

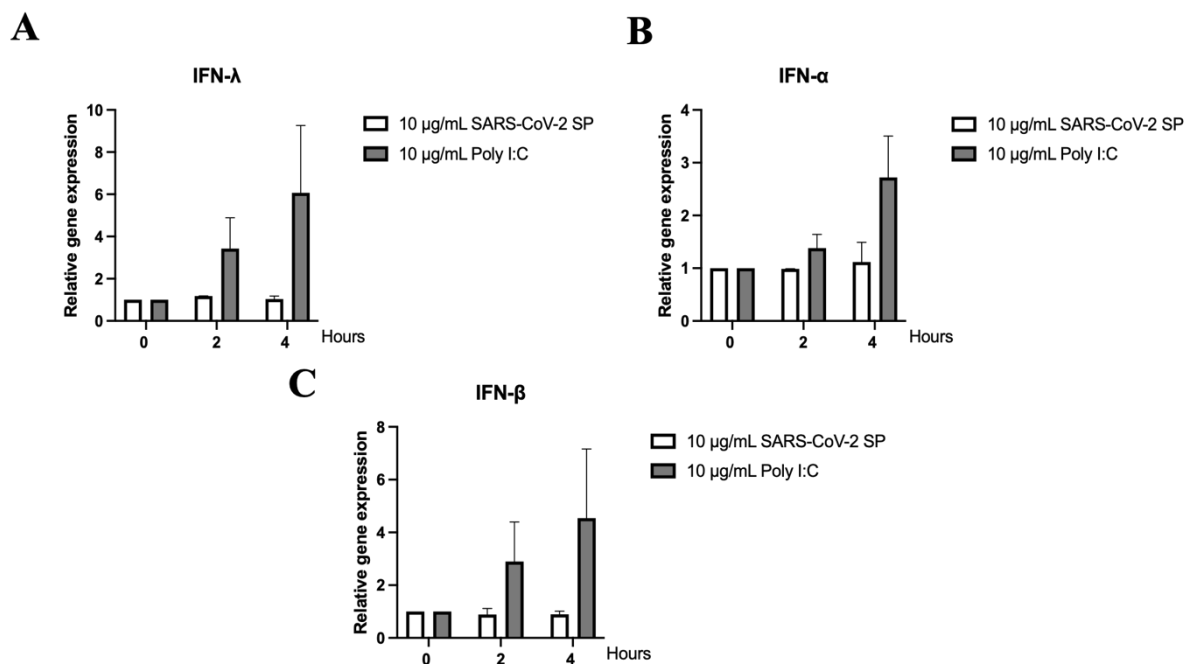


Figure 3.13 mRNA expression of IFNs in PMNs treated with SARS-CoV-2 SP or poly I:C.

Human PMNs were obtained by density gradient isolation (n=3). PMNs (1×10^6) were treated with 10 μ g/mL SARS-CoV-2 SP or 10 μ g/mL poly I:C for 0, 2, and 4 hours. mRNA levels of IFN- λ (A), IFN- α (B), and IFN- β (C) were measured by qRT-PCR. Gene expression was normalized to the housekeeping gene β -actin and SARS-CoV-2 SP and Poly I:C treated

samples were compared to the untreated controls (0 hour), which was normalized to 1. Data represent mean $\pm$ SEM from, with statistical analysis performed using two-way ANOVA.

3.4.13 Human Primary PMNs Do Not Express ACE2 and Show Limited Induction Under Inflammatory Conditions

Next, we investigated whether the lack of ACE2 expression in PMNs could explain their unresponsiveness to SARS-CoV-2 SP on functional assays. ACE2 plays a crucial role in the renin-angiotensin system and serves as the primary receptor for SARS-CoV-2 [461]. ACE2 expression varies across tissues and is influenced by factors such as age, sex, ethnicity, and comorbidities [462].

Therefore, we next determined ACE2 expression of PMNs by qPCR and Western blotting. As a positive control, we assessed ACE2 expression in HEK-293 cells that stably express human ACE2. As a negative control, we used wild type HEK-293 cells. While ACE2 expression was observed in ACE2/HEK-293 cells (**Fig. 3.14A and B**), we did not detect mRNA (**Fig. 3.14A**) nor protein expression (**Fig. 3.14B**) of ACE2 in PMNs.

Since viral infection causes inflammation, we next sought to investigate if the inflammatory stimuli could upregulate ACE2 expression of PMNs. Thus, we treated isolated PMNs with LPS (100 ng/mL), Pam3csk4 (1ug/mL), CL075 (1 ug/mL) or IFN- α (1000 U) for 0, 2 and 4 hours, before measuring ACE2 mRNA levels. While not statically significant, LPS (**Fig. 3.14C**) induced ACE2 mRNA expression $\sim$ 2 fold. However, we did not observe any induction of ACE2 mRNA with Pam3csk4 (**Fig. 3.14D**) CL075 (**Fig. 3.14E**), and IFN- α (**Fig.3.14F**).

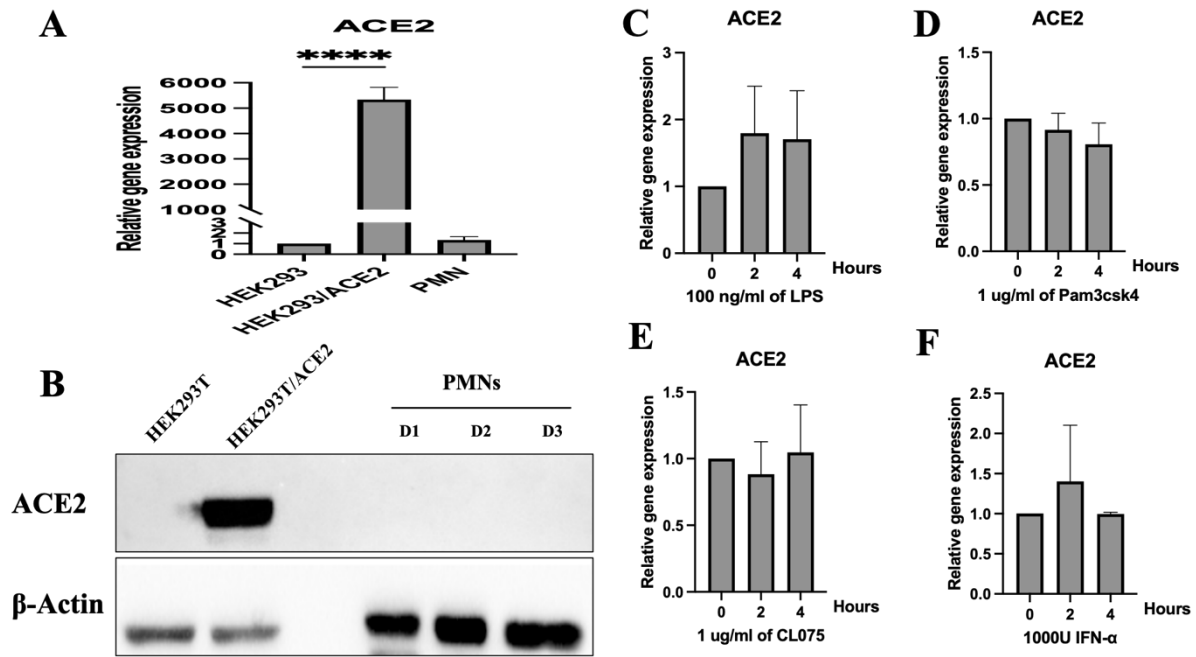


Figure 3.14 ACE2 expression in PMNs under baseline and inflammatory conditions.

Human PMNs from healthy donor blood were obtained by density gradient isolation (n=3) and basal levels of ACE2 mRNA and protein were analyzed by A) qRT-PCR and B) Western blot, respectively. HEK-293 cells and ACE2/HEK-293 cells lines were used as controls. All blots were probed for β-actin. PMNs were treated with C) LPS (100 ng/mL), D) Pam3csk4 (1ug/mL), E) CL075 (1 ug/mL) and F) IFN-α (1000 U) for 0, 2 and 4 hours. Total RNA was extracted, reverse transcribed to cDNA, and ACE2 mRNA expression was analyzed by qRT-PCR using gene-specific primers. Gene expression was normalized to the housekeeping gene β-actin and calculated using the $2^{-(\Delta\Delta Ct)}$ method relative to untreated controls (0h). Data represent mean $\pm$ SEM from, with statistical analysis performed using one-way ANOVA.

3.4.14 IL-10 and TGF-β treatments did not induce ACE2 mRNA in human Primary PMNs

Albini A, et al., found that IL-10 induces ACE2 expression in Calu-3 epithelial cells [463]. In view of this, we wanted to determine if IL-10 or TGF-β could influence ACE2 expression in PMNs at the transcriptional level. To this end, PMNs were treated with 25 ng/mL of IL-10 (**Fig. 3.15A**) or 5 ng/mL of TGF-β (**Fig. 3.15B**) for 0, 2, and 4 hours and then ACE2 mRNA was examined by qRT-PCR techniques. Unlike the findings in epithelial cells, our data demonstrated that neither IL-10 nor TGF-β significantly induced ACE2 mRNA expression in PMNs at any of the tested time points. Specifically, we observed no substantial

fold induction of ACE2 mRNA following treatment with either cytokine at 2 or 4 hours. Therefore, while Albini A, et al., found that IL-10 enhanced ACE2 expression in epithelial cells, our findings indicate this was not the case for neutrophils, thus illustrating the regulation of ACE2 expression may be cell type specific (**Fig. 3.15**).

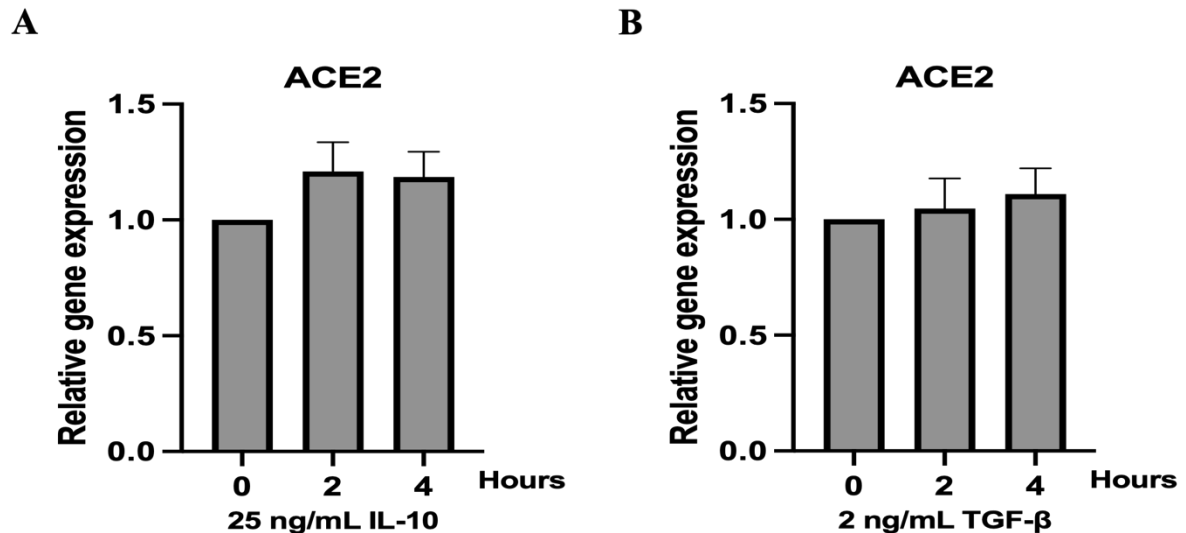


Figure 3.15 Effect of IL-10 and TGF-β on ACE2 mRNA expression in PMNs.

PMNs were isolated by density gradient centrifugation from healthy donor blood (n=6). PMNs (1×10^6) were stimulated with IL-10 (25 ng/mL) (A) or TGF-β (5 ng/mL) (B) for the indicated time points. Total RNA was extracted, reverse transcribed to cDNA, and ACE2 mRNA expression was analyzed by qRT-PCR using gene-specific primers. Gene expression was normalized to the housekeeping gene β-actin and calculated using the $2^{(-\Delta\Delta Ct)}$ method relative to untreated controls (0h). Data represent mean fold change $\pm$ SEM. Statistical significance was determined using one-way ANOVA.

3.4.15 SARS-CoV-2 SP enhances human primary PMNs Survival

Viral infections have evolved mechanisms to manipulate host immune responses, often targeting apoptotic pathways to prolong cell survival or induce premature cell death. Apoptosis, or programmed cell death, is a mechanism used to control the lifespan of neutrophils and regulate inflammation. However, apoptosis control can drastically change immune responses in pathological conditions, particularly during viral infection. By controlling the lifespan of neutrophils and clearing histotoxic contents, apoptosis is involved in limiting tissue damage and resolving inflammation [464]. EBV can infect neutrophils, inducing apoptosis via Fas/Fas ligand pathways, which may suppress immune responses during infections [465]. In this context, certain viruses can alter apoptotic pathways to

control immune responses. IAV accelerates neutrophil apoptosis and enhances the apoptotic effects of bacterial superinfections, influencing inflammation dynamics [466]. Also the Core protein of HCV prevents apoptosis induced by Fas or TNF- α by activating NF- κ B, which sustains the expression of anti-apoptotic factors [467]. In COVID-19, research shows that SARS-CoV-2 infection induces apoptosis [468-470]. Both intrinsic and extrinsic apoptotic pathways are induced during infection, and they influence a number of cellular phenotypes in the lungs, especially alveolar and endothelial cells [469]. The SARS-CoV-2 SP, particularly its C-terminal domain, is sufficient to trigger apoptosis Vero E6 cells [471]. Thus, we aimed to investigate whether SARS-CoV-2 SP induces apoptosis in PMNs. In order to determine the effect of the SARS-CoV-2 SP on PMN apoptosis, PMNs were treated with SP (0, 10, 50, 100 μ g/mL; 6 h) and stained with Annexin V and propidium iodide (PI) for flow cytometric analysis. Viable cells were defined as Annexin V⁻/PI⁻, early apoptotic cells as Annexin V⁺/PI⁻, late apoptotic cells as Annexin V⁺/PI⁺, and necrotic cells as Annexin V⁻/PI⁺. SARS-CoV-2 SP treatment significantly reduced late apoptosis at 10 μ g/mL compared with untreated controls, with similar but non-significant reductions observed at 50 and 100 μ g/mL. In parallel, viability was significantly increased at 100 μ g/mL SP. Necrosis levels remained low and unchanged across all treatments, as shown in (Fig.3.16).

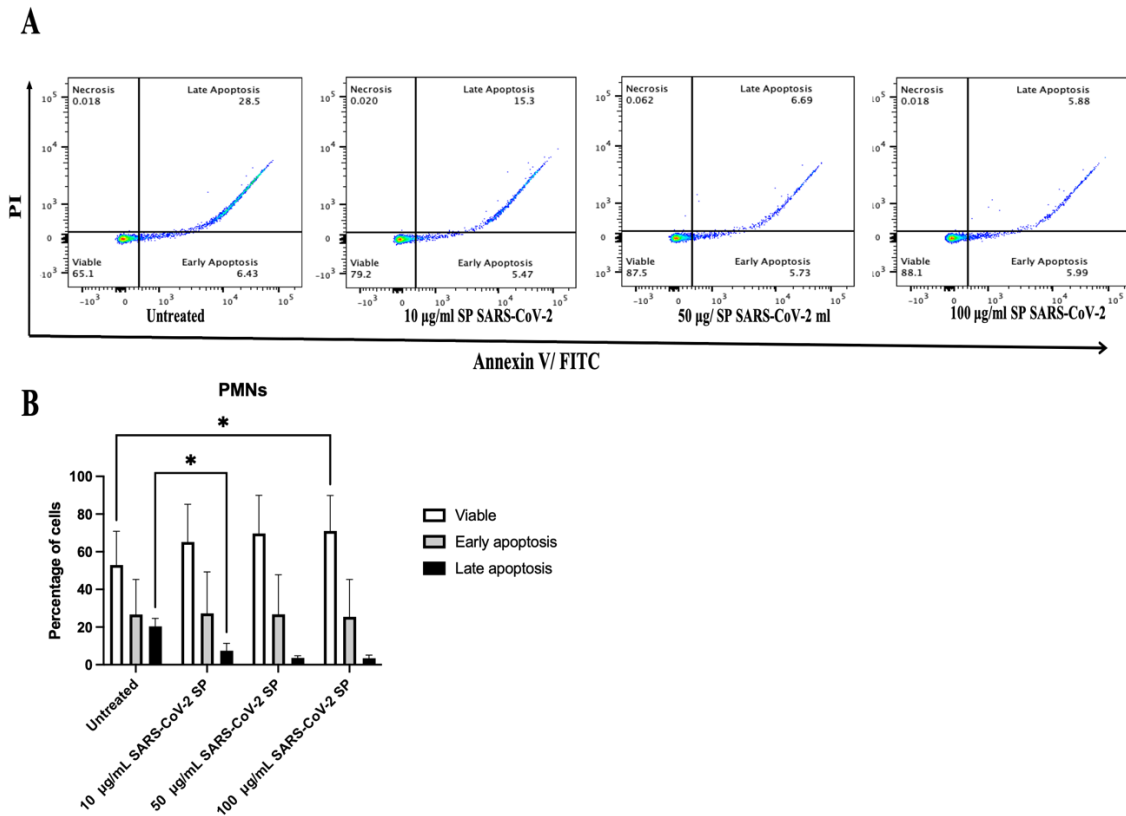


Figure 3.16 SARS-CoV-2 SP Modulates Apoptosis in PMNs

Human PMNs were obtained by density gradient isolation of healthy human blood. PMNs (1×10^6) were treated with increasing concentrations (0, 10, 50, and 100 $\mu\text{g/mL}$) of SARS-CoV-2 SP for 6 hours. Cells were subsequently stained with Annexin V-FITC and PI and analyzed by flow cytometry to distinguish cell populations: viable cells (Annexin V⁻/PI⁻), early apoptotic cells (Annexin V⁺/PI⁻), late apoptotic cells (Annexin V⁺/PI⁺), and necrotic cells (Annexin V⁻/PI⁺). (A) Representative Annexin V/PI flow cytometry dot plots from a single donor. (B) Quantitative analysis of viable and late apoptotic neutrophil populations pooled from multiple independent experiments. Data are presented as mean $\pm$ SEM. Statistical significance was determined by two-way ANOVA * $p < 0.05$.

3.4.16 SARS-CoV-2 SP Induces IL-8 but Not IL-1 β or IL-6 production in human Primary PMNs

Previous studies have shown that SARS-CoV-2 SP interacts with TLRs, specifically TLR4 and TLR2 to induce production of proinflammatory cytokines, IL-8, IL-6, IL-1 β and TNF- α in murine and human macrophages [472, 473]. Stimulation of isolated Peripheral blood mononuclear cells (PBMCs) with SARS-CoV-2 SP S1 unit (100 ng/mL) resulted in

significant elevation in the production of TNF- α , IL-6, IL-1 β and IL-8 [474]. However, the effects of SARS-CoV-2 SP on PMN cytokine production, have not been fully elucidated. Therefore, to investigate the effects of SARS-CoV-2 SP on neutrophil cytokine production, we treated isolated PMNs with 10 μ g/mL SARS-CoV-2 SP, or 100 ng/mL LPS as a positive control, for 18 hours. Following stimulation, the levels of IL-1 β , IL-6, and IL-8 were quantified using ELISA. The results showed that LPS, as expected, significantly induced the levels of IL-8 (**Fig. 3.17A**), IL-6 (**Fig. 3.17B**), and IL-1 β (**Fig. 3.17C**). In contrast, treatment with SARS-CoV-2 SP selectively induced a significant increase in IL-8 levels, with no substantial effect on IL-1 β or IL-6 production. (**Fig. 3.17**).

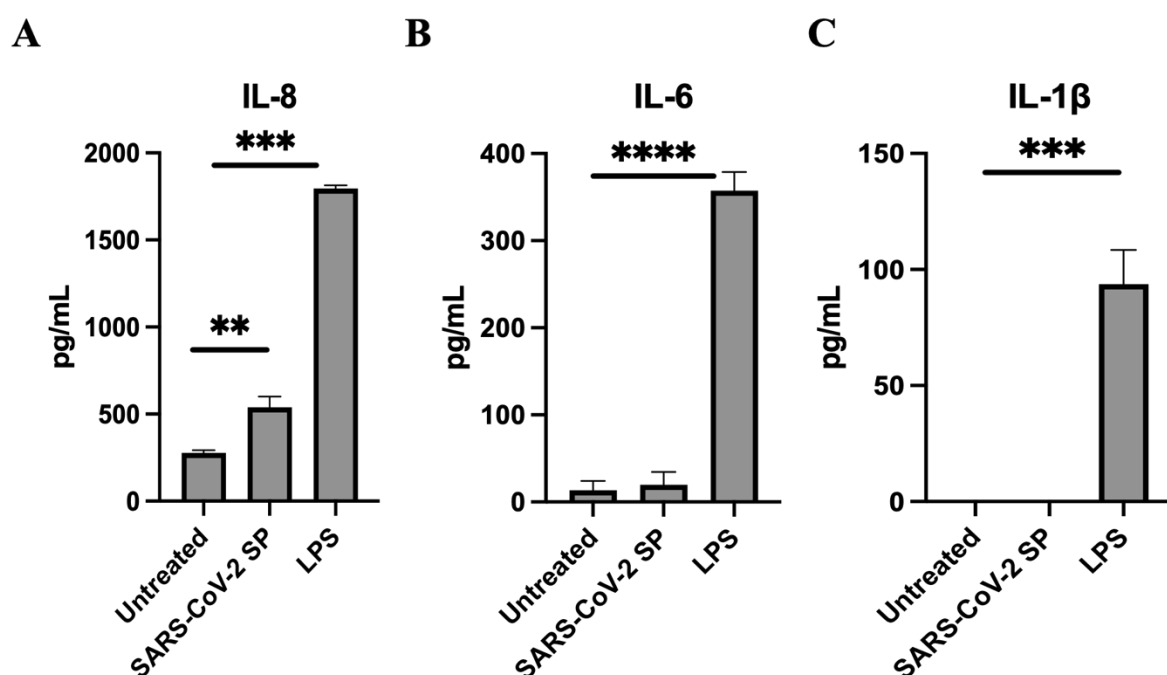


Figure 3.17: SARS-CoV-2 SP Selectively Induces IL-8 Production in Human PMNs.

PMNs were obtained by density gradient isolation of healthy human blood. PMNs (1×10^6) were treated with SARS-CoV-2 SP (10 μ g/mL) or LPS (100 ng/mL) for 18 hours, and cytokine levels IL-8 (A), IL-6 (B), and IL-1 β (C) were measured by ELISA. Cytokine levels in SARS-CoV-2 SP- and LPS-treated samples were compared to the untreated control (0 hour). Data represent mean $\pm$ SEM, with statistical analysis performed using one-way ANOVA. Statistical significance was determined as follows: ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$

3.4.17 SARS-CoV-2 SP-Induced IL-8 Production in Human Primary PMNs is Partially Reduced by TLR4 and TLR2 Inhibition

Having found that SARS-CoV-2 SP increases IL-8 production from human primary PMNs (**Fig. 3.17A**), we next sought to determine the involvement of TLR4 and TLR2 in mediating this response. Previous studies have demonstrated that SARS-CoV-2 SP is able to interact with these receptors and trigger proinflammatory signalling pathways in human and mouse macrophages, as well as human and mouse lung epithelial cells [409, 475]. SP from different SARS-CoV-2 variants can directly interact with NK cells, mainly stimulating the CD56bright subset of NK cells, leading to enhanced cytokine secretion and cytotoxicity. Importantly, SP does not engage ACE2 or classical NK activating receptors, but it does interact with TLR2 and TLR4 to trigger NK cell activation via the NF- κ B signalling pathway. Silencing or blocking these receptors greatly attenuates SP-stimulated NK cell activation and cytokine secretion [476]. Given that TLR2 and TLR4 have also been identified as key receptors for SP-induced NK cell activation, we extended our investigation to assess whether similar mechanisms underlie PMNs activation.

To further investigate the role of TLR4 and TLR2 in SP-induced IL-8 production in PMNs, we used CLI-095, a selective TLR4 inhibitor, and TL2-C29, a TLR2 inhibitor. LPS and Pam3CSK4 were used as positive controls for TLR4 and TLR2 activation, respectively.

PMNs were pre-treated with CLI-095 (1 μ g/mL) or TL2-C29 (10 μ M) for 30 minutes before stimulation with SARS-CoV-2 SP (10 μ g/mL), LPS (100 ng/mL), or Pam3CSK4 (1 μ g/mL) for 18 hours. Also, a control group were kept without treatment or treated with CLI-095 or TL2-C29 alone, to establish baseline IL-8 levels. Following incubation, supernatants were collected, and IL-8 production was quantified using ELISA.

We found that LPS-induced IL-8 production was significantly reduced by CLI-095 (**Fig. 3.18A**), confirming its dependence on TLR4, while Pam3CSK4-induced IL-8 production was significantly reduced by TL2-C29 (**Fig. 3.18B**), validating its dependence on TLR2.

Regarding SARS-CoV-2 SP, pre-treatment with CLI-095 (TLR4 inhibitor) completely abolished IL-8 production (571 to 0 pg/mL), while TL2-C29 (TLR2 inhibitor) nearly abolished IL-8 levels by approximately 8.7-fold (571 to 66 pg/mL)s. These findings suggest that SP-induced IL-8 production is mediated through TLR4 and TLR2 (**Fig. 3.18**).

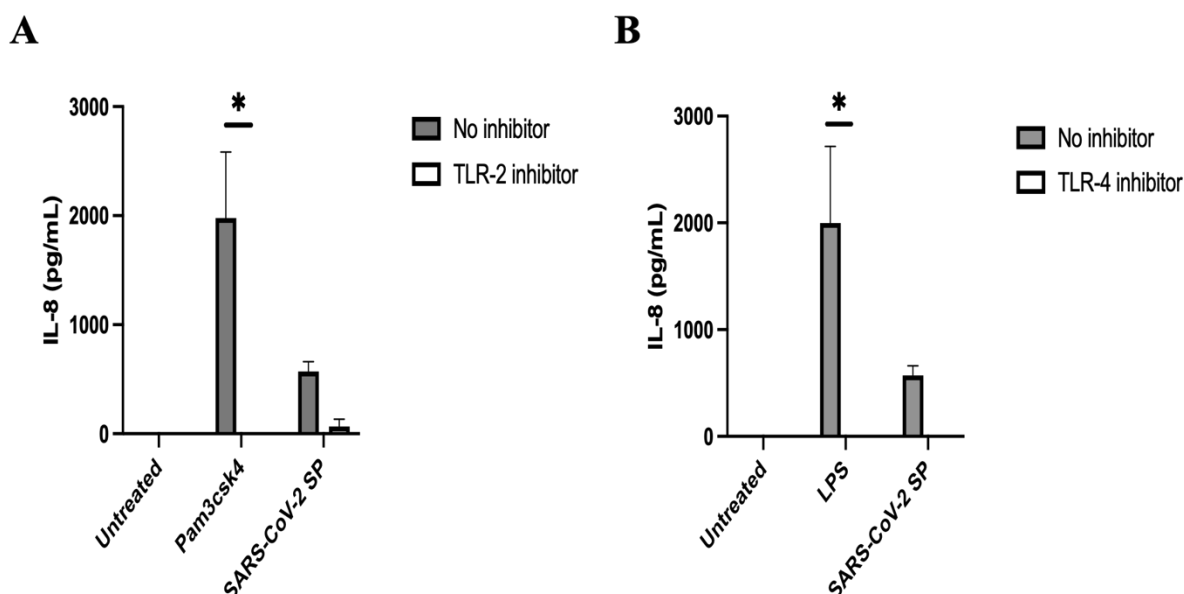


Figure 3.18 Effect of TLR4 and TLR2 Inhibition on IL-8 Production Induced by SARS-CoV-2 SP in Human Primary PMNs.

PMNs were obtained by density gradient isolation of healthy human blood (n=3). PMNs (1×10^6) were pre-treated with CLI-095 (1 μ g/mL) or TL2-C29 (10 μ M for 30 minutes before stimulation with SARS-CoV-2 SP, (10 μ g/mL) LPS (100 ng/mL), or Pam3CSK4 (1 μ g/mL) for 18 hours. IL-8 production was measured by ELISA. Data represent mean $\pm$ SEM from, with statistical analysis performed using two-way ANOVA. Statistical significance was determined as follows: (* $p < 0.05$)

3.4.18 Role of NF- κ B, p38 MAPK, and ERK1/2 Pathways in SARS-CoV-2 SP-Induced IL-8 Production in Human Primary PMNs

PMNs employ several intracellular signalling pathways to control their immune responses, including the production of proinflammatory cytokines. IL-8 is one of the most important chemoattractant of neutrophils, and is often upregulated by microbial stimuli through well characterized signalling cascades that lead to activation of NF- κ B, p38 MAPK, and ERK1/2 [296]. These pathways are known to be activated by various PRRs, including TLR2 and TLR4 [477], which we have previously identified as mediators of SARS-CoV-2 SP-induced IL-8 production (**Fig. 3.18**). However, the precise contribution of these intracellular signalling mechanisms to the activation of PMNs by SARS-CoV-2 SP is still unknown.

Previous studies have shown that NF- κ B is a key inducer of IL-8 transcription in response to microbial and viral stimuli and that the p38 MAPK and ERK1/2 pathways are also involved in the regulation of IL-8 production [478, 479]

Therefore, we investigated that SARS-CoV-2 SP could lead to the release of IL-8 from PMNs through any of the above mentioned pathways.

To further analyse the role of these signalling pathways in IL-8 production induced by LPS and SP, we used BAY 11-7082 (an NF- κ B inhibitor) (**Fig. 3.19A**), SB203580 (a p38 MAPK inhibitor) (**Fig. 3.19B**), and PD98059 (an ERK1/2 inhibitor) (**Fig. 3.19C**). PMNs were pre-treated with BAY 11-7082 (10 μ M), SB203580 (10 μ M), or PD98059 (10 μ M) for 30 minutes, followed by stimulation with LPS (100 ng/mL) or SP (10 μ g/mL) for 18 hours. Also, a control group was kept without treatment or treated with BAY 11-708, or PD98059 or SB203580 alone to establish baseline IL-8 levels. The results showed that pre-treatment of PMNs with BAY 11-7082 significantly reduced LPS-induced IL-8 production, highlighting the crucial role of the NF- κ B pathway in this response (**Fig. 3.19A**). Regarding SARS-CoV-2 SP, pre-treatment with NF- κ B inhibitor reduced IL-8 levels from 571 to 0 pg/mL; however, this reduction was not statistically significant compared to untreated controls, indicating that while NF- κ B may be involved, alternative signalling pathways likely play a more dominant role in SP-mediated IL-8 secretion. Pre-treatment with SB203580 (p38 inhibitor) reduced IL-8 levels from 571 to 67 pg/mL (**Fig. 3.19B**) and PD98059 (ERK inhibitor) reduced IL-8 levels from 571 to 221 pg/mL; however, these reductions were not statistically significant compared to untreated controls (**Fig. 3.19C**).

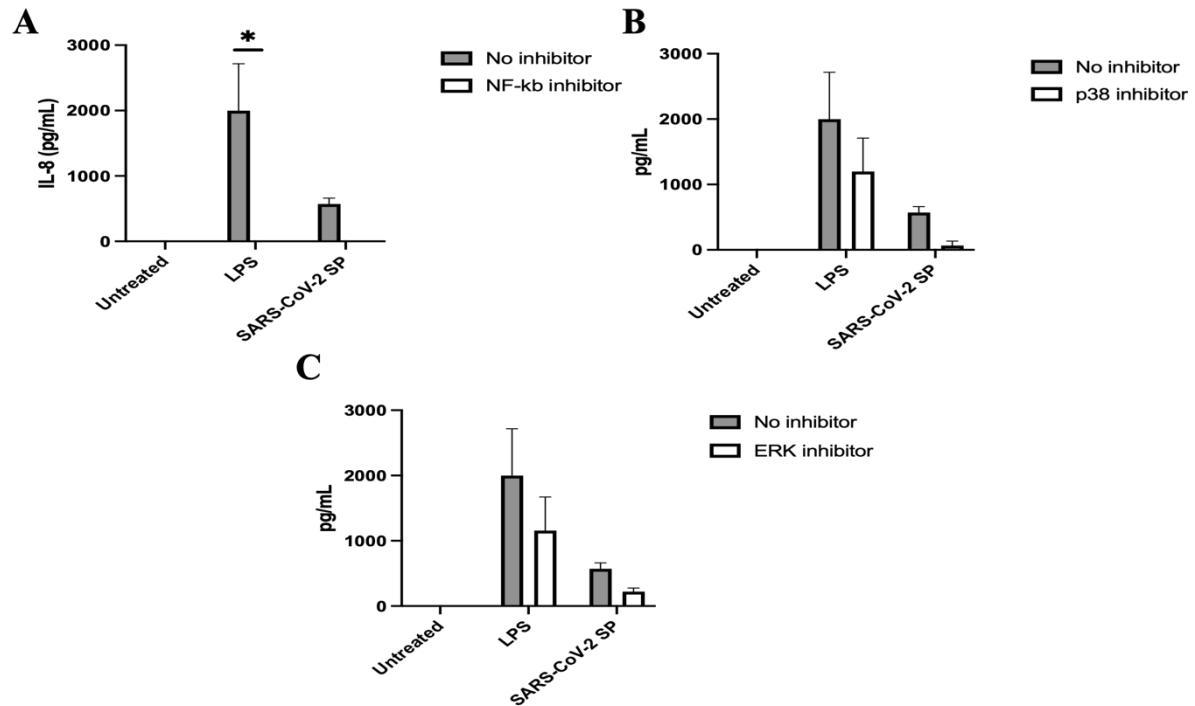


Figure 3.19 Effect of NF- κ B, p38 MAPK, and ERK1/2 Inhibition on IL-8 Production Induced by SARS-CoV-2 SP in Human Primary PMNs.

PMNs were obtained by density gradient isolation of healthy human blood ($n=3$). PMNs (1×10^6) were pre-treated with BAY 11-7082 (NF- κ B inhibitor), SB203580 (p38 MAPK inhibitor), or PD98059 (ERK1/2 inhibitor) before stimulation with SARS-CoV-2 SP (SP, $10 \mu\text{g/mL}$) or LPS (100 ng/mL) for 18 hours. IL-8 production was measured by ELISA. Data represent mean $\pm$ SEM from, with statistical analysis performed using two-way ANOVA.

3.4.19 TGF- β Induction by SARS-CoV-2 SP in Human Primary PMNs

As SARS-CoV-2 SP did not induce a strong proinflammatory response in PMNs, we next sought to determine whether it instead promotes an immunoregulatory or a pro-fibrotic response via TGF- β . TGF- β is a multifunctional cytokine that plays a critical role in immune regulation, tissue remodelling and fibrosis. It is also modulated during viral infections and plays a dual role as an immunosuppressive and pro-fibrotic factor in the host–pathogen interaction [265]. Certain viral proteins, such as those from influenza and Epstein-Barr virus, are known to influence TGF- β pathways to modulate immune responses [480]. The SARS-CoV-2 SP can trigger barrier dysfunction and vascular leak through interactions with glycosaminoglycans, integrins, and TGF- β signalling, independent of viral replication and ACE2 [481].

Some viral infections are known to alter TGF- β signalling and control of immune responses and disease progression through it. HIV-1 Tat protein increases TGF- β production in human articular chondrocytes, which may play a role in joint destruction [482]. LCMV chronic infection results in the increase of TGF- β production in DCs, which can help the virus to survive [483]. The HBx protein of HBV blocks TGF- β -induced apoptosis by the PI3K/Akt pathway, to evade immune elimination [484]. In the case of Hepatitis Delta Virus (HDV), the large antigen (LHDAg) boosts TGF- β signalling and leads to liver fibrosis [485]. Conversely, the Tax protein of HTLV-1 activates TGF- β 1 production but inhibits its signalling, which is leukemogenic [486]. However, TGF- β regulation by SARS-CoV-2 SP in neutrophils remains unknown. To evaluate whether SARS-CoV-2 SP induces TGF- β production, PMNs were treated with SP at 0, 1, 5, and 10 μ g/ml and TGF- β levels were analysed using qRT-PCR and ELISA. qRT-PCR, analysis of TGF- β mRNA levels at 0, 2, and 4 hours SP treatment demonstrated a significant upregulation at 4 hours post-treatment with 10 μ g/mL SP (Fig. 3.20A). Similarly ELISA, results showed a significant increase in TGF- β secretion at 10 μ g/mL after 18 hours (Fig. 3.20B). These findings suggest that SARS-CoV-2 SP induces TGF- β production significantly in human neutrophils, and especially at higher concentrations which may play a role in immune modulation and fibrosis in COVID-19 (Fig. 3.20).

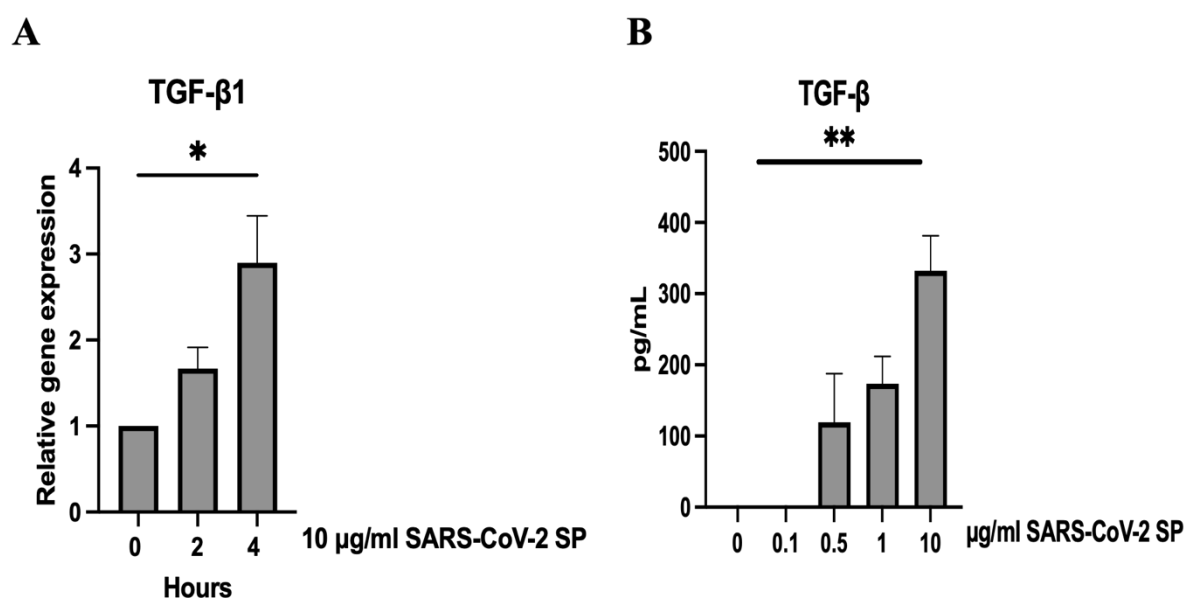


Figure 3.20 Induction of TGF- β by SARS-CoV-2 SP in Human Primary PMNs.

Human PMNs were obtained by density gradient isolation of healthy human blood (n=3).

(A) PMNs (1×10^6) were treated with SARS-CoV-2 SP at 10 μ g/mL for 0, 2, 4 hours; RNA was then extracted and analysed by qRT-PCR for TGF- β mRNA expression. (B) PMNs were

treated with SARS-CoV-2 SP at 0, 1, 5, and 10 $\mu\text{g/mL}$ for 18 hours, then supernatants were collected for ELISA quantification. Data represent mean $\pm$ SEM, with statistical analysis performed using one-way ANOVA. Statistical significance was determined as follows: (* $p < 0.05$ and ** $P < 0.01$)

3.4.20 SARS-CoV-2 SP Induces TGF- β Production in Human Primary PMNs via Integrin-Dependent Mechanisms

Having found that the SARS-CoV-2 SP induced TGF- β production in human primary PMNs, we next aimed to determine whether this occurred via integrin-dependent mechanisms. The role of the RGD motif in TGF- β activation remains unclear, although the SARS-CoV-2 SP is known to interact with integrins like $\alpha\text{v}\beta 3$ and $\alpha\text{v}\beta 6$. The aim of this study is to determine whether this TGF- β production is occurring via integrin-dependent mechanisms [295, 487]. To address this, our next steps include the inhibition of SARS-CoV-2 SP and integrins interactions, and the examination of their impact on the production of TGF- β .

To determine whether integrin-mediated pathways contribute to SARS-CoV-2 SP-induced TGF- β production, primary PMNs were pre-treated with 1 μM ATN-161, a selective RGD-binding integrin inhibitor, for 30 minutes, or left untreated, before stimulation with SARS-CoV-2 SP for 18 hours. ELISA results revealed that ATN-161 significantly reduced TGF- β secretion in PMNs treated with 10 $\mu\text{g/mL}$ SP. This indicated that SP-induced TGF- β production was integrin-dependent (Fig. 3.21).

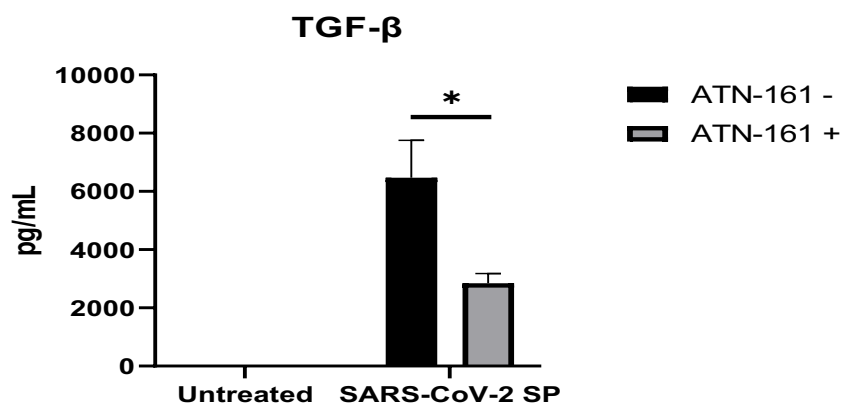


Figure 3.21 Integrin-Dependent Mechanism of SARS-CoV-2 SP-Induced TGF- β Production Human Primary PMNs.

Human PMNs were isolated from healthy donor blood using density gradient centrifugation ($n=3$). PMNs (1×10^6 cells/mL) were pre-treated with ATN-161 (1 μM), a selective RGD-binding integrin inhibitor, for 30 minutes or left untreated as controls, followed by

stimulation with SARS-CoV-2 SP for 18 hours. TGF- β levels in culture supernatants were measured by ELISA. Data represent mean $\pm$ SEM with statistical analysis performed using two-way ANOVA, with * $p < 0.05$ considered significant.

3.4.21 TGF- β does not induce the expression of Human Primary PMNs Activation Markers (CD66b, CD11b, CD16 and CD66b)

Our findings show that SARS-CoV-2 SP stimulates production of TGF- β by human primary PMNs. However, the role of TGF- β on PMN function is not well documented in the current literature. PMNs are of significance in the innate immune response and participate in pathogen eradication by means of the generation of ROS, NETosis, phagocytosis, and degranulation. Since TGF- β is a potent immunoregulatory cytokine, it is important to know its impact on these crucial neutrophil responses. Therefore, the purpose of our next series of experiments was to determine the effect of TGF- β on PMN functions and gain new insights into its possible role in the regulation of immune responses in SARS-CoV-2 infection and other inflammatory diseases.

Thus, we first investigated whether TGF- β directly affects PMNs activation marker expression. PMNs were treated with varying concentrations of recombinant TGF- β 1 (0, 5, 10, and 50 ng/mL) for 2 hours, after which the expression of key activation markers, including CD66b (**Fig. 3.22A**), CD11b (**Fig. 3.22B**), CD62L (**Fig. 3.22C**), and CD16 (**Fig. 3.22D**), were analysed by flow cytometry.

We found no significant changes in the expression of the activation markers at any of the concentrations tested. The lack of response suggests that TGF- β does not directly modulate neutrophil activation, as measured by these particular markers.

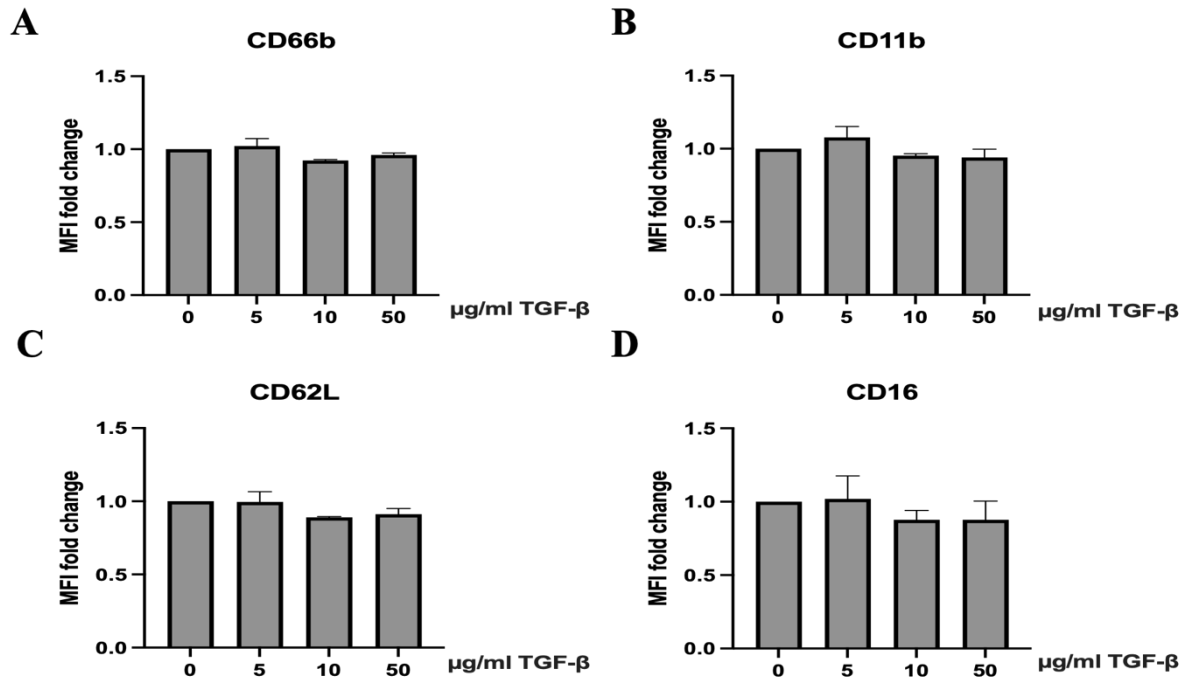


Figure 3.22 Assessment of TGF-β Effects on PMN Activation Marker Expression Human Primary PMNs.

Human PMNs were isolated from healthy donor blood using density gradient centrifugation ($n=3$). PMNs (1×10^6) were treated with recombinant human TGF-β1 at concentrations of 0, 5, 10, and 50 ng/mL for 2 hours at 37°C in a humidified 5% CO₂ atmosphere. Surface expression of neutrophil activation markers was analysed by flow cytometry: (A) CD66b, (B) CD11b, (C) CD62L, and (D) CD16. Flow cytometric analysis was performed using a standard gating strategy: cells were first gated based on FSC and SSC properties to exclude debris and select the PMNs population, followed by singlet discrimination using SSC-Area vs. SSC-Height, and finally gated for live cells using live/dead stain exclusion. Results are expressed as MFI fold change relative to untreated controls (normalized to 1.0). Data represent mean $\pm$ SEM from, with statistical analysis performed using one-way ANOVA.

3.4.22 TGF-β Does Not Modulate Basal or tBuOOH-Induced ROS Production in Human Primary PMNs

TGF-β and ROS exhibit a complex interplay in human cell lines. TGF-β increases ROS production primarily through NADPH oxidase 4 (Nox4), and decreases antioxidant systems in human endothelial cells [488-490]. Conversely, ROS can activate TGF-β and mediate its signalling effects [491]. However, the impact of TGF-β on ROS production in PMNs remains unclear. To address this, we conducted two separate experiments. In the first PMNs were treated with recombinant TGF-β at 0, 5, 10, and 50 ng/mL for 2 hours, and ROS levels

were measured by flow cytometry (**Fig. 3.23A**) . The results showed no significant changes in basal ROS levels at any of the tested concentrations, indicating that TGF- β does not directly regulate ROS generation in PMNs. In the second experiment, PMNs were pre-treated with TGF- β at the same concentrations for 2 hours, followed by stimulation with tBuOOH (10 nM), an organic peroxide used to induce reactive ROS and mimic oxidative stress, for an additional 2 hours, after which ROS levels were measured (**Fig. 3.23B**). Pre-treatment with TGF- β did not affect tBuOOH-induced ROS production, with no significant changes observed across all conditions.

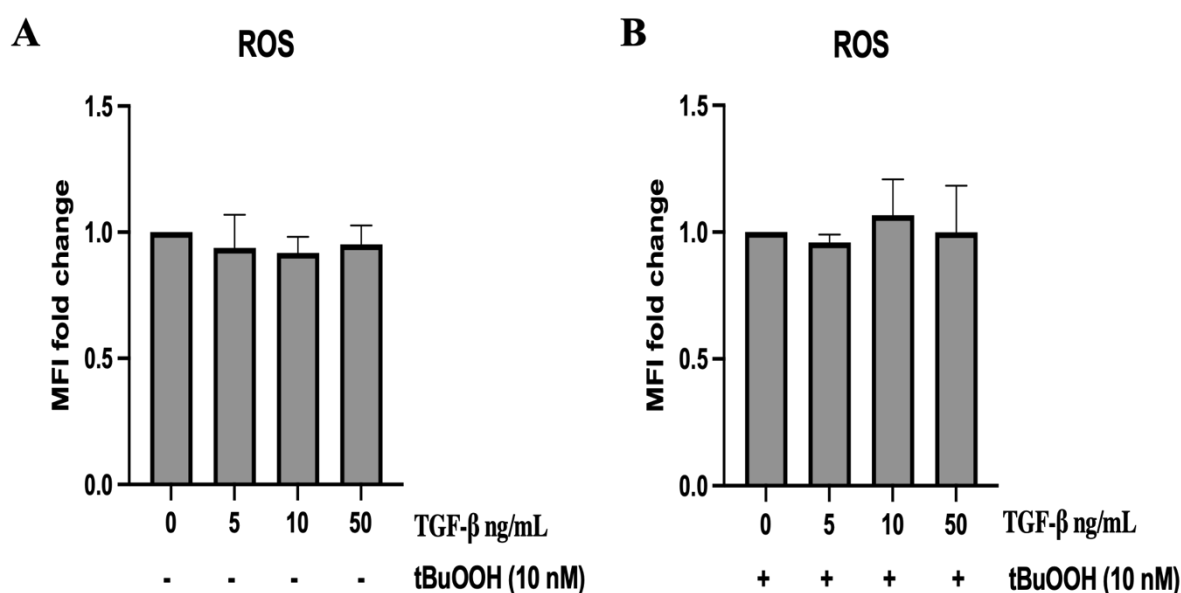


Figure 3.23 Effect of TGF- β on Basal and tBuOOH-Induced ROS Production in Human Primary PMNs.

Human PMNs were isolated from healthy donor blood using density gradient centrifugation (n=4). **(A)** Basal ROS production: Freshly isolated PMNs (1×10^6) were treated with recombinant human TGF- β 1 at concentrations of 0, 5, 10, and 50 ng/mL for 2 hours at 37°C in a humidified 5% CO₂ atmosphere. ROS levels were measured by flow cytometry using CellROX Green Reagent. **(B)** Stimulated ROS production: PMNs were pre-treated with TGF- β at the same concentrations for 2 hours, followed by stimulation with 10 μ M tBuOOH, a known ROS inducer, for an additional 2 hours. ROS levels were then measured by flow cytometry using the same CellROX Green Reagent. Flow cytometric analysis was performed using a standard gating strategy: cells were first gated based on FSC and SSC properties to exclude debris and select the PMNs population, followed by singlet

discrimination using SSC-Area vs. SSC-Height, and finally gated for live cells using live/dead stain exclusion. Results are expressed as MFI fold change relative to untreated controls (normalized to 1.0). Data represent mean $\pm$ SEM, with statistical analysis performed using one-way ANOVA.

3.4.23 TGF- β Does Not Modulate Basal nor PMA-Induced NETosis in Human Primary PMNs

TGF- β has a complex role in NET formation and associated pathologies and is involved in the regulation of their formation. TGF- β may induce NET formation in oral lichen planus and may be involved in the development of oral cancer [492]. In breast cancer lung metastasis, NETs are activated by chemotherapy induced TGF- β , which in turn promotes chemoresistance [493]. Also TGF- β 1 secreted by platelets enhances the formation of NETs and deep vein thrombosis [494]. Despite these findings, the direct role of TGF- β in PMNs NET formation remains unclear. To investigate this, we performed two distinct experiments using flow cytometry to assess Sytox Orange fluorescence, which specifically binds extracellular DNA. In the first experiment, PMNs were treated with recombinant TGF- β at 0, 5, 10, and 50 ng/mL for 4 hours to determine whether TGF- β alone could induce NETosis (**Fig. 3.24A**). The results showed no significant increase in Sytox Orange fluorescence across any of the TGF- β concentrations, indicating that TGF- β does not directly stimulate NET formation in resting PMNs. In the second experiment, we assessed whether TGF- β could modulate NETosis induced PMA (20 nM), a potent activator of neutrophils. PMNs were pre-treated with TGF- β at 0, 5, 10, and 50 ng/mL for 2 hours, followed by stimulation with PMA for an additional 4 hours. Flow cytometry analysis revealed no significant differences in Sytox Orange fluorescence between TGF- β pre-treated groups and controls, indicating that TGF- β did not affect PMA-induced NETosis (**Fig. 3.24B**).

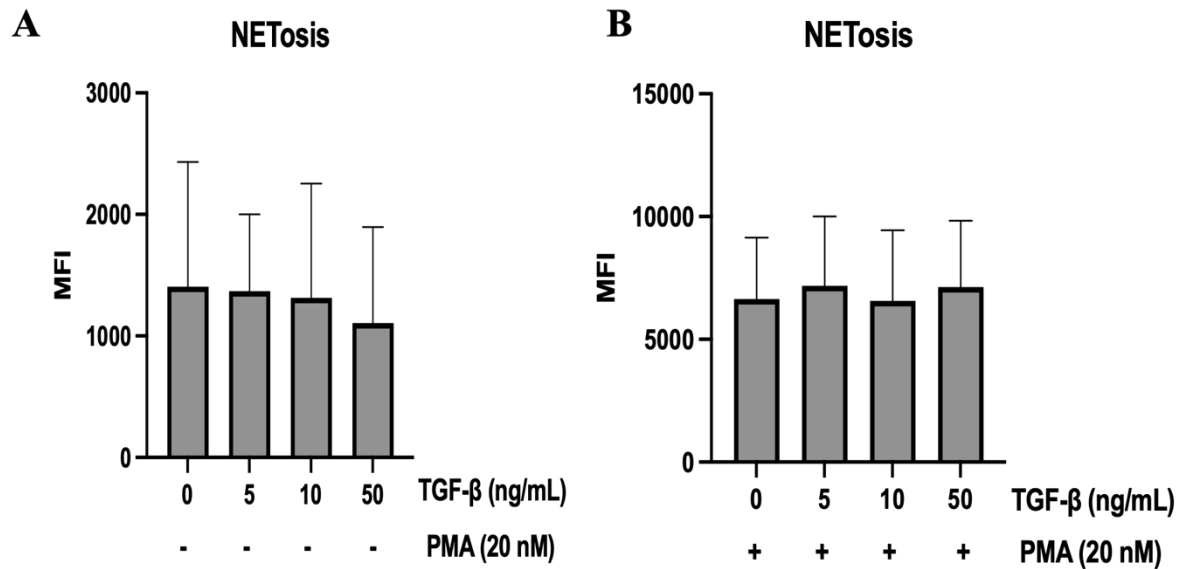


Figure 3.24 Effect of TGF- β on Basal and PMA-Induced NETosis in Neutrophils Assessed by Flow Cytometry.

Human PMNs were isolated from healthy donor blood using density gradient centrifugation (n=3). **(A)** Spontaneous NETosis: Freshly isolated PMNs (1×10^6) were treated with recombinant human TGF- β 1 at concentrations of 0, 5, 10, and 50 ng/mL for 4 hours at 37°C in a humidified 5% CO₂ atmosphere. NETosis was quantified by flow cytometry using SYTOX Orange nucleic acid stain, which selectively binds to extracellular DNA released during NET formation. **(B)** PMA-induced NETosis: PMNs were pre-treated with TGF- β 1 at the same concentrations for 2 hours, followed by stimulation with PM (20 nM) for an additional 4 hours. NET formation was analysed by flow cytometry using SYTOX Orange staining. Flow cytometric analysis employed the following gating strategy: FSC/SSC gating for neutrophil population selection, singlet discrimination, live/dead cell exclusion, CD66b⁺ gating to confirm neutrophil identity, and finally SYTOX Orange⁺ gating to identify cells undergoing NETosis. Results are expressed as MFI indicating the level of extracellular DNA release. Data represent mean $\pm$ SEM from, with statistical analysis performed using one-way ANOVA.

3.4.24 TGF- β Does Not Significantly Alter Human Primary PMNs Phagocytosis of GFP-Expressing *Staphylococcus aureus*

TGF- β plays a complex role in regulating phagocytosis and immune responses in human cells. In retinal pigment epithelium, TGF- β upregulates phagocytosis through a PKC-dependent pathway [495]. Also in myeloid cells, TGF- β suppresses phagocytosis by

reducing surface expression of Fc γ receptors and their associated γ -subunit [496]. TGF- β also promotes tumour-associated macrophage infiltration and enhances their phagocytic capacity [497]. Despite these insights, the direct effect of TGF- β on neutrophil phagocytosis remains poorly understood.

To investigate the potential role of TGF- β in PMNs phagocytosis, we conducted an experiment using flow cytometry to assess the uptake of *S. aureus* expressing GFP at a MOI of 1:100. PMNs were pre-treated with recombinant TGF- β at 0, 5, 10, and 50 ng/mL for 2 hours, followed by infection with SA-GFP for 1 hour. Phagocytosis was quantified as the MFI of GFP in PMNs, reflecting the amount of bacteria internalized.

The results demonstrated no significant changes in GFP MFI at 5 or 10 ng/mL TGF- β compared to the untreated control. However, at 50 ng/mL TGF- β there was a slight increase in GFP MFI (1.5-fold) compared to the untreated control, suggesting enhanced bacterial internalization, although this change did not reach statistical significance.

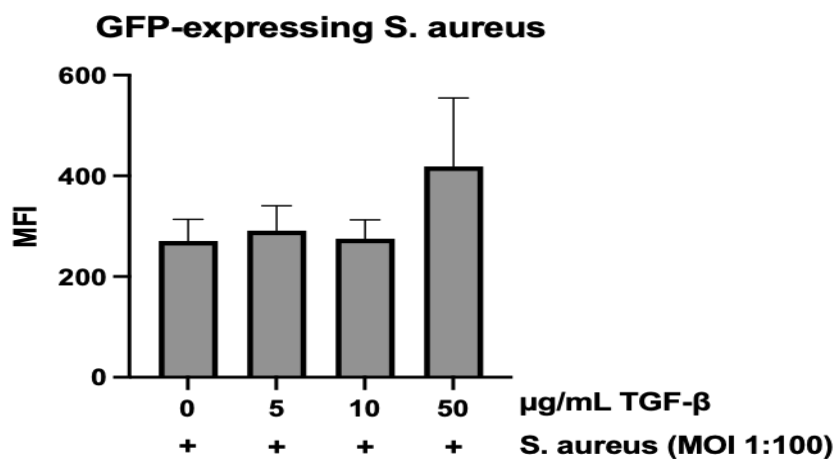


Figure 3.25 Effect of TGF- β on Neutrophil Phagocytosis of GFP-Expressing *S. aureus*.

Human PMNs were obtained by density gradient isolation of healthy human blood (n=3). PMNs (1×10^6) were pre-treated with recombinant TGF- β at 0, 5, 10, and 50 ng/mL for 2 hours, followed by infection with GFP-expressing *Staphylococcus aureus* (MOI 1:100) for 1 hour. Phagocytosis was assessed by flow cytometry and quantified as the MFI of GFP in PMNs, reflecting bacterial internalization. Flow cytometric analysis was performed using a standard gating strategy: cells were first gated based on FSC and SSC properties to exclude debris and select the PMNs population, followed by singlet discrimination using SSC-Area vs. SSC-Height, and finally gated for live cells using live/dead stain exclusion. Data represent mean $\pm$ SEM from, with statistical analysis performed using one-way ANOVA.

3.4.25 TGF- β does not effect MPO and NE Release in PMNs

TGF- β exhibits diverse effects on immune cell degranulation. In eosinophils, TGF- β 1 inhibits cytokine-enhanced survival and degranulation [498]. Similarly, TGF- β 1 suppresses human skin mast cell mediator release and Kit expression through a Smad-dependent pathway [499]. In neutrophils, TGF- β 1 significantly reduces lactoferrin secretion in response to various stimuli [430]. To investigate the role of TGF- β in regulating PMN degranulation, we measured the release of MPO, and NE, by ELISA in two distinct experiments. In the first experiment, PMNs were treated with recombinant TGF- β at 0, 5, 10, and 50 ng/mL for 2 hours to evaluate its effect on MPO and NE secretion. The results showed no significant change in MPO release with increasing TGF- β concentrations, indicating that TGF- β has minimal impact on MPO secretion from resting PMNs, while TGF- β treatment also showed no significant change in NE levels, suggesting no substantial effect on degranulation markers (**Fig. 3.26 A & B**). In the second experiment, we assessed the impact of TGF- β on degranulation in PMNs activated with PMA (20 nM). PMNs were pre-treated with TGF- β at 0, 5, 10, and 50 ng/mL for 2 hours, followed by stimulation with PMA for an additional 2 hours. The ELISA data showed no significant change in PMA-induced MPO release when PMNs were pre-incubated with TGF- β at different concentrations. Similarly, NE levels showed no significant change following TGF- β pre-treatment and PMA stimulation, indicating that TGF- β does not substantially affect degranulation of either primary or secondary granules in activated PMNs (**Fig. 3.26 C & D**).

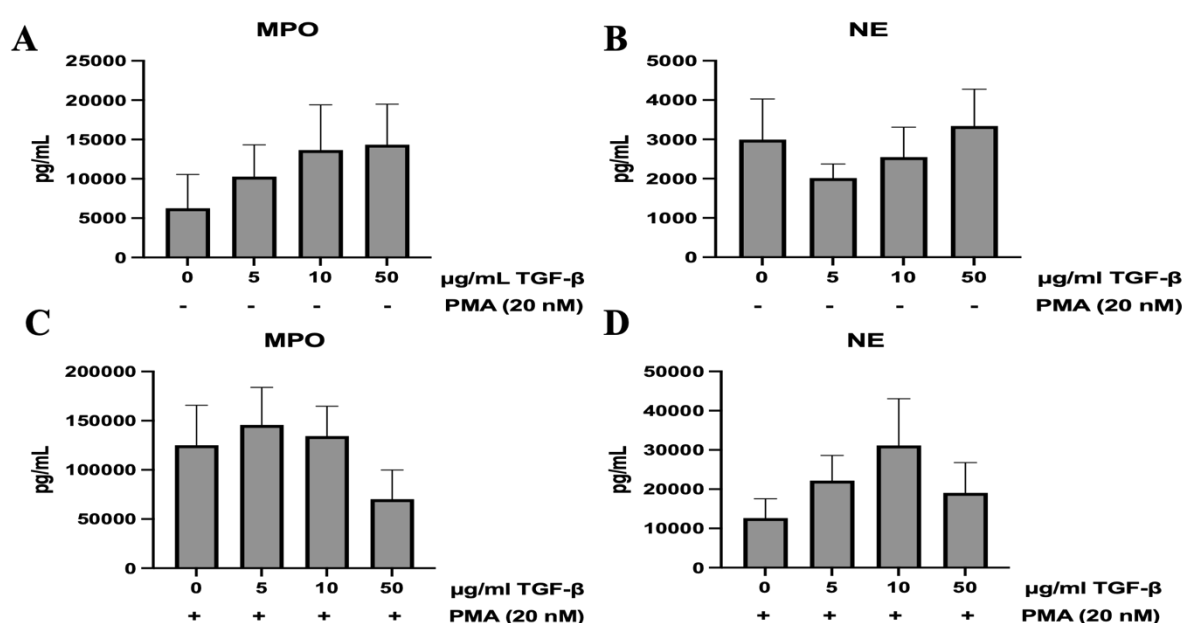


Figure 3.26: Effects of TGF- β on Human Primary PMN Degranulation.

Human PMNs were isolated from healthy donor blood using density gradient centrifugation (n=4). PMNs (1×10^6) were cultured under two experimental conditions to evaluate TGF- β effects on MPO and NE release. A and B: PMNs were treated with TGF- β (0, 5, 10, or 50 $\mu\text{g/mL}$) alone for 2 hours to assess baseline effects on MPO (A) and NE (B) release.

C and D: PMNs were pre-treated with TGF- β (0, 5, 10, or 50 $\mu\text{g/mL}$) for 2 hours, followed by PMA (20 nM) stimulation for an additional 2 hours to evaluate TGF- β modulation of stimulated MPO (C) and NE (D) release. Enzyme concentrations were measured in cell culture supernatants by ELISA. Data represent mean $\pm$ SEM. Statistical analysis was performed using one-way ANOVA

3.5 Discussion

The pronounced neutrophilia observed in COVID-19 patients suggests a role for these leukocytes in the pathology of the disease. Therefore, we aimed by this study to provides a comprehensive analysis of the effects of SARS-CoV-2 SP on neutrophil activation, immune responses, and functional dynamics. These findings are significant because they are explored across various aspects of neutrophil activity, such as surface marker expression, cytokine production, ROS generation, and phagocytosis, to understand how SARS-CoV-2 impacts neutrophil function. In addition, this research assists in the identification of potential pathways that may be involved in COVID-19 pathogenesis and extends our knowledge of virus-driven immune dysregulation.

3.5.1 Differential Expression of Neutrophil Activation Markers CD11b and CD66b in COVID-19 Disease Severity

In our analysis of Nanostring data from Hadjadj *et al.*, [434] , we gained further understanding of the differential expression of the key neutrophil activation markers, CD11b and CD66b, in COVID-19 patients. CD66b was elevated only in critically ill patients, while CD11b was increased across all disease severities. These findings align with previous studies identifying CD11b as a strong marker of neutrophil activation in various inflammatory diseases and thus support its role in the systemic immune response to SARS-CoV-2 infection [54]. The upregulation of CD66b in critically ill patients suggests that it may play a more significant role in later disease stages rather than in early infection. This aligns with previous research, which has established CD66b as a clear marker of neutrophil activation in bacterial infections [55].

3.5.2 Flow Cytometry Analysis of CD11b and CD66b Expression in COVID-19

To further explore neutrophil activation in COVID-19, we analysed CD11b and CD66b expression using flow cytometry in PMNs isolated from COVID-19 patients and healthy controls. Our results showed that CD11b expression was greatly increased in COVID-19 patients, while the level of CD66b expression remained similar between patients and controls. The observed increase in CD11b expression is consistent with our Nanostring data analysis, which also showed CD11b as a consistent marker of neutrophil activation in COVID-19 across different levels of disease severity. However, these findings contradict other studies. For example, *Karawajczyk et al.*, examined the expression of CD11b and CD66b on neutrophils and monocytes in severe COVID-19 patients admitted to the ICU. Flow cytometry analysis revealed increased CD66b expression in neutrophils, while CD11b remained unchanged. In monocytes, CD11b were upregulated [500]. Similarly, another study shows that CD11b expression was significantly reduced and CD66b expression remained unchanged in non-ICU COVID-19 patients [501]. Moreover, *Loyer et al.*, demonstrated the increased expression of CD11b in a cohort of COVID-19 patients treated in the ICU in comparison with CAP and healthy controls [442]. These inconsistencies across studies may reflect differences in disease severity, patient cohorts, or methodological variations in data collection and analysis. More studies are required to shed light on the functions of CD11b and CD66b in COVID-19 and to establish if they are good predictors of disease severity or progression.

3.5.3 SARS-CoV-2 SP Fails to Induce PMN Activation Markers

Despite pronounced neutrophil activation in COVID-19 patients, our experiments indicate that the SARS-CoV-2 SP alone does not directly trigger neutrophil activation in vitro. In severe COVID-19 cases, circulating neutrophils show increased expression of activation markers such as CD11b and CD66b, reflecting heightened activation [502]. In contrast, purified SARS-CoV-2 SP did not increase these surface markers on neutrophils in vitro. Spike protein stimulation also failed to induce activation-dependent shedding of CD16 or CD62L. The SP alone did not reproduce the activated neutrophil phenotype seen in vivo, indicating it is insufficient as a standalone activation stimulus. This discrepancy suggests that neutrophil hyperactivation in COVID-19 results mainly from indirect pathways rather than direct SP engagement. Severe SARS-CoV-2 infection produces an intense pro-inflammatory environment, with elevated levels of neutrophil-stimulating cytokines (e.g., IL-8, G-CSF) and other inflammatory mediators [503, 504]. These factors prime and activate neutrophils independently of direct viral binding.

3.5.4 SARS-CoV-2 SP Does Not Induce ROS Production in PMNs

Oxidative stress is a significant factor in COVID-19 severity, driving inflammation, cytokine storms, and tissue damage, while high ROS levels and iron overload further weaken immune function in critically ill patients [505]. Recent research suggests that the SARS-CoV-2 SP interacts with copper (Cu(II)) ions, leading to excessive ROS production and DNA damage. Experiments with SARS-CoV-2 SP-derived peptides (L1 and L2) showed they bind Cu(II), forming complexes that trigger mitochondrial ROS overproduction in A549 cells [506]. Also using mouse models and human lung cells, SARS-CoV-2 SP triggers ROS, DNA damage, TGF- β signalling, and cellular senescence [507]. However, our results show that SARS-CoV-2 SP did not trigger ROS production in neutrophils, suggesting that SP alone does not directly contribute to oxidative stress in neutrophils. One study reported no effect on ROS generation in neutrophils stimulated with the Wuhan S1 protein, which aligns with our experimental results [448]. They also analyzed neutrophil responses to the Omicron S1 protein. Their data showed that Omicron S1 enhances ROS production by neutrophils. The Omicron variant has distinct immunogenicity and antigenicity compared to the wild-type Wuhan strain, driven by multiple mutations in the RBD. These structural changes may alter receptor interactions or immune recognition, explaining its enhanced ability to induce ROS [508]. Recent studies show that immune complexes containing SARS-CoV-2 SP and anti-SP antibodies trigger neutrophil oxidative burst and degranulation, whereas SP alone produces minimal stimulation under the same conditions [509].

3.5.5 SARS-CoV-2 SP Alone Does Not Induce NETosis

Studies have demonstrated that SARS-CoV-2 SP can activate the process of neutrophil NETosis. This process is mediated by increased ROS production and involves spleen tyrosine kinase (SYK) signalling [448]. SARS-CoV-2 SP and its immune complexes stimulate neutrophils to produce NETs, which R406, a SYK inhibitor, can inhibit [510]. While studies have shown that SARS-CoV-2 SP and its immune complexes induce NETosis formation in neutrophils, our experimental conditions showed a substantial decrease in NET formation when neutrophils were exposed to SARS-CoV-2 SP alone. This discrepancy might be explained by several key methodological and experimental differences. First, the referenced study used SP-specific immune complexes created by combining SP-trimer with SARS-CoV-2 patient plasma, whereas our experiments utilized purified SARS-CoV-2 SP alone without immune complex formation. Second, methodological differences in NET detection could contribute to varying results - our study utilized SYTOX Orange staining for NET quantification, while the referenced study employed live cell imaging (Incucyte S3) to visualize and quantify NETs in real-time. Third, the presence of patient-derived antibodies

forming immune complexes likely triggers Fc receptor-mediated activation pathways that are absent when using purified SP alone. Fourth, the inflammatory potential of the SARS-CoV-2 SP preparation itself may be crucial - *Cinquegrani et al.*, demonstrated that LPS-free, glycosylated SARS-CoV-2 SP has no inflammation-stimulating capacity in human primary macrophages, suggesting that the purified form of the protein does not appear to activate innate immune responses on its own [511].

3.5.6 SARS-CoV-2 SP Does Not Affect PMN Phagocytic Activity.

The SP of SARS-CoV-2 is known to have an impact on phagocytic activity during viral infection. It stimulates human lung macrophages to secrete cytokines and enhances phagocytosis [512]. Also, SARS-CoV-2 SP-specific antibodies can either enhance or reduce SP-bead phagocytosis by monocytes, suggesting non-neutralising antibodies could confer protection against SARS-CoV-2 infection [457]. Our results suggest that SARS-CoV-2 SP does not affect neutrophil phagocytosis, as neutrophils pretreated with 10 µg/mL SP showed no significant difference in *S. aureus* uptake compared to controls, as measured by flow cytometry.

3.5.7 SARS-CoV-2 SP Selectively Induces IL-8 Production in PMNs via TLR2/TLR4-NF-κB Signalling

Regarding the role of SARS-CoV-2 SP in inducing pro-inflammatory cytokines, one study found that the SARS-CoV-2 SP induces inflammatory cytokines and chemokines, including IL-6, IL-1β, TNF-α, CXCL1, CXCL2, and CCL2, in human and mouse macrophages, while other structural proteins (M, E, N) do not. Lung epithelial cells also produce cytokines in response to extracellular SP but remain non-inflammatory when expressing SP intracellularly. The inflammatory response is mediated through NF-κB activation via a MyD88-dependent pathway and requires TLR2, as inflammation is absent in TLR2-deficient macrophages and mice [409]. Another study found that the SARS-CoV-2 SP S1 subunit (S1) induces pro-inflammatory responses in murine and human macrophages. S1 activates NF-κB, and selective inhibitors of these pathways suppress JNK signalling pathways and cytokine induction. Additionally, blocking TLR4 with antagonists or siRNA significantly reduces S1-induced inflammation, while TLR2 neutralisation has no effect. These findings suggest that while the full-length S protein activates inflammation through

TLR2 and TLR4, the S1 subunit specifically triggers inflammatory signalling via TLR4 [513].

In our study, we explore the impact of the SARS-CoV-2 SP on neutrophil cytokine production. We observed that SARS-CoV-2 SP stimulates IL-8 production in neutrophils specifically, and we did not observe an induction for other proinflammatory cytokines like IL-6, TNF- α , and IL-1 β . This induction of IL-8 depended on both TLR2 and TLR4 but was independent of MAPK signalling, relying primarily on NF- κ B. This suggests SP enhances neutrophil migration without broadly activating inflammation.

3.5.8 SARS-CoV-2 SP Induces TGF- β Activation in PMNs Through Integrin-Dependent Mechanisms

TGF- β is a key player in tissue remodelling and fibrosis, with prior studies linking its upregulation to the development of long-term complications such as pulmonary fibrosis in SARS-CoV-2 infection [514]. The SP of SARS-CoV-2 activates TGF- β signalling via multiple pathways through interactions with ACE2 and integrins, thereby contributing to COVID-19 pathogenesis [481]. This activation results in barrier dysfunction, extracellular matrix reorganization, and fibrosis in various organs [515, 516]. SARS-CoV-2 SP has an RGD (Arg-Gly-Asp) motif in its RBD, which enables it to interact with integrins α 5 β 1 and other RGD-binding integrins. Integrins are known to control the activation of TGF- β by interacting with the latent TGF- β complex. Also, blocking integrins (with ATN-161) reduced TGF- β activation [481]. However, our results showed that SARS-CoV-2 SP induces TGF- β activation in neutrophils in a dose-dependent manner, with a significant increase at 10 μ g/mL. Pre-treatment with ATN-161 reduced TGF- β secretion, confirming an integrin-dependent mechanism. These findings suggest that SP may contribute to immune modulation and fibrosis in COVID-19 through integrin-mediated TGF- β activation.

3.5.9 SARS-CoV-2 SP Inhibits PMNs Apoptosis and Promotes Prolonged Inflammatory Responses

A particularly notable observation is the SARS-CoV-2 SP's ability to inhibit neutrophil apoptosis, thereby prolonging cellular survival. Apoptosis is a crucial regulatory mechanism that maintains immune homeostasis and prevents excessive inflammation by removing aged or activated neutrophils [517]. In our study, the SARS-CoV-2 SP significantly reduced neutrophil apoptosis in a dose-dependent manner, particularly at higher concentrations. This

observation aligns with previous research demonstrating how other viruses employ similar strategies to evade immune clearance. For instance, the core protein of hepatitis HCV inhibits apoptosis by activating the NF- κ B pathway, sustaining the expression of anti-apoptotic factors [518]. Similarly, HIV-1 promotes the survival of infected macrophages by upregulating anti-apoptotic proteins such as Bcl-2 and Bcl-xL, leading to chronic immune activation [519]. These parallels suggest that SARS-CoV-2 may have evolved similar strategies to suppress apoptotic pathways in immune cells, particularly neutrophils, to promote inflammation and enhance viral survival. The delayed apoptosis observed in neutrophils treated with SARS-CoV-2 SP may also contribute to neutrophil NETs formation, which is implicated in severe COVID-19. Although protective during early infection, if dysregulated, extended neutrophil longevity increases the risk of NETosis, which is involved in the development of thromboinflammation, tissue injury, and fibrosis [404]. Additionally, the extended survival of neutrophils may also facilitate the enhanced release of cytokines and chemokines, sustaining inflammatory responses and creating a positive feedback loop that continues to drive tissue destruction. This is consistent with previous findings showing that the delayed apoptosis of neutrophils is associated with increased levels of inflammatory cytokines like IL-8 [520], both of which were robustly induced by SP in this study.

3.5.10 TGF- β Has Minimal Direct Effects on PMNs Activation and Effector Functions.

The effects of TGF- β on neutrophils, as observed in this study, stand in contrast to its more prominent roles in other immune cells. For instance, TGF- β is a key regulator of T cell function, promoting the development of regulatory T cells (Tregs) while suppressing effector T cell responses, thus maintaining immune tolerance and controlling inflammation [521]. Similarly, in macrophages, TGF- β encourages a shift toward the anti-inflammatory M2 phenotype, supporting tissue repair but potentially limiting adequate pathogen clearance [522]. This cytokine works in DCs by impairing their maturation and the presentation of antigens, leading to inefficient initiation of adaptive immune responses [523]. The reported effects of TGF- β on neutrophils have so far been minor in *in vitro* tests under conditions allowing for very low activities of such events as degranulation or phagocytosis. A study revealed that TGF- β 1 triggers SMAD3 and ERK1/2 signalling in neutrophils, influencing gene expression to enhance pro-tumour factors such as oncostatin M and VEGFA. Despite these effects, it does not drive neutrophil migration, pointing to its indirect role in supporting tumour progression within the microenvironment [433]. Another paper showed that TGF- β 1 inhibits neutrophil degranulation by reducing lactoferrin secretion in response to various

stimuli through the ALK5 receptor, without affecting chemotaxis [524]. However, our data show that TGF- β treatment does not modify neutrophil activation markers, degranulation, ROS production or NETosis. We noticed a slight increase in bacterial phagocytosis at higher concentrations.

3.6 Future Directions

This study generates future research questions and opportunities for future work:

Mechanistic Insights: The mechanisms that underlie SP-mediated inhibition of apoptosis and selective production of cytokines need to be further clarified. Transcriptomic and proteomic studies may reveal the key regulators.

Therapeutic Potential: Given the involvement of TLRs and integrins in SP-induced responses, targeted inhibitors could be explored as therapeutic strategies to mitigate neutrophil-driven pathology in severe COVID-19.

Neutrophil Heterogeneity: Single-cell analyses could uncover functional subsets of neutrophils with distinct responses to SP, which allowed a more nuanced understanding of their role in COVID-19.

Comparative Studies: examining SP effects in relation to other viral proteins may reveal conserved or unique strategies that SARS-CoV-2 is using to evade and modulate host immunity.

Elucidate Pathways: Investigate the signalling pathways involved in delayed apoptosis, focusing on anti-apoptotic regulators like Bcl-2, NF- κ B, and PI3K/Akt. Inhibitor studies may reveal therapeutic targets for mitigating prolonged neutrophil survival.

Explore Viral Mimicry: Compare SARS-CoV-2 SP with other viral proteins that inhibit apoptosis, such as CMV and HCV proteins, to identify conserved strategies and pathways.

Evaluate Functional Consequences: Assess whether prolonged neutrophil survival enhances secondary immune functions, such as sustained NETosis, cytokine release, or immune complex formation.

Therapeutic Modulation: Investigate whether agents targeting apoptotic pathways can reverse neutrophil dysregulation without compromising host defence mechanisms.

3.7 Conclusion

The SARS-CoV-2 SP selectively modulates PMN functions, inhibiting apoptosis and inducing IL-8 production without robustly activating classical markers or functional responses like ROS generation, degranulation, or NETosis. These findings highlight SP's unique immune-modulatory properties, which may contribute to the dysregulated

inflammatory responses observed in COVID-19. By comparing the effects observed in neutrophil behaviour with other known viruses, this study sets the stage to appreciate the broader impacts of neutrophil dynamics in viral diseases while highlighting the prospect for targeting potential therapeutic approaches.

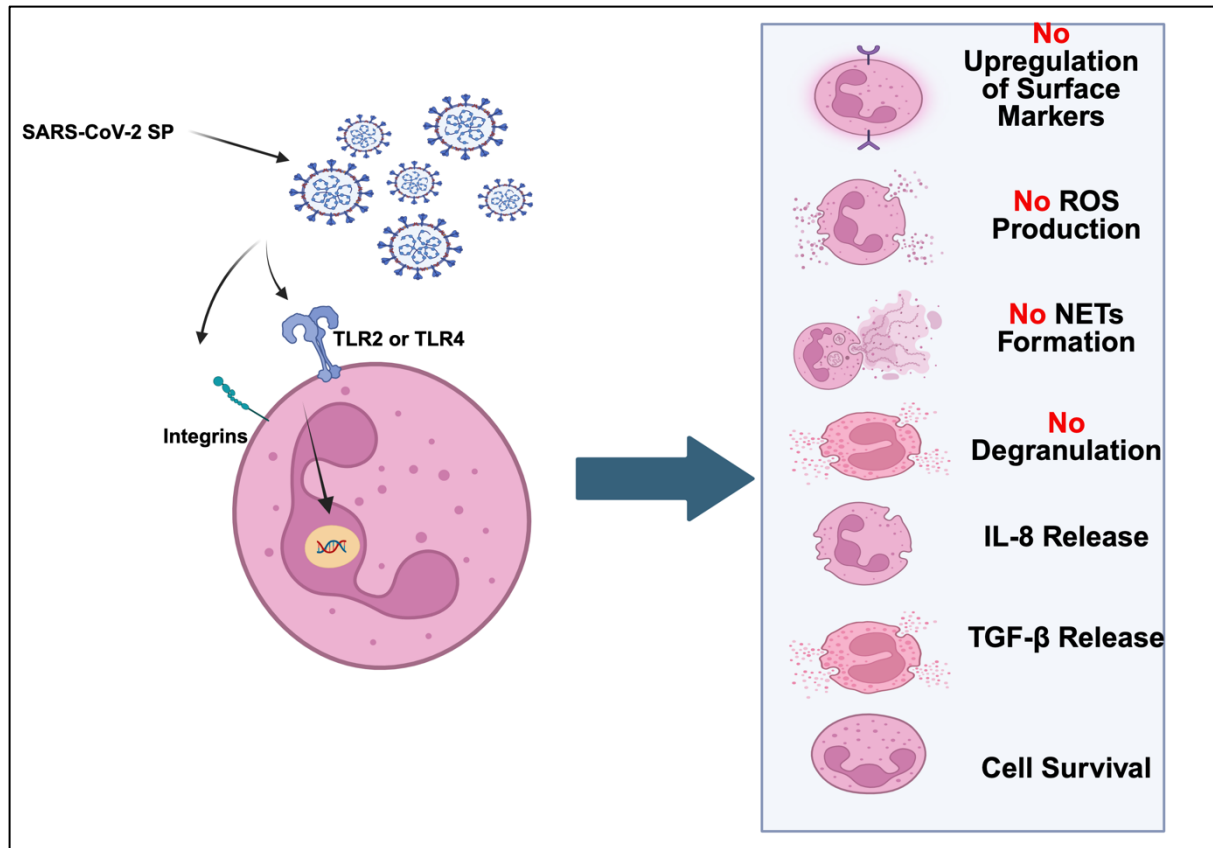


Figure 3.27: Selective Modulation of PMN Functions by SARS-CoV-2 SP.

SARS-CoV-2 SP selectively modulates PMN functions through distinct receptor-mediated signalling pathways. Following SP exposure, PMN exhibit a unique functional profile characterized by the absence of classical activation responses including upregulation of surface markers, ROS production, NETs formation, and degranulation. In contrast, SP treatment significantly enhances IL-8 release through TLR2 and TLR4 and TGF-β release through integrin-mediated signalling, while promoting cell survival through apoptosis inhibition.

Chapter 4:

TLR8 Recognition of SARS-CoV-2-Associated ssRNAs and Its Role in Inflammatory Responses in Human PMNs

4.1 Introduction

SARS-CoV-2 is a positive-sense single-stranded RNA virus. Severe COVID-19 is characterized by excessive pro-inflammatory cytokine secretion that drives immune-mediated pathology [525, 526]. Neutrophils accumulate in affected tissues, releasing proteolytic enzymes, ROS, and NETs that worsen lung injury and promote microthrombosis [527]. The innate immune system detects viral RNA through pattern recognition receptors, triggering immune responses. Type I IFN production provides antiviral protection [528], but impaired IFN responses worsen disease outcomes [529, 530]. Understanding how SARS-CoV-2 RNA activates neutrophils through TLR8 signaling is critical for elucidating inflammatory mechanisms in severe COVID-19. TLRs are essential components of the immune system; they recognise viral components and trigger the immune responses [531]. TLR7 and TLR8, located in endosomes, recognise GU-rich ssRNA and activate signalling through MyD88-dependent pathways. These pathways induce the production of various inflammatory cytokines and type I IFN, which are crucial components of the antiviral response mechanisms. [532]. Studies have demonstrated that SARS-CoV-2-associated ssRNAs can directly stimulate TLR7/8, thereby initiating a cascade of immune responses. This activation signals through the MyD88 pathway to induce the production of IFN-I and proinflammatory cytokines. [533]. Although these responses are necessary for controlling viral replication, they may exacerbate the over-inflammation observed in severe COVID-19. This chapter will examine the specific effects of SARS-CoV-2 ssRNAs on primary human PMNs and TLR7/8-mediated activation and inflammatory responses. By investigating these pathways, the research aims uncover the mechanism of the immune response to COVID-19 and may even identify a explanation for the excessive inflammation often observed during SARS-CoV-2 infections.

4.1.1 Mechanisms of TLR7/8 Activation by ssRNAs

TLR7 and TLR8 are endosomal PRRs that play a crucial role in recognising ssRNA viruses, including SARS-CoV-2. These receptors are located within endosomes to detect viral RNA released during the endocytosis of infected cells or taken up with free virions. TLR7 and TLR8 sense ssRNA oligonucleotides that contain guanosine- and uridine-rich sequences from RNA viruses. TLR7 agonists activate pDCs and B cells, preferentially producing type I IFN and IFN-regulated cytokines. TLR8 is closely related to TLR7, both phylogenetically and functionally, and it recognises ssRNA. Agonists specific to TLR8 activate myeloid DCs, monocytes, and monocyte-derived DCs, primarily leading to the production of pro-inflammatory cytokines and chemokines such as TNF- α , IL-12, and MIP-1 α . [534, 535].

However, upon recognising ssRNA from viruses, these receptors undergo conformational changes that enable dimerization of their ectodomains. This structural rearrangement facilitates the recruitment of the adaptor protein MyD88 to the TIR domain. This ligand-induced activation is crucial for the initiation of downstream signalling cascades, thus making TLR7/8 essential sensors of viral threats infections [532].

4.1.2 Role of MyD88 Signalling in Cytokine Production and Immune Activation

The signalling pathways downstream of TLR7/8 are activated through the MyD88 adaptor protein, which is a central component. Following ligand binding and receptor dimerization, MyD88 interacts with the TIR domain of the receptors and triggers the formation of a signalosome complex. It also recruits IRAKs, including IRAK1 and IRAK4, which activate downstream kinases such as TRAF6. This cascade results in the activation of transcription factors like NF- κ B and IRFs, including IRF7. These transcription factors stimulate the production of proinflammatory cytokines, including TNF- α , IL-6, and IFN- α . These cytokines and IFN are crucial for antiviral defence, promoting immune cell recruitment, antiviral gene expression, and coordination of the adaptive immune response responses [536] (Fig. 4.1). In summary, the TLR7/8-dependent recognition of ssRNAs and the consequent MyD88-dependent signalling pathways are fundamental to the innate immune response against RNA viruses. These processes facilitate rapid immune activation while maintaining a balance between antiviral defence and pathological inflammation.

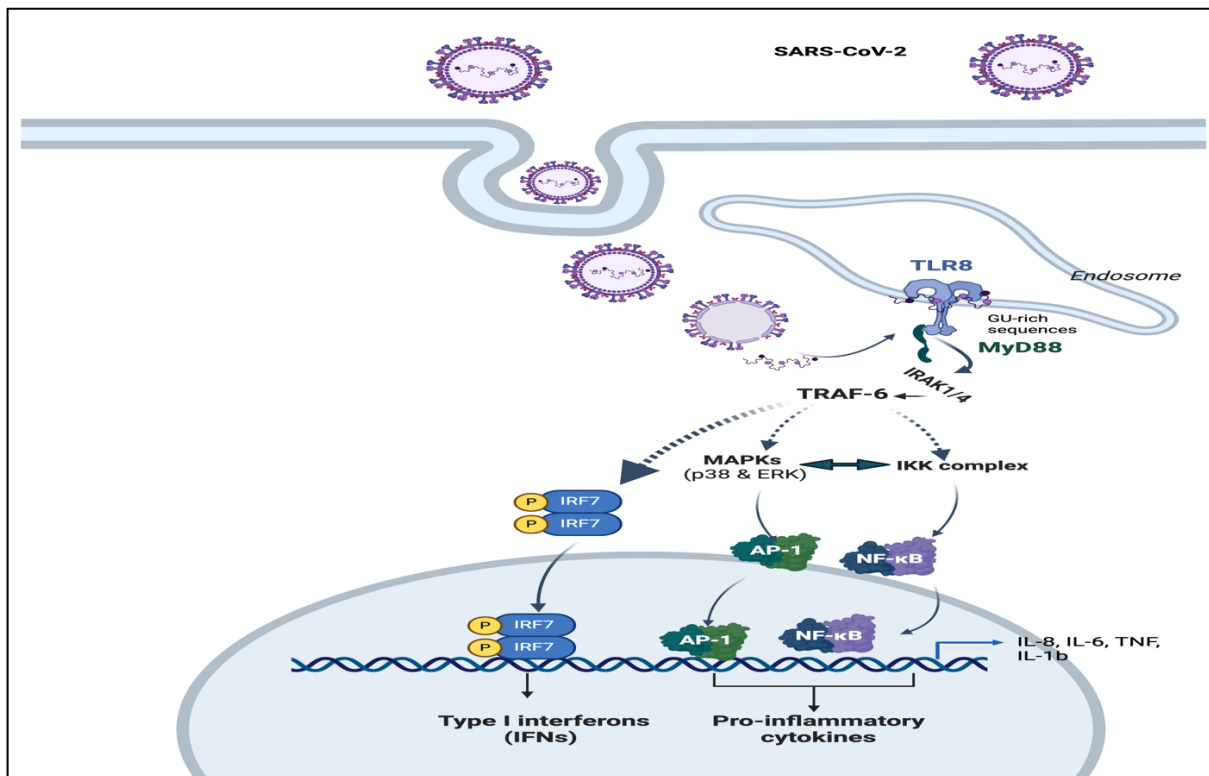


Figure 4. 1 SARS-CoV-2 Detection by TLR8 and Induction of Immune Responses.

This diagram illustrates how TLR8 recognises guanosine- and uridine-rich (GU)-rich single-stranded RNA sequences from SARS-CoV-2 within endosomes. Upon recognition, TLR8 interacts with MyD88, launching a signalling pathway that involves IRAK1/4 and TRAF6. This initiates MAPKs (p38 and ERK) and the IKK complex, leading to the activation of transcription factors AP-1 and NF-κB. These transcription factors subsequently induce cytokines such as IL-8, IL-6, TNF-α, and IL-1β. Simultaneously, IRF7 is phosphorylated, resulting in the expression of Type IFNs. These two processes form a significant part of the innate immune response against viral diseases, including SARS-CoV-2.

4.1.3 TLR7/8 in viral infections

Several reports have emerged highlighting the significance of TLR7 and TLR8 in viral infections. For instance, a study revealed that certain GU-rich sequences in the HCV genome can activate APCs via TLR7/8, leading to the production of pro-inflammatory cytokines. However, while HCV particles can stimulate macrophages in a TLR7/8-dependent manner, this interaction fails to induce IFN responses, thus evading immunity [537]. HCV has discovered to have developed methods for evading the TLR7/8 mediated immune responses that result in chronic infection [538]. Variations in TLR7/8 genes are linked to susceptibility to HCV infection and treatment outcomes [84]. Moreover, HCV can diminish the expression and functionality of TLR7 in hepatocytes, thereby aiding the persistence of viral particles

[539]. However, the response to viral infection following TLR8 activation can be dependent on the type of cell that is infected. For instance, HIV-1 triggers TLR8 signal transduction upon its internalisation by human primary CD4⁺ T cells. This response induces a cytokine reaction that facilitates viral replication and the reactivation of latent HIV-1 through pro-inflammatory stimuli [540]. Additionally, TLR7/8 activation in monocytes and lymphoid tissue is known to suppress HIV replication, potentially slowing disease progression. TLR8 activation consistently inhibited HIV replication in both PBMCs and tonsillar cells, while TLR7 was only effective in tonsillar cells. DCs facilitate NK and CD8⁺ T-cell-mediated HIV suppression. Moreover, the role of soluble factors is also highly significant, as direct cell contact is unnecessary for involvement [541]. Nevertheless, in the case of HIV, the protein SNAPIN inhibits the colocalization of TLR8 with HIV [81]. Moreover, TLR8 expression has been shown to rise approximately 2 to 6 hours after infection with human parechovirus 1 (HPEV-1) and influenza virus (H5N1) [542, 543]. Another example of viral activation of TLR8 is provided by the Coxsackie B virus (CBV), which primarily stimulated an inflammatory response through TLR8 and, to a limited extent, through TLR7 [544].

4.1.4 Structural and Functional Properties of SARS-CoV-2 ssRNAs

SARS-CoV-2 is a positive-sense ssRNA virus with a ~30 kb genome encoding structural proteins (S, E, M, N) and non-structural proteins involved in replication and immune evasion [545]. The SARS-CoV-2 ssRNA genome features GU-rich regions and secondary structures that are highly immunostimulatory. When these sequences are exposed to host immune cells during viral replication or endosomal processing, they function as PAMPs. The GU-rich regions and specific secondary structures of SARS-CoV-2 ssRNA serve as ligands for TLR7/8 [533]. Through a bioinformatics analysis, this study aimed to identify ssRNA motifs in the genomes of SARS-CoV-2, SARS-CoV, and MERS-CoV that are recognised by TLR7/8. The researchers reported that SARS-CoV-2 possesses more TLR7/8-binding ssRNA fragments than SARS-CoV, suggesting that it may elicit a stronger innate immune response. Such hyperactivation of signalling may be accompanied by excessive inflammation and could contribute to immune pathology, including acute lung injury [546]. Sequence-specific SARS-CoV-2 ssRNAs have been experimentally validated as activators of TLR7/8, resulting in the induction of cytokine release from human macrophages and microglia [547]. Wang *et al.*, examined the effect of TLR7 and IRF7 deficiencies on severity of COVID-19 in mice and revealed that these deficiencies lead to reduced IFN activation, higher viral loads, and more severe lung injury. Their study demonstrated that SARS-CoV-2 infection upregulates TLR7, IRF7 and IFN pathways in the lungs, but TLR7 or IRF7

knockout mice have attenuated innate immune responses and delayed antibody production, which in turn hampers viral clearance [90]. However, the dysregulation of TLR7/8 activation can lead to severe inflammation and the pathogenesis of COVID-19, emphasising their dual role in immunity[548]. In their study, Arefinia *et al.*, quantified TLR3, TLR7 and TLR8 gene expression in COVID-19 patients and found that the levels were significantly higher in the severe and critical cases as compared to the mild cases and healthy controls (p value < 0.001). Moreover, TLR8 was found to be significantly increased in male patients with severe and critical disease (p value = 0.008, p value = 0.02), which suggests a role in the gender-based severity differences [549]. Gómez-Carballea *et al.*, aimed to explore the expression of TLR7 during severe COVID-19 cases and found that TLR7 is expressed at lower levels in men than in women, especially in the most critical and ICU settings. Using a transcriptomic and epigenomic analysis, they found that males have increased methylation at the CpG sites near TLR7 and this may result in reduced expression of TLR7 and hence a reduced immune response. This work suggests that the escape of TLR7 from X-chromosome inactivation in females results in a stronger antiviral response, which may account for the greater severity and death rate of COVID-19 in males [550].

4.1.5 The Role of TLR7 and TLR8 in Neutrophil Activation and Function

Human studies have established that neutrophils lack TLR7, as both the mRNA and the protein for this receptor are absent in these cells. This absence also emphasizes a crucial distinction between neutrophils and other immune cells, such as pDCs and B cells, which possess TLR7 to recognise viral RNA and trigger immune responses [551, 552]. This research has demonstrated that TLR8 is expressed in neutrophils, employing qRT-PCR to detect TLR8 mRNA and flow cytometry to assess TLR8 protein expression. Flow cytometry analysis further revealed that stimulation with synthetic TLR8 agonists enhanced the expression of activation markers CD11b and CD62L shedding and induced neutrophil degranulation and respiratory burst. Functional assays established that TLR8 activation led to the production of proinflammatory cytokines, TNF- α and IL-8, which were quantified using ELISA. These findings suggest that TLR8 is not only present but also active in neutrophils, regulating degranulation, respiratory burst, and cytokine production [551]. Researchers have also demonstrated that TLR8, but not TLR7, plays a critical role in priming the activation of NADPH oxidase in human neutrophils. It has been shown that stimulation of TLR8 by its ligands triggers signalling pathways that lead to the accumulation of neutrophils for oxidative burst, a major antibacterial process. This priming effect enhances the production of ROS upon subsequent stimulation, thereby enabling neutrophils to combat

pathogens more effectively. In contrast, TLR7 lacks this priming activity in neutrophils, suggesting a difference in the functions of these two receptors in innate immunity. This finding establishes the specific importance of TLR8 in enhancing the NADPH oxidase activation of neutrophils for antimicrobial activity function [552]. Another study also indicated that TLR8 activation stimulates neutrophils to produce NETs, rather than engage in phagocytosis by shedding FcγRIIA through the furin-dependent pathway. When TLR8 is activated, furin activity increases, leading to the cleavage of FcγRIIA and consequently reducing the neutrophils' phagocytic capacity. Furthermore, TLR8 signalling enhances the formation of NETs. This shift in neutrophil function highlights the role of TLR8 in altering the regulation of these functions during the immune response [553]. Furthermore, research has demonstrated that IFNα amplifies the production of IL-6 in human neutrophils activated via TLR8. When TLR8 is engaged by its ligands, it initiates the production of pro-inflammatory cytokines, including IL-6. The presence of IFNα further enhances this response by upregulating the signalling pathways linked to TLR8 activation. This synergistic effect leads to significantly higher IL-6 production, underscoring the interplay between IFN-I and TLR8 in modulating neutrophil-mediated inflammation. This mechanism is particularly pertinent in inflammatory conditions, where IFNα and TLR8 ligands are present in abundance, thereby resulting in an enhanced immune response [554]. All these researches make TLR8 the primary sensor by which neutrophils can recognize SARS-CoV-2 RNA.

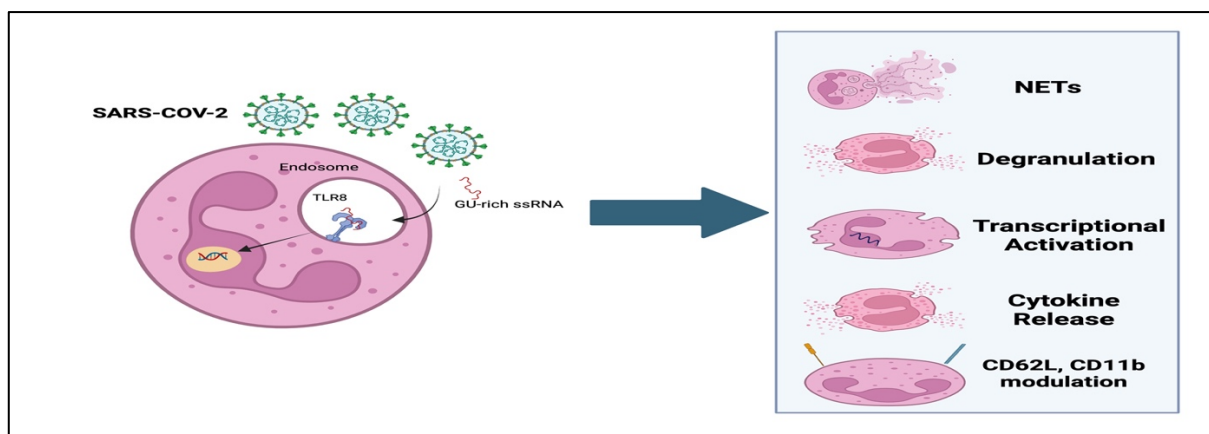


Figure 4. 2 TLR8 Activation in Neutrophils by SARS-CoV-2 ssRNA and Associated Cellular Responses

This illustration shows the activation of neutrophils upon recognising GU-rich ssRNA from SARS-CoV-2 via endosomal TLR8. The subsequent responses include the release of NETs,

degranulation, transcriptional activation, cytokine release, and modulation of surface markers such as CD62L and CD11b, all contributing to the immune response.

4.1.6 Neutrophil Apoptosis and Lifespan

The lifespan of neutrophils is tightly regulated by apoptosis (programmed cell death), which ensures that these potent inflammatory cells are removed once their job is done. Normally neutrophils survive only ~8–20 h in the blood and then undergo spontaneous apoptosis [555]. Neutrophil apoptosis is tightly controlled by overlapping extrinsic and intrinsic pathways [556]. In cell and animal models, intrinsic events such as caspase activation, mitochondrial dysfunction, and chromatin cleavage set the stage, while extrinsic signals, including Fas receptor engagement, natural killer cell contact, and tumour necrosis factor signalling, further promote the process [557]. Studies document that regulators such as Bcl-2 family proteins, ROS, cathepsin D, resolvin E1, and TNF-related apoptosis-inducing ligand modulate how quickly and effectively apoptosis proceeds [558]. Apoptotic neutrophils are cleared mainly by macrophages through integrin- and receptor-mediated recognition, a process that removes cytotoxic cell remnants and limits inflammatory damage [559]. These findings show that timely induction and efficient clearance of neutrophils help restrict tissue injury and restore homeostasis, underlining a central role for apoptosis in balancing immune responses and resolving inflammation [560]. However, many viruses dysregulate this apoptotic program. For example, RSV markedly delays neutrophil apoptosis: RSV particles activate PI3K/Akt and NF- κ B pathways (likely via endosomal TLR7/8 and IL-6) to upregulate the anti-apoptotic protein Mcl-1. This prolonged PMN survival drives the massive neutrophil accumulation and mucus hypersecretion seen in severe RSV bronchiolitis. In contrast, HIV and SIV infections accelerate neutrophil turnover. SIV particles binding the cell surface prime a Bax/Bak-independent, calpain-dependent mitochondrial apoptosis, causing early neutropenia [561].

4.1.7 TGF- β Role in Neutrophil Function

TGF- β plays a significant role in neutrophil function and inflammation. TGF- β 1 enhances neutrophil survival, activation, and chemotaxis [562, 563]. Neutrophils express TGF- β receptors and can synthesize and release TGF- β , particularly TGF- β 1 and TGF- β 2 [563–565]. TGF- β 1 enhances neutrophil survival, activation, and chemotaxis [562], but inhibits their degranulation [430]. During viral infections, TGF- β can have both protective and detrimental effects. It suppresses early innate responses, enhancing rhinovirus and respiratory syncytial virus replication [566, 567].

4.1.8 Poly(I:C) as a Mimic of SARS-CoV-2 for Studying Neutrophil Responses

Poly(I:C) is widely used as a viral mimic because it is a synthetic dsRNA that resembles viral dsRNA produced during replication of many RNA viruses [568]. During SARS-CoV-2 replication, the viral positive-sense genome is transcribed into negative-sense RNA, forming dsRNA intermediates [569]. Cells sense dsRNA via endosomal TLR3 and cytosolic RIG-I and MDA5, triggering innate antiviral signalling. Poly(I:C) engages these receptors, with TLR3 recognizing it in endosomes while RIG-I and MDA5 detect it in the cytosol [570]. This activation leads to the production of pro-inflammatory cytokines and type I IFNs [570]. In neutrophils, poly(I:C) induces activation, infiltration, and NET formation. Markers of NETs, including extracellular DNA decorated with MPO and citrullinated histone H3, are elevated after poly(I:C) exposure [568]. NET formation in response to poly(I:C) requires the enzyme PAD4. Inhibition or knockdown of PAD4 abolishes poly(I:C)-induced NET release [568]. Blocking TLR3 signalling reduces poly(I:C)-driven neutrophil recruitment and NETosis [571]. In summary, poly(I:C) mimics SARS-CoV-2 dsRNA replication intermediates, activating endosomal TLR3 and cytosolic RIG-I/MDA5 pathways that drive PAD4-dependent NETosis in neutrophils.

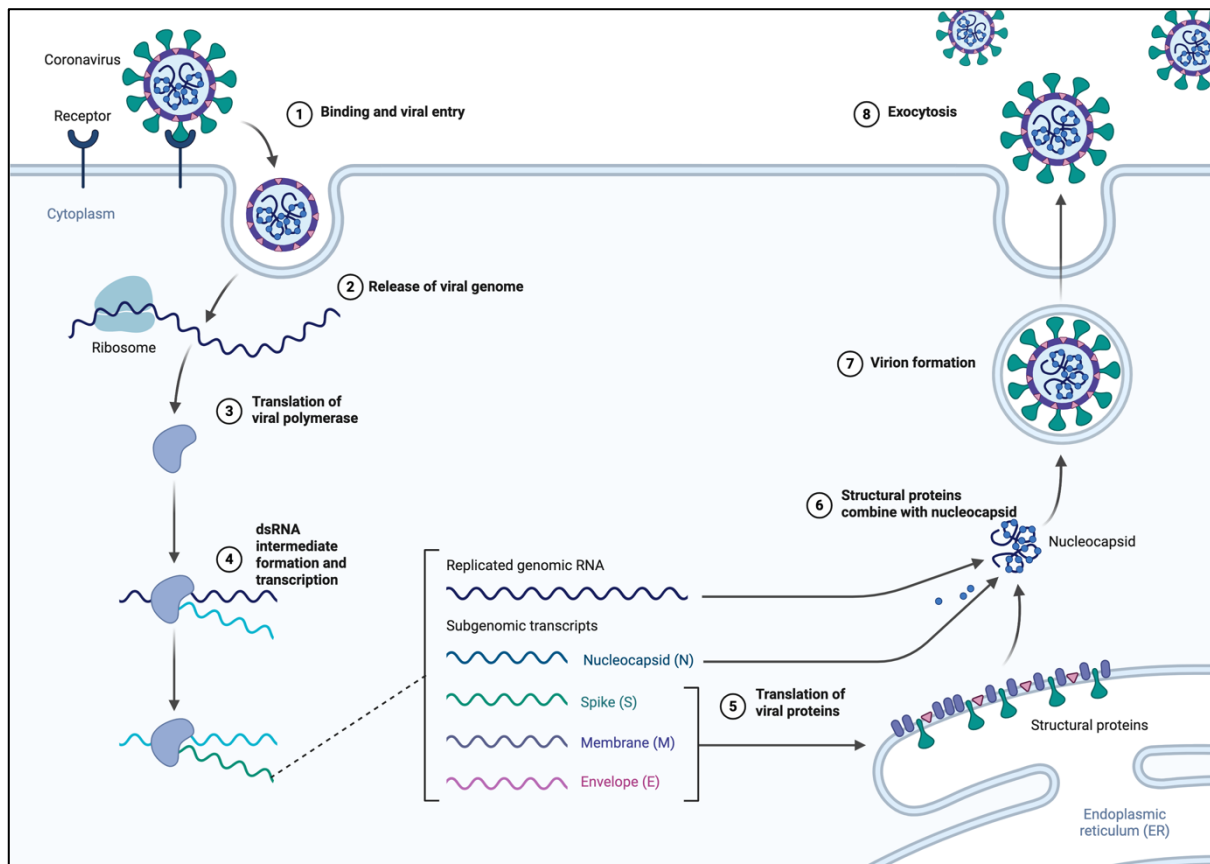


Figure 4.3 SARS-CoV-2 replication cycle showing dsRNA intermediate formation

The viral life cycle begins with (1) receptor binding and endocytic entry, followed by (2) genomic RNA release into the cytoplasm. (3) Host ribosomes translate viral polymerase and replicase proteins. (4) During replication and transcription, synthesis of negative-sense RNA from the positive-sense template creates transient dsRNA intermediates. This generates replicated genomic RNA and subgenomic transcripts encoding nucleocapsid (N), spike (S), membrane (M), and envelope (E) proteins. (5) Subgenomic mRNAs are translated to produce structural proteins. (6) Structural proteins combine with nucleocapsid-packaged genomic RNA to assemble virions. (7) Virions undergo maturation and (8) are released via exocytosis. The dsRNA intermediates formed during replication serve as pathogen-associated molecular patterns recognized by host innate immune sensors.

4.1.8 Rationale and Knowledge Gaps

While TLR7 and TLR8 both recognize viral ssRNA, human neutrophils lack TLR7 expression, making TLR8 the primary endosomal receptor for detecting viral RNA in these cells [551, 552]. TLR8 activation in neutrophils triggers multiple effector functions including degranulation, ROS production, NET formation, and cytokine secretion [551-554]. The SARS-CoV-2 genome contains numerous GU-rich regions that serve as potent TLR7/8 ligands, with SARS-CoV-2 possessing more TLR7/8-binding fragments than SARS-CoV [533, 546]. These viral RNA sequences have been validated as TLR7/8 activators in macrophages and microglia [572], and clinical studies show elevated TLR8 expression in severe COVID-19 cases, particularly in males [549]. Despite this evidence, the direct effects of SARS-CoV-2 ssRNA on neutrophil TLR8 signaling remain poorly characterized. Given that neutrophils accumulate massively in COVID-19 lungs and contribute to hyperinflammation through NET release and cytokine production, understanding how SARS-CoV-2 RNA specifically activates neutrophils via TLR8 is crucial. Furthermore, the interplay between TLR8-mediated activation, neutrophil survival, and immunomodulatory factors like TGF- β in the context of SARS-CoV-2 infection has not been systematically investigated. Addressing these gaps may reveal mechanisms underlying neutrophil-driven pathology in COVID-19 and identify potential therapeutic targets.

4.2 Hypothesis

TLR8 is a key endosomal receptor that plays a role in recognising SARS-CoV-2-associated ssRNAs in PMNs. We suggest that the engagement of SARS-CoV-2-derived GU-rich ssRNAs with TLR8 results in a robust inflammatory response involving cytokine secretion, transcription activation, degranulation, modulation of surface markers, and the induction of

NETosis. As the function of TLR8 in mediating ssRNA virus-induced immune responses has previously been demonstrated, we propose that SARS-CoV-2 ssRNA triggers TLR8 signalling, which in turn leads to dysregulated PMN responses, including aberrant NETosis, thereby contributing to the inflammatory pathology observed in COVID-19. Thus, the current study aims to gain new insights into the role of neutrophil-driven inflammation in COVID-19 by exploring the molecular mechanisms of TLR8-mediated signalling and NETosis in PMNs treated with ssRNA SARS-CoV-2.

4.3 Specific Aims

Aim 1: Investigate the role of TLR8 in PMN activation associated with SARS-CoV-2

Hypothesis: TLR8 expression and activation in PMNs, along with downstream effects such as cytokine release and transcriptional activation, are upregulated in severe cases of COVID-19 and contribute to hyperinflammation.

Approach: Utilise Nanostring gene expression profiling of whole blood samples from COVID-19 patients with varying disease severities to measure TLR8 expression levels and associated signalling molecules.

Aim 2: Investigate the effect of SARS-CoV-2 ssRNA on PMN activation markers (CD66b, CD11b, and CD16b) and their dependency on TLR8, utilising flow cytometry.

Hypothesis: SARS-CoV-2 ssRNA activates PMNs through TLR8, modulating surface markers CD66b, CD11b, and CD16b, which indicate neutrophil activation, adhesion, and immune function. These alterations may correlate with disease severity in COVID-19.

Approach: Isolate primary human PMNs and treat them with SARS-CoV-2 ssRNA. Measure marker expression using flow cytometry and confirm TLR8's role with specific inhibitors. Correlate *in vitro* findings with clinical markers of COVID-19 severity, such as cytokine profiles and inflammatory biomarkers.

Aim 3: Evaluate whether SARS-CoV-2 ssRNA increases TLR8 expression in PMNs and its role in inflammation responses.

Hypothesis: SARS-CoV-2 ssRNA enhances TLR8 expression in PMNs.

Approach: Primary human PMNs will be isolated and treated with SARS-CoV-2 ssRNA *in vitro*. TLR8 expression levels will be measured using flow cytometry at various time points. The relationship between TLR8 expression and functional neutrophil responses, such as cytokine release and activation marker expression, will be analysed to explore the potential role of TLR8 upregulation in driving inflammation.

Aim 4: Assess the capacity of SARS-CoV-2 ssRNA to induce ROS production in PMNs.

Hypothesis: Exposure to SARS-CoV-2 ssRNA stimulates ROS production in PMNs, leading to oxidative stress and neutrophil activation during the immune response to the virus infection.

Approach: Primary human PMNs will be isolated and treated with SARS-CoV-2 ssRNA in a controlled *in vitro* environment. ROS production will be measured using flow cytometry with a fluorescent probe that is sensitive to oxidative activity. The results will be compared to untreated controls to ascertain the extent and timing of ROS generation. Data will also be analysed alongside other neutrophil activation markers to enhance our understanding of the role of ROS in the inflammatory response triggered by SARS-CoV-2 ssRNA.

Aim 5: Investigate if SARS-CoV-2 ssRNA induces the degranulation of MPO in PMNs.

Hypothesis: Treatment with SARS-CoV-2 ssRNA enhances the release of MPO, a vital enzyme involved in neutrophil-mediated antimicrobial defence and inflammatory processes.

Approach: Isolate primary human PMNs and treat them with SARS-CoV-2 ssRNA *in vitro*. MPO degranulation will be assessed by quantifying MPO levels in cell culture supernatants using ELISA. Results will be compared between treated neutrophils and untreated controls to evaluate the effect of SARS-CoV-2 ssRNA on degranulation.

Aim 6: Evaluate the capacity of SARS-CoV-2 ssRNA to stimulate PMN NET formation employing SYTOX Orange.

Hypothesis: SARS-CoV-2 ssRNA triggers the formation of NETs in PMNs.

Approach: Primary human PMNs will be isolated and exposed to SARS-CoV-2 ssRNA *in vitro*. NETosis will be evaluated by quantifying extracellular DNA using SYTOX Orange, a DNA-binding fluorescent dye that selectively stains extracellular DNA. Fluorescence will be measured using flow cytometry to assess the extent of NET formation. Results from treated neutrophils will be compared with untreated controls to determine whether SARS-CoV-2 ssRNA significantly induces NETosis.

Aim 7: Assess whether SARS-CoV-2 ssRNA stimulates the production of TGF- β in human PMNs.

Hypothesis: Exposure to SARS-CoV-2 ssRNA enhances TGF- β secretion by PMNs, thereby contributing to the immunopathology observed in COVID-19.

Approach: Isolate primary human PMNs and incubate them with SARS-CoV-2 ssRNA *in vitro*. Measure the levels of TGF- β in the culture supernatant using an ELISA. Compare TGF- β production between ssRNA-treated neutrophils and untreated controls to assess the stimulatory effect of viral RNA.

Aim 8: Assess the effect of SARS-CoV-2 ssRNA on apoptosis in human PMNs.

Hypothesis: Exposure to SARS-CoV-2 ssRNA triggers apoptotic cell death in PMNs, contributing to the immunopathology observed in COVID-19.

Approach: Isolate primary human PMNs and incubate them with SARS-CoV-2 ssRNA *in vitro*. Assess apoptosis by detecting phosphatidylserine externalisation using Annexin V staining, combined with PI to differentiate apoptotic from necrotic cells. Analyse the samples via flow cytometry to determine the percentage of cells undergoing apoptosis. Compare the proportion of apoptotic cells between ssRNA-treated neutrophils and untreated controls to evaluate the pro-apoptotic effect of viral RNA.

Aim 9: Examine if SARS-CoV-2 ssRNA stimulates proinflammatory cytokine production in human PMNs and identify the signalling pathways involved by employing specific inhibitors.

Hypothesis: SARS-CoV-2 ssRNA stimulates PMNs to produce pro-inflammatory cytokines via specific signalling pathways, which can be identified and modulated with targeted inhibitors.

Approach: Isolate primary human PMNs and expose them to SARS-CoV-2 ssRNA *in vitro*. Measure levels of pro-inflammatory cytokines, such as IL-6, IL-8, TNF- α , and IL-1 β , in the supernatants using ELISA. To identify the involved signalling pathways, pre-treat the neutrophils with specific inhibitors targeting:

- **NF- κ B pathway:** Bay 11-7082.
- **MAPK pathways:** PD98059 for ERK and SB203580 for p38.
- **TLR8:** CU-CPT9a.

Following the inhibitor pre-treatment, stimulate PMNs with SARS-CoV-2 ssRNA and compare cytokine production to that of untreated controls. This will assist in determining which pathways are involved in the ssRNA-induced cytokine response.

Aim 10: Investigate the effects of dsRNA analogue poly I:C on human PMN activation, concentrating on ROS production and pro-inflammatory cytokine secretion.

Hypothesis: Poly I:C encourages PMN to generate ROS and release pro-inflammatory cytokines.

Approach: Isolate primary human PMNs and expose them to poly I:C *in vitro*. Assess ROS production using appropriate detection methods. Measure levels of pro-inflammatory cytokines, including IL-6, IL-1 β TNF- α and IL-8, in the culture supernatants. Compare these responses with untreated controls to ascertain the effect of poly I:C on neutrophil activation.

4.4 Experiments & Discoveries:

4.4.1: TLR7 and TLR8 Expression and Transcriptional Activation in Whole Blood Are Associated with the Severity of COVID-19

To further understand the molecular mechanisms of immune responses in COVID-19, we first focused on TLR7/8, which act as sensors of viral RNA and initiators of antiviral responses. As SARS-CoV-2 is a single-stranded RNA virus, TLR7 and TLR8 serve as primary immune sensors that activate downstream signalling cascades, including MyD88, NF- κ B, IRF7, and IRF3, to induce cytokine production and inflammation. The dysregulation of these pathways has been shown to contribute to the severity of COVID-19 by inducing hyperinflammation and immune pathology. To understand these dynamics, we reviewed Nanostring gene expression data from Hadjadj *et al.*, [25] which analysed blood samples from moderate, severe, and critical COVID-19 patients. The aim of our study was to determine the expression of TLR7/8 and their effector molecules to understand their role in the progression of immune dysfunction in SARS-CoV-2 infection. Our findings indicated that while TLR7 was overexpressed in patients with moderate disease compared to healthy controls, there was no significant change in severe or critical cases (**Fig. 4.4A**). Conversely, TLR8 expression progressively increased with the severity of the disease and was significantly higher in moderate, severe, and critically ill patients than in healthy controls (**Fig. 4.4B**). These patterns suggest that TLR7 is involved in the initial immune response during moderate infection, while TLR8 assumes a central role in driving inflammation as the disease severity increases. Among the downstream signalling molecules, MyD88, a central adaptor protein in TLR signalling, was significantly elevated in moderate, severe, and critical disease compared to healthy controls, indicating its role across all stages of disease progression (**Fig. 4.4C**). NF- κ B expression was significantly upregulated in severe and critical cases, consistent with its role in promoting pro-inflammatory cytokine production during advanced disease (**Fig. 4.4D**). The TLR7/8 signalling-associated transcription factor IRF7 was found to be significantly increased in moderate and severe illness but did not increase in critical patients, suggesting its role in antiviral responses during the initial and moderate infection stages (**Fig. 4.4E**). On the other hand, IRF3 expression did not show a significant change among all, indicating it does not play a significant role in SARS-CoV-2-induced immune responses in this dataset (**Fig. 4.4F**). These findings demonstrate the distinct roles of TLR7 and TLR8 in sensing ssRNA and activating downstream signalling pathways in COVID-19. TLR7 and MyD88 are implicated in early immune activation during moderate disease, whilst TLR8 and NF- κ B drive the

heightened inflammatory response observed in severe and critical cases. IRF7 appears to mediate antiviral responses during the early to moderate stages of the disease, whereas IRF3 shows minimal involvement in the SARS-CoV-2-induced immune response.

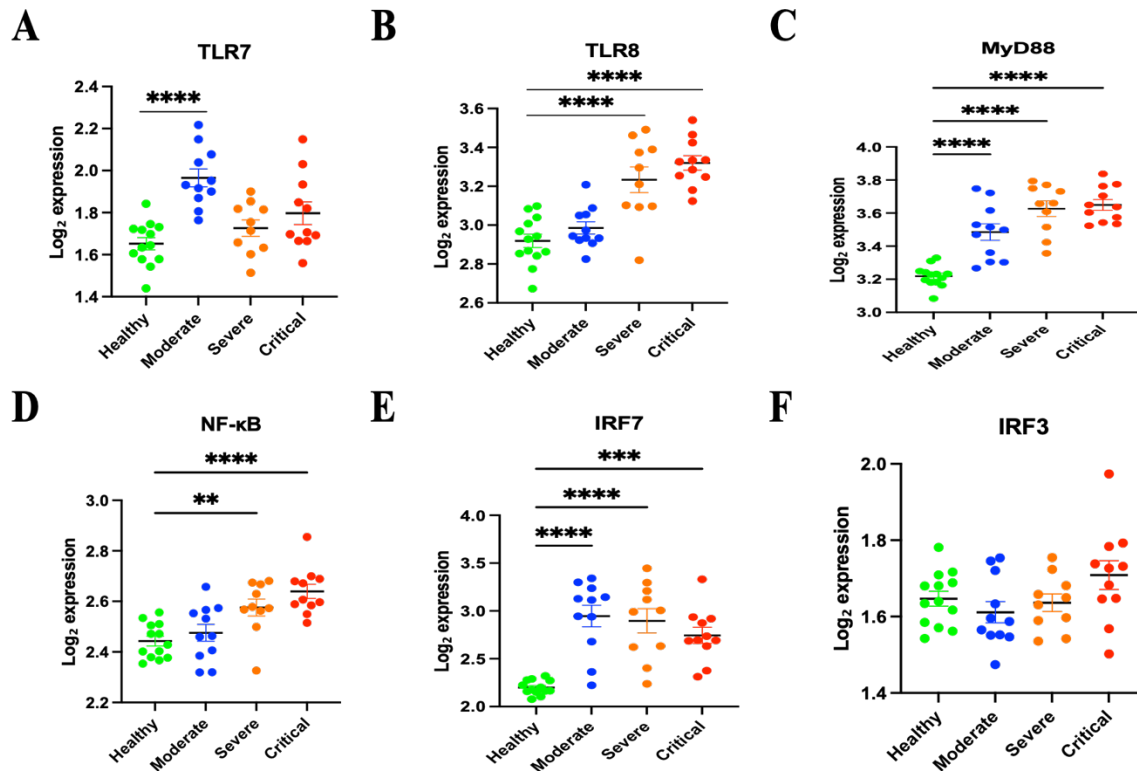


Figure 4.4 TLR7 and TLR8 Expression and Their Downstream Signalling Molecules Correlate with Disease Severity in COVID-19 Patient.

Normalized log₂ expression of TLR7 (A), TLR8 (B), MyD88 (C), NF-κB (D), IRF7 (E), and IRF3 (F) in healthy controls (n = 13) and patients with mild-to-moderate (n = 11), severe (n = 10), and critical (n = 11) COVID-19. Data are presented as mean ± SEM. Statistical analysis was performed using one-way ANOVA. Significant differences compared with the healthy group are indicated as: ****P < 0.001 and **P < 0.0001

4.4.2 The Role of Cytokines in COVID-19 Severity: Focus on IL-1β, TNF-α, IL-6, and IL-8

Next, we analysed the mRNA dataset above for the expression of IL-1β, TNF-α, IL-6, and IL-8 to further characterise the pro-inflammatory response in COVID-19 patients across various disease severities. These cytokines were selected due to their central roles in inflammation, immune cell recruitment, and the progression of cytokine-mediated pathology. To aid in understanding disease progression, we compared their mRNA levels in patients with moderate, severe, and critical illness. The analysis demonstrated that IL-1β (Fig. 4.5A) was significantly elevated in patients with severe and critical disease compared

to healthy controls. In contrast, the levels of TNF- α (Fig. 4.5B), IL-6 (Fig. 4.5C), and IL-8 (Fig. 4.5D) did not exhibit significant changes across the various severity groups.

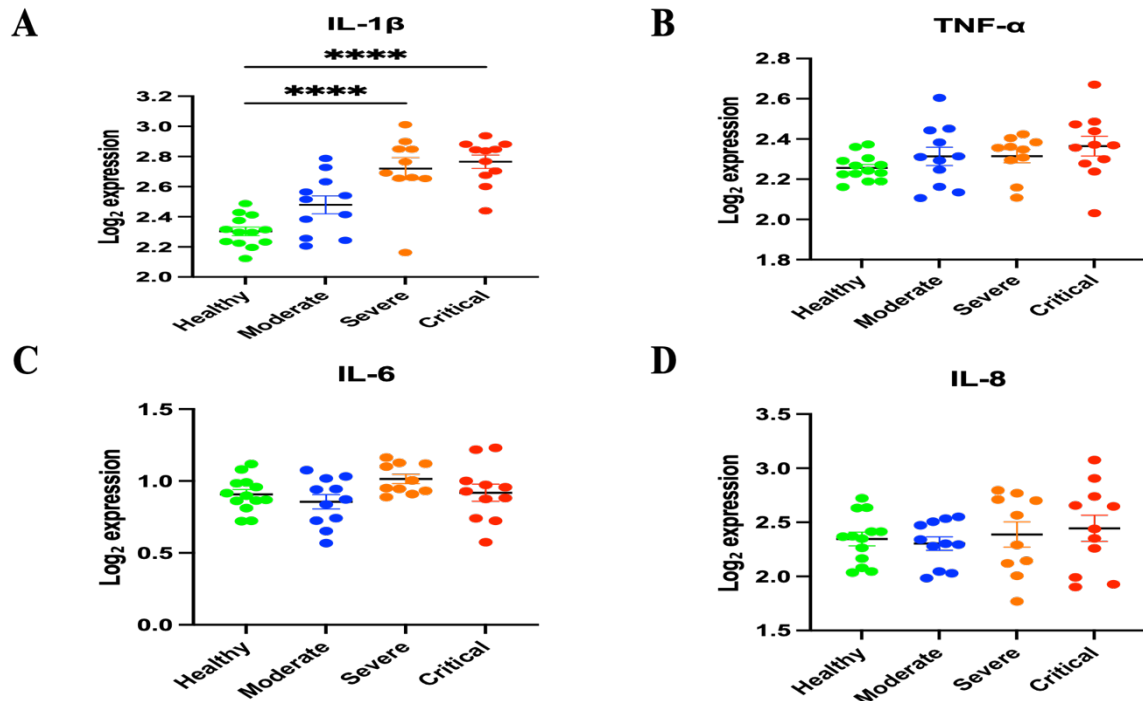


Figure 4.5 Expression of Key Pro-Inflammatory Cytokines in Relation to COVID-19 Disease Severity.

Normalized log₂ expression of IL-1 β (A), TNF- α (B), IL-6 (C), and IL-8 (D) in healthy controls (n = 13) and patients with mild-to-moderate (n = 11), severe (n = 10), and critical (n = 11) COVID-19. Data are presented as mean $\pm$ SEM. Statistical analysis was performed using one-way ANOVA. Significant differences compared with the healthy group are indicated as: **P < 0.0001.

4.4.3 SARS-CoV-2 GU-Rich ssRNAs does not induce the expression of CD66b in human primary PMNs:

Based on the study by Thieme *et al.*, (2021), the SARS-CoV-2 genome (NC_045512, positive strand) was analysed to identify GU-rich ssRNA fragments using the SequenceSearcher tool in Fuzzy mode. GU-enriched sequences were defined as RNA strings up to 20 nucleotides in length, with GU and/or UG pairs comprising more than 40% of their length. This analysis identified 491 GU-rich sequences, which were further filtered for the presence of at least one UGUGU immune-instructive motif (IIM) as described in the study (see Supplemental Table 1 in Thieme *et al.*, (2021). Among these, specific sequences with notable IIM enrichment were selected and synthesised by IDT. These included V2-RNA1

5'-UGCUGUUGUGUGUU*U-3' (genome position: 15692-15706); SCV2-RNA2 5'-GUGUGUGUGUUCUGUUAUU*G-3' (genome position: 20456-20475; *indicates a phosphorothioate linkage). To ensure their specificity, these sequences were validated using BLAST searches targeting RNA viruses (taxid: 2559587). Additionally, a mutated version of SCV2-RNA2, designated SCV2-RNA2A, was synthesised by substituting uracil (U) with adenine (A) to impair TLR7/8 stimulation, serving as a negative control [533]. PMNs play a key role in the innate immune response to viral infections such as SARS-CoV-2. Upon activation, they can enhance either antiviral immunity or immunopathology, making it crucial to understand how viral components regulate neutrophil functions. Given that TLR7 and TLR8 are activated by ssRNA and can stimulate the immune system, we aimed to determine whether these SARS-CoV-2 ssRNA sequences can activate neutrophils and consequently lead to CD66b expression, suggesting the involvement of TLR7/8 in neutrophil-mediated immune responses to SARS-CoV-2. PMNs were treated with 10 µg/ml of SCV2-RNA2 or SCV2-RNA2A, while CL075, a known TLR7/8 agonist, served as a positive control at 1 µg/ml. After a 2-hour incubation, CD66b expression was evaluated using flow cytometry. The results indicated that CL075 strongly enhanced the expression of CD66b, reflecting significant neutrophil activation. In contrast, neither SCV2-RNA2 nor SCV2-RNA2A induced a notable increase in CD66b levels (**Fig.4.6**).

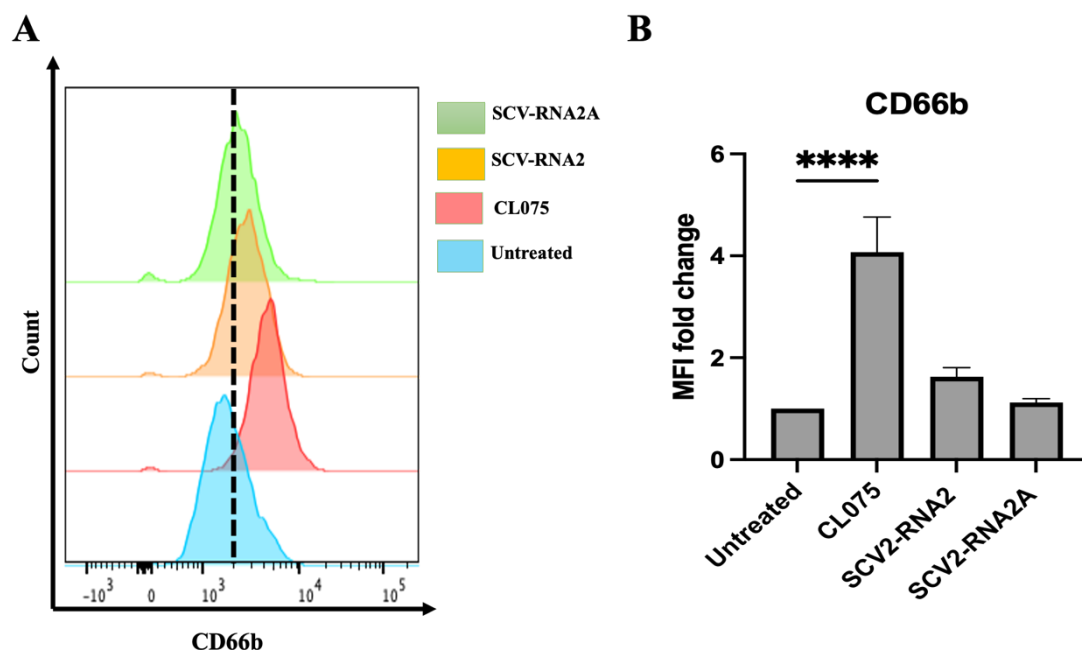


Figure 4.6 Evaluation of CD66b Expression as a Marker of Neutrophil Activation Following Treatment with SARS-CoV-2 ssRNAs.

Human PMNs were obtained from healthy donors (n=10) using density gradient isolation method. PMNs (1×10^6 cells/mL) were treated with SCV2-RNA2 (10 μ g/ml), SCV2-RNA2A (10 μ g/ml), or CL075 (1 μ g/ml) for 2 hours and CD66b expression was measured by flow cytometry. Flow cytometric analysis was performed using a standard gating strategy: cells were first gated based on FSC and SSC properties to exclude debris and select the PMNs population, followed by singlet discrimination using SSC-Area vs. SSC-Height, and finally gated for live cells using live/dead stain exclusion. (A) Representative flow cytometry histograms showing CD66b fluorescence intensity distribution. (B) Quantitative analysis showing MFI fold change relative to untreated control. Data represent mean $\pm$ SEM. Statistical analysis was performed using one-way ANOVA. (**** $P < 0.00001$)

4.4.4 SARS-CoV-2 ssRNA SCV2-RNA1 Induces CD11b Expression in human Primary PMNs:

Next, we tested the capacity of CL075 and SARS-CoV-2-associated ssRNA sequences to activate PMNs, assessing the expression of CD11b, a key marker of PMN activation and adhesion in immune responses. SCV2-RNA1 or its mutated counterpart SCV2-RNA1A, at a concentration of 10 μ g/ml were incubated with PMNs. Additionally, CL075, a TLR7/8 agonist, was used as a positive control at a concentration of 1 μ g/ml. Following a 2 hour incubation, the level of CD11b was measured to determine whether the PMNs were activated. The results indicated that both CL075 and SCV2-RNA1 triggered the expression of CD11b, suggesting their capability to activate neutrophils and enhance their adhesion. In contrast, SCV2-RNA1A, which features a single-nucleotide change that inactivates TLR8 stimulation, did not elicit a similar response.

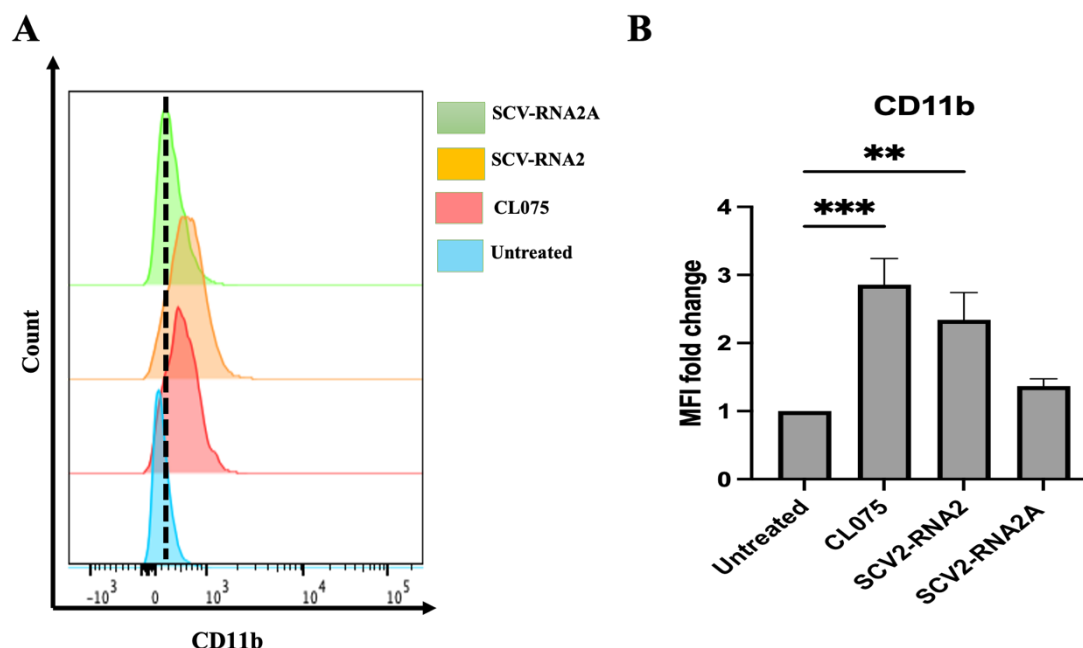


Figure 4.7 Induction of CD11b Expression in PMNs by SARS-CoV-2 ssRNA SCV2-RNA1.

Human PMNs were obtained from healthy donors (n=10) using density gradient isolation method. PMNs (1×10^6) were treated with SCV2-RNA2 (10 $\mu\text{g/ml}$), SCV2-RNA2A (10 $\mu\text{g/ml}$), or CL075 (1 $\mu\text{g/ml}$) for 2 hours and CD11b expression was measured by flow cytometry. Flow cytometric analysis was performed using a standard gating strategy: cells were first gated based on FSC and SSC properties to exclude debris and select the PMNs population, followed by singlet discrimination using SSC-Area vs. SSC-Height, and finally gated for live cells using live/dead stain exclusion. (A) Representative flow cytometry histograms showing CD11b fluorescence intensity distribution. (B) Quantitative analysis showing MFI fold change relative to untreated control. Data represent mean $\pm$ SEM. Statistical analysis was performed using one-way ANOVA. (** $P < 0.01$; *** $P < 0.001$).

4.4.5 CD16 Shedding in human primary PMNs Remains Unaffected by SARS-CoV-2 ssRNA SCV2-RNA1

We also assessed the ability of CL075 and SARS-CoV-2-associated ssRNA sequences to regulate the shedding of CD16, a receptor that mediates neutrophil function in antibody-dependent cellular cytotoxicity. Neutrophils were incubated with 10 $\mu\text{g/ml}$ of SCV2-RNA1 or SCV2-RNA1A, while CL075 was used at 1 $\mu\text{g/ml}$ as a positive control. Two hours post-incubation, the amounts of CD16 were measured to determine whether there was any shedding. The results indicated an increase in CD16 expression across all treatment groups,

rather than shedding. CL075 stimulated a three fold increase, SCV2-RNA1 caused a four fold increase, and SCV2-RNA1A resulted in a two fold increase in CD16 levels.

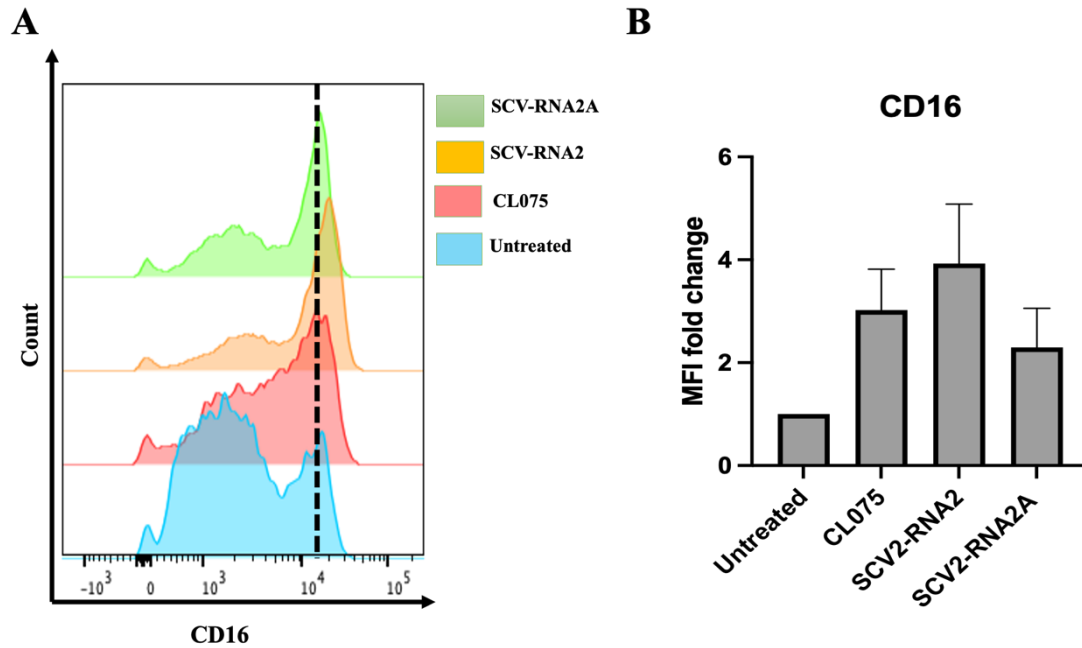


Figure 4.8 Evaluation of CD16 Shedding in PMNs Following Treatment with SARS-CoV-2 ssRNA SCV2-RNA1.

Human PMNs were obtained from healthy donors (n=10) using density gradient isolation method. PMNs (1×10^6) were treated with SCV2-RNA2 (10 $\mu\text{g/ml}$), SCV2-RNA2A (10 $\mu\text{g/ml}$), or CL075 (1 $\mu\text{g/ml}$) for 2 hours and CD16 expression was measured by flow cytometry. Flow cytometric analysis was performed using a standard gating strategy: cells were first gated based on FSC and SSC properties to exclude debris and select the PMNs population, followed by singlet discrimination using SSC-Area vs. SSC-Height, and finally gated for live cells using live/dead stain exclusion. (A) Representative flow cytometry histograms showing CD16 fluorescence intensity distribution. (B) Quantitative analysis showing MFI fold change relative to untreated control. Data represent mean $\pm$ SEM. Statistical analysis was performed using one-way ANOVA.

4.4.6 SARS-CoV-2 ssRNA SCV2-RNA1 Induces TLR8 Expression in human Primary PMNs:

TLR8 is a sensor of ssRNA and initiates innate immune responses through its stimulation, primarily in monocytes and neutrophils. Given that TLR8 signalling can influence PMNs activation and cytokine production, we aimed to determine whether specific SARS-CoV-2 RNA sequences affect TLR8 expression in PMNs. While previous experiments assessed

neutrophil activation markers such as CD66b, CD11b, and CD16, it remains uncertain whether these responses correlate with alterations in TLR8 expression levels. By treating neutrophils with SCV2-RNA2 and its modified form (SCV2-RNA2A) and comparing their effects to CL075, we intended to investigate whether viral RNA sequences directly influence TLR8 expression, providing insights into their potential role in neutrophil-mediated immune responses. The results indicated that both CL075 and SCV2-RNA2 significantly increased TLR8 expression, highlighting their ability to activate signalling pathways involving this receptor. However, the modified sequence SCV2-RNA2A did not elicit a similar increase in TLR8 levels.

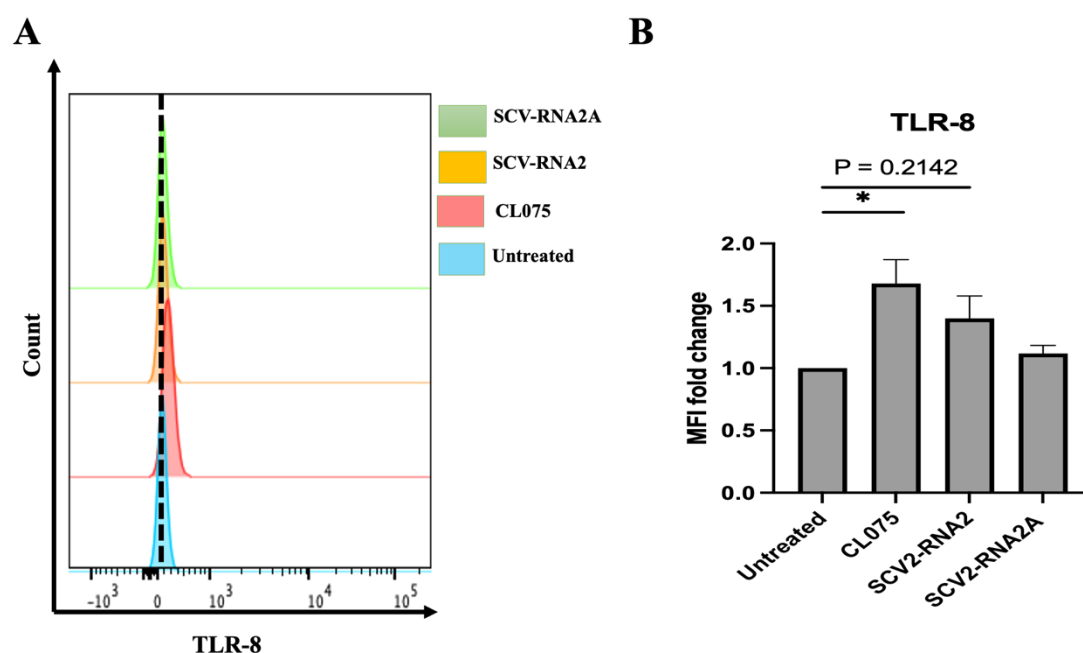


Figure 4.9 TLR8 expression in PMNs following treatment with CL075, SCV2-RNA2, and SCV2-RNA2A.

Human PMNs were obtained from healthy donors ($n=4$) using density gradient isolation method. PMNs (1×10^6) were treated with SCV2-RNA2 ($10 \mu\text{g/ml}$), SCV2-RNA2A ($10 \mu\text{g/ml}$), or CL075 ($1 \mu\text{g/ml}$) for 2 hours and TLR8 expression was measured by flow cytometry. Flow cytometric analysis was performed using a standard gating strategy: cells were first gated based on FSC and SSC properties to exclude debris and select the PMNs population, followed by singlet discrimination using SSC-Area vs. SSC-Height, and finally gated for live cells using live/dead stain exclusion. (A) Representative flow cytometry histograms showing TLR8 fluorescence intensity distribution. (B) Quantitative analysis showing MFI fold change relative to untreated control. Data represent mean $\pm$ SEM. Statistical analysis was performed using one-way ANOVA. * $P < 0.05$

4.4.7: ROS Production in human primary PMNs Remains Unaffected by SARS-CoV-2 ssRNA SCV2-RNA1

PMNs produce ROS that assists in pathogen elimination through the oxidative burst. TLR8 activation has been linked to PMNs activation and inflammatory responses; however, the role of TLR8 in modulating ROS production in response to SARS-CoV-2 ssRNA sequences remains unclear. Given that TLR7/8 activation is known to influence PMNs effector functions, we sought to determine whether specific SARS-CoV-2 ssRNA sequences could stimulate ROS production. By treating neutrophils with SCV2-RNA2 and its modified form (SCV2-RNA2A) and comparing their effects to CL075, we aimed to evaluate whether viral RNA could induce oxidative bursts through TLR8 signalling. This experiment aids in understanding the role of SARS-CoV-2 ssRNA in modulating PMNs functions and consequently the inflammatory process in COVID-19. The results indicated that there was no significant ROS production by PMNs in response to any of the treatments, including SCV2-RNA2, SCV2-RNA2A, or CL075. This suggests that under the given conditions, neither the viral ssRNA sequences nor the positive control was capable of activating the pathways leading to PMNs ROS production.

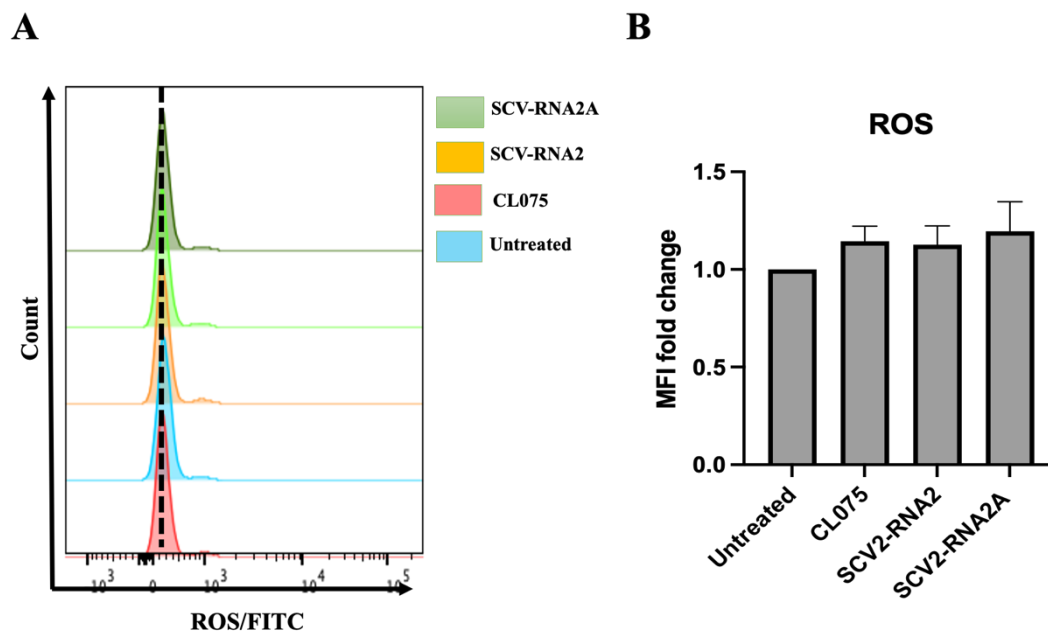


Figure 4.10 Assessment of ROS Production in PMNs Treated with SARS-CoV-2 ssRNA and TLR7/8 Agonist CL075.

Human PMNs were obtained from healthy donors (n=5) using density gradient isolation method. PMNs (1×10^6) were treated with SCV2-RNA2 (10 μ g/ml), SCV2-RNA2A (10 μ g/ml), or CL075 (1 μ g/ml) for 2 hours and ROS expression was measured by flow

cytometry. Flow cytometric analysis was performed using a standard gating strategy: cells were first gated based on FSC and SSC properties to exclude debris and select the PMNs population, followed by singlet discrimination using SSC-Area vs. SSC-Height, and finally gated for live cells using live/dead stain exclusion. (A) Representative flow cytometry histograms showing ROS fluorescence intensity distribution. (B) Quantitative analysis MFI fold change relative to untreated control. Data represent mean $\pm$ SEM. Statistical analysis was performed using one-way ANOVA.

4.4.8 MPO Production in human primary PMNs Remains Unaffected by SARS-CoV-2 ssRNA SCV2-RNA1

The next experiment assessed the impact of SARS-CoV-2 ssRNA on PMN degranulation, specifically focusing on the release of MPO, a crucial enzyme stored in the granules of PMNs. PMNs were stimulated with 10 μ g/ml of SCV2-RNA2 or SCV2-RNA2A, while CL075, was used at 1 μ g/ml as a positive control. After incubating the PMNs at 37°C for 2 hours, MPO release into the supernatant was evaluated using ELISA. While not statistically significant, the findings indicated a slight reduction in MPO release across all treatment groups, including SCV2-RNA2, SCV2-RNA2A, and CL075, compared to untreated controls.

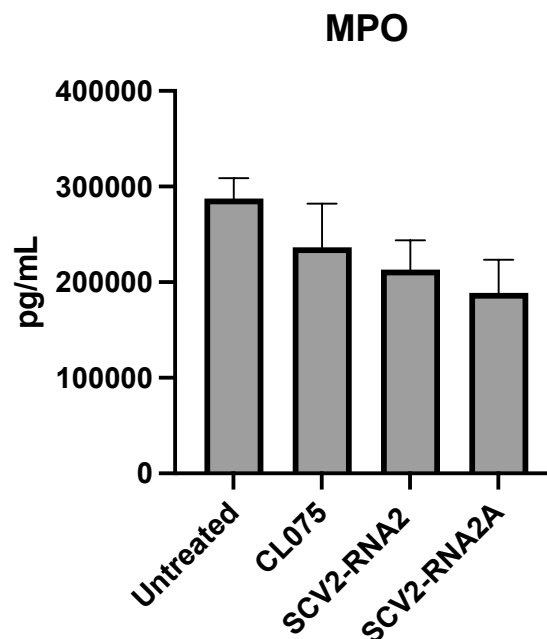


Figure 4.11 Analysis of MPO Degranulation in PMNs Treated with SARS-CoV-2 ssRNA.

Human PMNs were obtained by density gradient isolation (n=5). PMNs (1×10^6) were treated with 10 $\mu\text{g/ml}$ of SCV2-RNA2, SCV2-RNA2A, or 1 $\mu\text{g/ml}$ CL075 for 2 hours. MPO release, was measured in the supernatant ELISA. Results are presented as ng/ml. Data represent mean $\pm$ SEM. Statistical analysis was performed using one-way ANOVA.

4.4.9 NETosis Induction in human primary PMNs Remains Unaffected by SARS-CoV-2 ssRNA SCV2-RNA1

NETs are also part of an innate immune defence mechanism, whereby neutrophils release DNA, histones, and antimicrobial proteins to capture and neutralise pathogens. However, NETosis has been reported to lead to tissue damage, inflammation, and thrombotic events in the context of severe COVID-19. Since TLR7/8 signalling has been noted to activate PMNs, we aimed to investigate whether SARS-CoV-2 ssRNA sequences are capable of inducing NET formation and, therefore, escalating the hyperinflammatory response characteristic of critical COVID-19 cases. To assess this, PMNs were treated with 10 $\mu\text{g/ml}$ of SCV2-RNA2 or its modified version, SCV2-RNA2A, or CL075 (1 $\mu\text{g/ml}$). After 6 hours of incubation, NET formation was assessed using SYTOX Orange fluorescence, which selectively detects extracellular DNA. PMNs were also left untreated to serve as a baseline control. The results indicated that there was no increase in NET formation in any of the treatment groups; that is, SCV2-RNA2 and SCV2-RNA2A, compared to untreated controls. These findings suggest that, under the experimental conditions, the SARS-CoV-2 ssRNA sequences failed to induce NETosis in PMNs. This implies that these RNA sequences are not, on their own, sufficient to activate the pathways leading to NET formation.

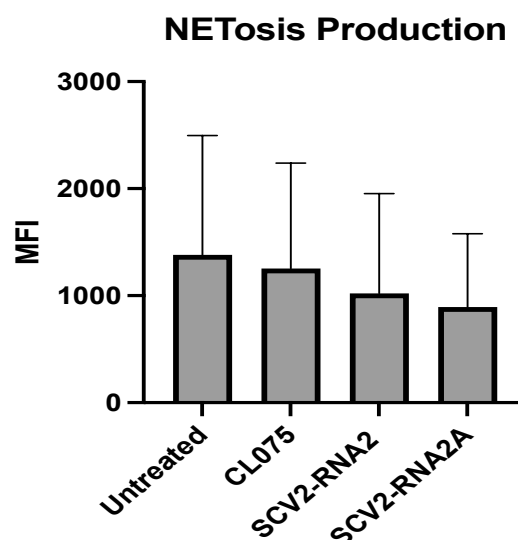


Figure 4.12 Assessment of NETosis in PMNs Treated with SARS-CoV-2 ssRNA Using SYTOX Orange Fluorescence.

Human PMNs were obtained through density gradient isolation. PMNs (1×10^6) were treated with 10 $\mu\text{g/ml}$ of SCV2-RNA2, SCV2-RNA2A 1 $\mu\text{g/ml}$ CL075 for 6 hours ($n=3$). NETosis was evaluated by measuring extracellular DNA release using the fluorescent dye SYTOX Orange. Untreated PMNs were included as a control. Results are expressed as mean relative FMI. Data represent mean $\pm$ SEM. Statistical analysis was performed using one-way ANOVA.

4.4.10 The TGF- β levels in human primary PMNs remain unchanged by SARS-CoV-2 ssRNA SCV2-RNA1.

TGF- β is a multifunctional cytokine involved in the regulation of immune responses, the resolution of inflammation, and tissue healing. Although TGF- β is necessary for maintaining immune homeostasis, its overproduction has been linked to fibrosis, immune suppression, and chronic inflammation, which are characteristic features of severe COVID-19 pathology. Since TLR7/8 activation impacts cytokine secretion in immune cells, including PMNs, we sought to determine whether SARS-CoV-2 ssRNA sequences can regulate TGF- β production to either suppress the immune response or promote tissue remodelling during the infection. To assess this, PMNs were treated with 10 $\mu\text{g/ml}$ of SCV2-RNA2 or its modified version, SCV2-RNA2A or CL075 (1 $\mu\text{g/ml}$) for 18 h, and TGF- β secretion in the supernatant was measured using an ELISA. PMNs were also left untreated to establish baseline TGF- β secretion. This experiment aimed to determine whether viral RNA can modulate TGF- β production in PMNs, which may help explain the development of fibrosis in COVID-19.

The results showed that there were no significant changes in TGF- β levels among the treatment groups compared to the control. Both SCV2-RNA2 and SCV2-RNA2A prompted similar amounts of TGF- β release, indicating that the SARS-CoV-2 ssRNA sequences investigated here had no effect on TGF- β production under the current conditions.

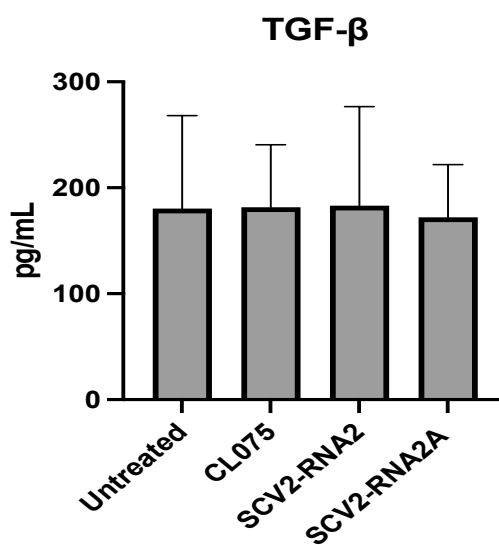


Figure 4.13 Assessment of TGF- β production in PMNs Treated with SARS-CoV-2 ssRNA.

Human PMNs were obtained through density gradient isolation (n=4). PMNs (1×10^6) were treated with 10 μ g/ml of SCV2-RNA2, SCV2-RNA2A or 1 μ g/ml CL075 for 18 hours. TGF- β levels in the supernatant were quantified using ELISA. Results are expressed as mean TGF- β concentration (pg/ml). Data represent mean $\pm$ SEM. Statistical analysis was performed using one-way ANOVA.

4.4.11 SCV2-RNA2 has no Effects on human primary PMN Apoptosis and Cell Survival

Neutrophil apoptosis is a crucial mechanism of immune system regulation, ensuring the timely resolution of inflammation and preventing excessive tissue damage. Delayed apoptosis of neutrophils has been shown to contribute to hyperinflammatory responses, prolonged neutrophil function, and immune dysregulation, which are the primary drivers of severe COVID-19 pathology. As viral RNA can modulate immune cell survival via PRRs, we aimed to determine whether the SARS-CoV-2 ssRNA sequence SCV2-RNA2 alters

neutrophil apoptosis and might contribute to the persistent inflammation observed in severe disease. To test this, PMNs were exposed to SCV2-RNA2 at concentrations of 0, 1, 5, and 10 $\mu\text{g/ml}$ for 6 hours, after which apoptosis was assessed by flow cytometry. We evaluated apoptosis using markers of cell death, including PI (for late apoptosis/necrosis) and annexin V (for early apoptosis) to distinguish between different stages of apoptosis. Untreated neutrophils served as a control to establish a baseline apoptotic rate. Our results show that SCV2-RNA2 has no significant effect on neutrophil apoptosis. Viability remained consistently high across all concentrations, ranging from 64% to 81%, comparable to untreated controls at 76% to 80%. Early apoptosis stayed between 9% and 34%, and late apoptosis ranged from 10% to 32%, with no clear dose-dependent pattern.

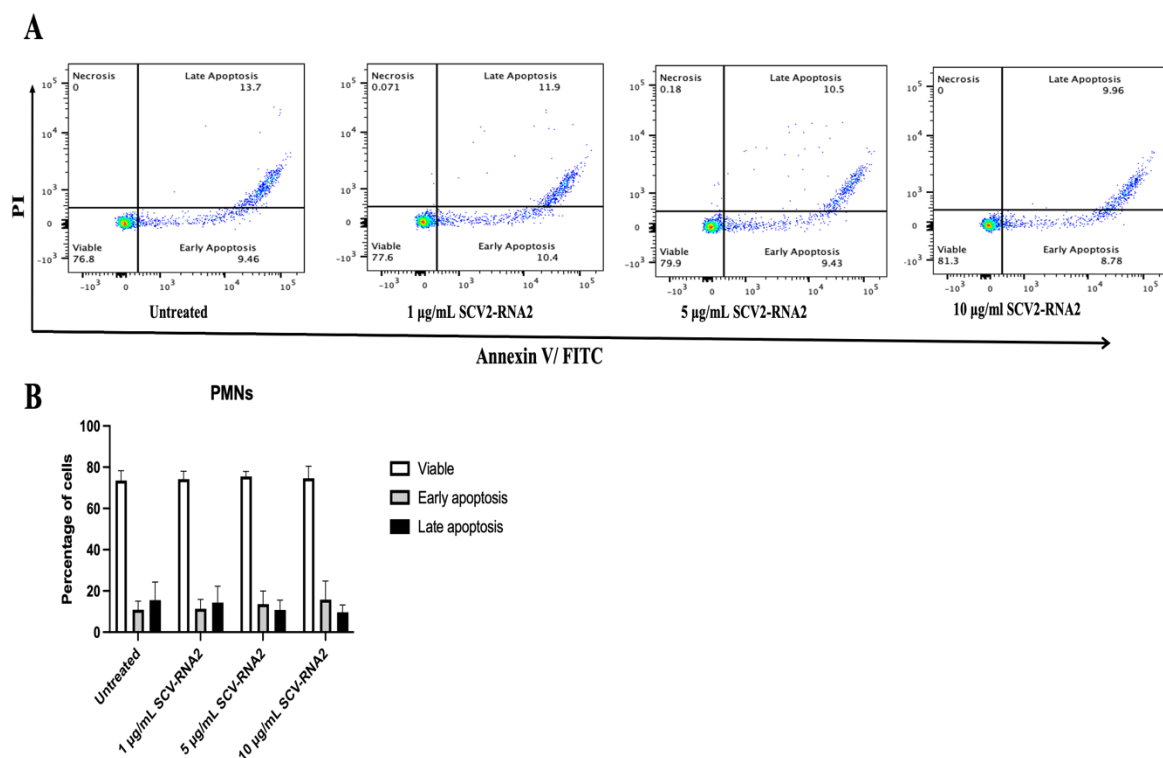


Figure 4.14 Flow Cytometric Analysis of Apoptosis in PMNs Treated with SCV2-RNA2.

PMNs were obtained through density gradient isolation and analysed by flow cytometry. PMNs (1×10^6) were treated with SCV2-RNA2 at concentrations of 0, 1, 5, and 10 $\mu\text{g/ml}$ for 6 hours. Apoptosis was assessed using flow cytometry with annexin V and PI staining. Annexin V-positive/PI-negative cells were identified as early apoptotic, while annexin V-

positive/PI-positive cells represented late apoptotic or necrotic populations. Untreated neutrophils served as the control. Results are presented as the percentage of dead cells. Data represent the mean $\pm$ SEM with statistical significance determined by one-way ANOVA.

4.4.12 SCV2-RNA2 has no Effects on human primary PMNs Phagocytosis

Phagocytosis by PMNs represents one of their primary defences against pathogens. Effective phagocytosis facilitates the clearance of invading microbes, whereas dysfunction in PMNs correlates with immune dysregulation, an increased incidence of secondary infections, and excessive inflammation, all of which characterise severe COVID-19. Given that the TLR7/8 signalling pathway regulates PMN function, we aimed to investigate whether SARS-CoV-2 ssRNA sequences can alter the phagocytic ability of neutrophils and consequently influence immune defence mechanisms. To assess this, PMNs were pre-treated with 10 μ g/mL of SCV2-RNA2 or its modified version, SCV2-RNA2A, for 4 hours before being incubated with GFP-labelled *Staphylococcus aureus* (MOI 1:100) for 1 hour. This experimental design enabled us to ascertain whether exposure to viral ssRNA affects the uptake of bacterial pathogens by neutrophils, thereby enhancing our understanding of how SARS-CoV-2 RNA may modulate neutrophil function and host defence during infection. Phagocytosis was analysed through flow cytometry of GFP-labelled bacteria uptake. The results indicated that there was no statistically significant change in the phagocytic index of untreated control neutrophils compared to those pre-treated with SCV2-RNA2 or SCV2-RNA2A. However, all groups exhibited similar bacterial uptake, suggesting that these stimuli do not influence the phagocytic capacity of neutrophils in this study (**Fig 4.15**).

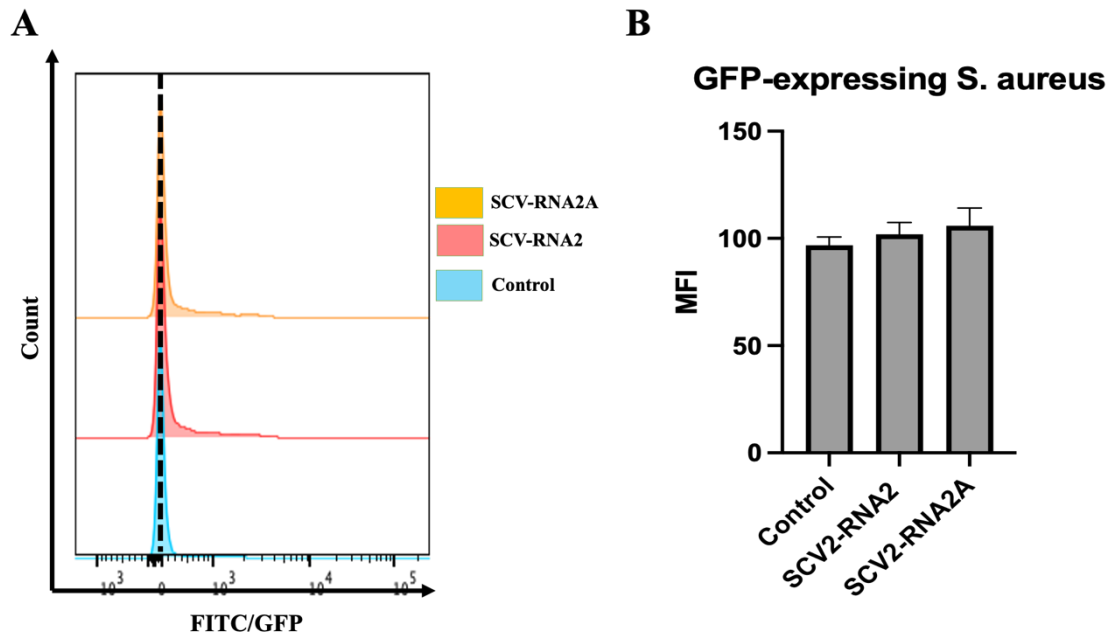


Figure 4.15 Analysis of PMN Phagocytosis Following Treatment with SARS-CoV-2 ssRNA Sequences.

Human PMNs were obtained through density gradient isolation and analysed by flow cytometry. PMNs (1×10^6) were pre-treated with $10 \mu\text{g/mL}$ of SCV2-RNA2 or SCV2-RNA2A for 4 hours and then incubated with GFP-labelled *Staphylococcus aureus* (MOI 1:100) for 1 hour. Phagocytosis was assessed using flow cytometry by quantifying the uptake of GFP-labelled bacteria. Untreated PMNs were included as a control. Flow cytometric analysis was performed using a standard gating strategy: cells were first gated based on FSC and SSC properties to exclude debris and select the PMNs population, followed by singlet discrimination using SSC-Area vs. SSC-Height, and finally gated for live cells using live/dead stain exclusion. (A) Representative flow cytometry histograms showing GFP fluorescence intensity distribution. (B) Quantitative analysis showing mean MFI of GFP uptake for each treatment condition. Data represent mean $\pm$ SEM. Statistical analysis was performed using one-way ANOVA.

4.4.13 SARS-CoV-2 ssRNA SCV2-RNA1 Induces Proinflammatory Cytokine Production in human Primary PMNs

PMNs produce pro-inflammatory cytokines that regulate disease progression and immune responses. Cytokine storm, or excessive cytokine production, is generally associated with severe COVID-19 pathology, characterised by systemic inflammation, tissue damage, and respiratory failure. Given that TLR7/8 recognises viral ssRNA and activates inflammatory

pathways, we aimed to verify whether SARS-CoV-2 ssRNA sequences can induce neutrophil cytokine production and thereby contribute to the cytokine storm. To assess this, PMNs were stimulated with 10 µg/ml of SARS-CoV-2 ssRNA sequences (SCV2-RNA2 and SCV2-RNA2A) or 1 µg/ml of the TLR7/8 agonist CL075 and cultured for 18 hours. Quantification of IL-6 (**Fig 4.16A**), IL-8 (**Fig 4.16B**), TNF-α (**Fig 4.16C**), and IL-1β (**Fig 4.16D**) secretion was carried out from the supernatants. This experiment was conducted to determine whether SARS-CoV-2 ssRNA can regulate inflammation mediated by neutrophils and may elucidate its role in immune dysregulation in COVID-19. The analysis revealed that IL-6 and IL-8 production was significantly enhanced in PMNs exposed to SCV2-RNA2 and CL075, while SCV2-RNA2A had no significant effect on these cytokines. TNF-α levels were also significantly increased in response to CL075 and showed a slight, but not statistically significant, increase with SCV2-RNA2 ($p=0.4$). Furthermore, the level of IL-1β was elevated in neutrophils exposed to SCV2-RNA2 ($p = 0.3$) and CL075 ($p = 0.2$); however, these increases were not statistically significant.

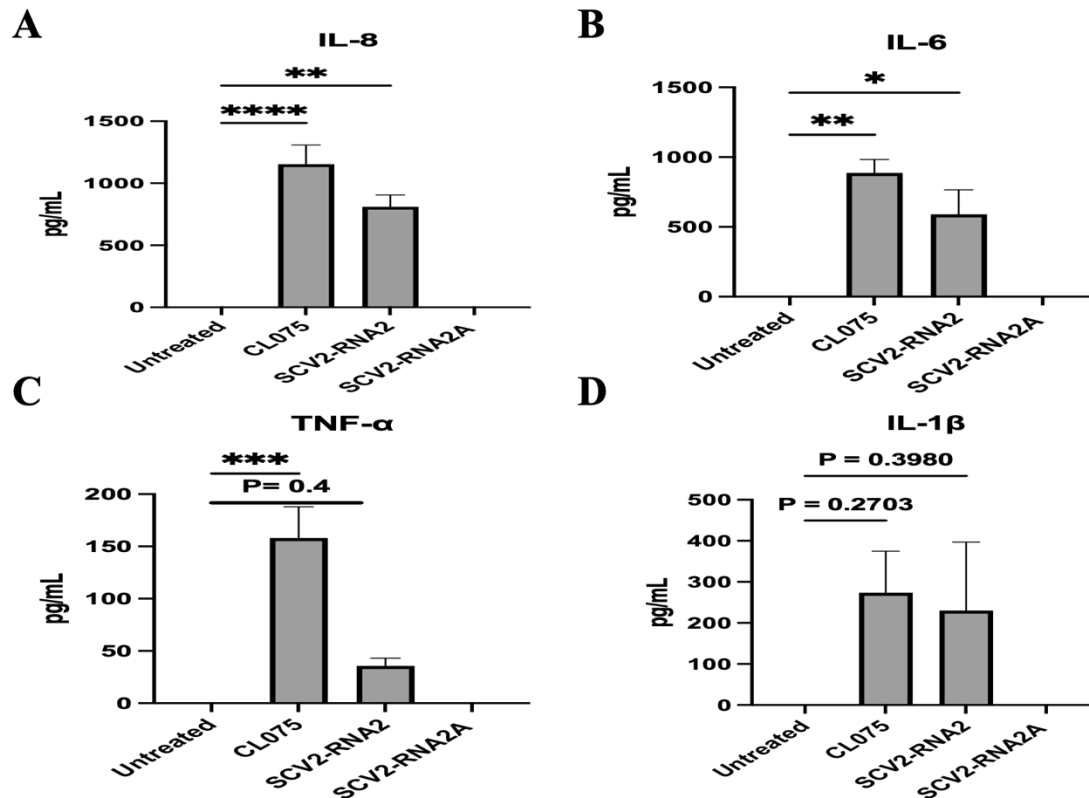


Figure 4.16 SARS-CoV-2 ssRNA Induces IL-8, IL-6, IL-1 β and TNF- α Production in Human PMNs

Human PMNs were obtained by density gradient isolation (n=4). PMNs (1×10^6) were treated with 10 μ g/ml of SCV2-RNA2, SCV2-RNA2A, or 1 μ g/ml CL075 for 18 hours. Levels of IL-8 (A), IL-6 (B), TNF- α (C), and IL-1 β in (D) the supernatant were measured ELISAs. Untreated neutrophils were included as a control. Results are expressed as mean cytokine concentration (pg/ml) $\pm$ Data represent the mean $\pm$ SEM with statistical significance determined by one-way ANOVA (*P < 0.05; **P < 0.01; ***P < 0.001 and ****P < 0.00001).

4.4.14 SCV2-RNA2-Induced Modulation of I κ B α Levels in PMNs: Activation and Recovery of NF- κ B Signalling

Since we observed that CV2-RNA2 stimulates proinflammatory cytokines, we subsequently evaluated the impact of SCV2-RNA2 on I κ B α levels in PMNs, a crucial regulator of NF- κ B pathway activation. The NF- κ B signalling pathway is a central regulator of inflammation and immune responses, and its activation is tightly controlled by I κ B α , which prevents NF- κ B from translocating to the nucleus under non-stimulated conditions. Upon stimulation by viral RNA through TLR7/8, I κ B α is degraded, which in turn activates NF- κ B and cytokine

production. To test this, PMNs were exposed to SCV2-RNA2 at 10 $\mu\text{g/mL}$ for 0, 2, and 4 hours, and I κ B α levels were analysed by western blot (**Fig 4.17**). The band intensity of I κ B α was normalised to the loading control (β -actin) to allow for a comparison of results between time points. The results indicated a decrease in I κ B α levels at 2 hours post-treatment compared to baseline (0 hours), suggesting the degradation of I κ B α and, consequently, the activation of the NF- κ B pathway. At 4 hours, however, I κ B α levels were significantly increased compared to 2 hours, indicating a recovery in I κ B α synthesis. This suggests that while SCV2-RNA2 initially triggers NF- κ B activation through I κ B α degradation, the pathway may begin to reset after prolonged exposure. The consistent loading control bands confirmed equal protein loading, thereby supporting the validity of the observed changes in I κ B α levels. The densitometry analysis supported these findings, showing a slight decrease at 2 hours followed by a significant increase at 4 hours.

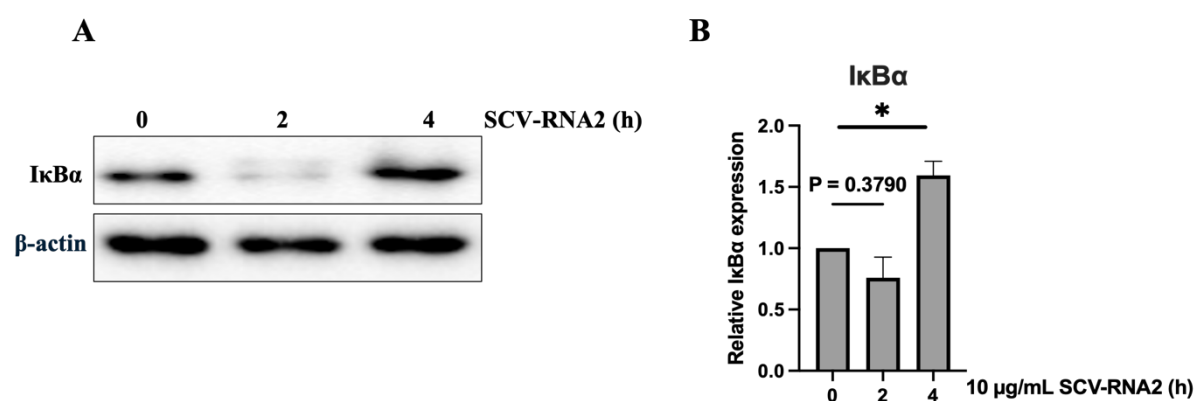


Figure 4.17 SCV2-RNA2-Induced I κ B α Degradation at 2 Hours and Restoration at 4 Hours in Human PMNs

Human PMNs were isolated by density gradient centrifugation (n=3) and treated with 10 $\mu\text{g/mL}$ SCV2-RNA2 for 0, 2, and 4 hours. (A) Representative western blot showing I κ B α protein levels with β -actin as a loading control. (B) Densitometric quantification of I κ B α expression normalized to β -actin, presented as mean $\pm$ SEM. Statistical analysis was performed using one-way ANOVA ($P < 0.05$).

4.4.15 NF- κ B, MAPK, and TLR8 Inhibitors Do Not Significantly Reduce Viability in Primary Human PMNs

To investigate the role of intracellular signalling pathways in the observed inflammatory cytokine production in human PMNs, we examined the effects of NF- κ B, MAPK, and TLR8 inhibitors on PMN viability. We initially tested the impact of these inhibitors on cell survival before analysing the cytokine pathways. PMNs were pre-incubated for 30 minutes with NF-

κ B (Bay-11) and MAPK inhibitors PD98059 for ERK and SB303580 for p38 at concentrations of 5 μ M and 20 μ M, respectively. CU-CPT9a, a TLR8 inhibitor, was also employed at 5 μ M. Following this, LPS (100 ng/mL) or CL075 (1 μ g/mL) was used to stimulate PMNs for 18 hours. Cell viability was assessed using fixable viability stain 510, with positive staining indicating dead cells, analysed by flow cytometry. When LPS or CL075 was used alone, viability was approximately 80% at 18 hours, indicating a moderate decrease in cell survival compared to the controls. No further reduction in cell viability was observed with NF- κ B, MAPK, or TLR8 inhibitors pre-treatment, with viability remaining around 80% in all conditions.

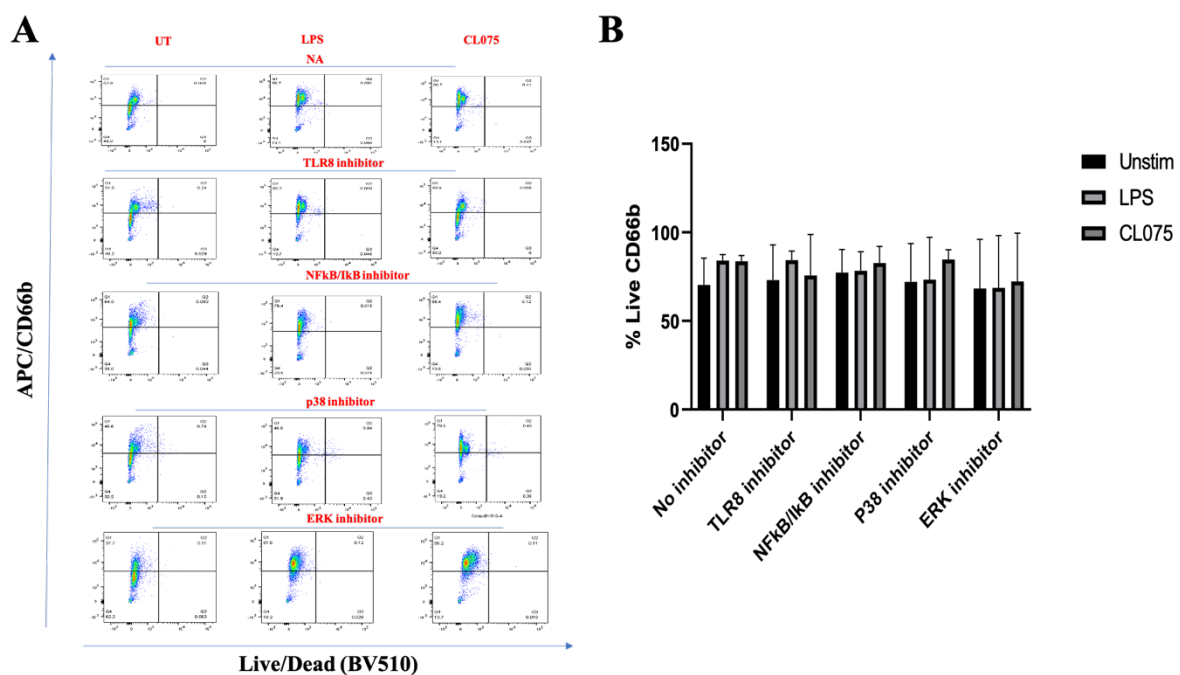


Figure 4.18 Impact of NF- κ B, MAPK, and TLR8 Inhibitors on PMN Viability After Stimulation with LPS or CL075.

Human PMNs were isolated using density gradient centrifugation (n=3). Cells (1×10^6) were pre-treated for 30 minutes with the indicated inhibitors: NF- κ B inhibitor Bay-11 (5 μ M), ERK inhibitor PD98059 (20 μ M), p38 MAPK inhibitor SB303580 (20 μ M), or TLR8 inhibitor CU-CPT9a (5 μ M). Following pre-treatment, cells were stimulated with LPS (100 ng/ml) or CL075 (1 μ g/ml) for 18 hours. Cell viability was assessed by fixable viability stain 510, with flow cytometric analysis identifying dead cells as positively stained events. (A) Representative flow cytometry dot plots illustrating cell viability across treatments and inhibitor conditions. (B) Quantification of cell viability expressed as the percentage of viable cells under each treatment condition. Results are presented as mean $\pm$ SEM.

4.4.16 SCV2-RNA2 Induces Proinflammatory Cytokine Production in PMNs via a TLR8-Dependent Mechanism

Having observed that the SARS-CoV-2 ssRNA sequence SCV2-RNA2 stimulates the production of pro-inflammatory cytokine proteins from primary human PMNs, we aimed to determine whether the increase in cytokine protein levels was TLR8-dependent. PMNs were pre-incubated with the TLR8 inhibitor CU-CPT9a for 30 minutes and subsequently stimulated with SCV2-RNA2 for 18 hours. Supernatants were collected for cytokine levels and analysed using ELISA. The results revealed an almost significant increase in the levels of TNF- α (Fig 4.19C) following 18 hours of stimulation with SCV2-RNA2. Additionally, IL-8 (Fig 4.19A), IL-6 (Fig 4.19B) and IL-1 β (Fig 4.19D) were significantly elevated after treatment with SCV2-RNA2. Pre-treatment with CU-CPT9a significantly reduced the production of IL-6, IL-8 and IL-1 β . Furthermore, CU-CPT9a nearly significantly reduced TNF- α levels. These findings demonstrate that the pro-inflammatory cytokine protein production induced by SCV2-RNA2 in PMNs occurs primarily through a TLR8-dependent mechanism. The data indicate that TLR8 inhibition resulted in significant decreases in IL-8, IL-1 β and IL-6 levels, with nearly significant reductions in TNF- α levels. (Fig 4.19).

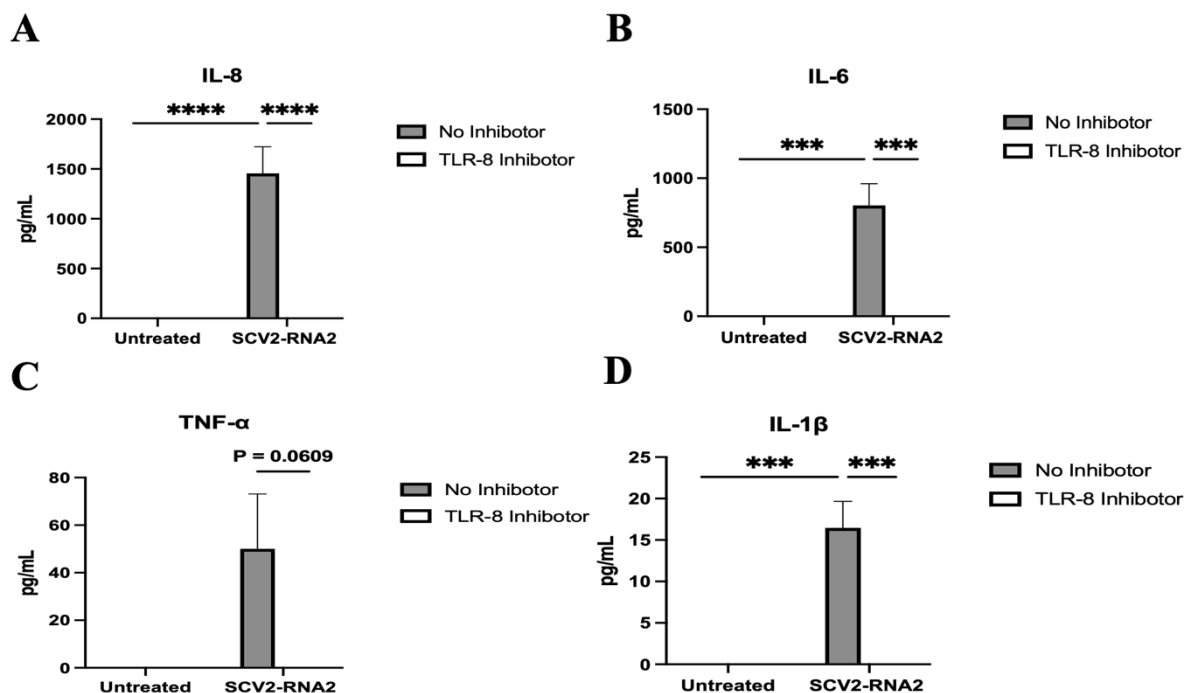


Figure 4.19 Proinflammatory Cytokine Production in PMNs Treated with SCV2-RNA2 and Inhibited by the TLR8 Inhibitor CU-CPT9a.

Human PMNs were obtained by density gradient isolation (n=3). PMNs (1×10^6) were treated with 10 $\mu\text{g/ml}$ of SCV2-RNA2 for 18 hours, with or without pre-treatment with the TLR8 inhibitor CU-CPT9a (30 minutes prior to stimulation). Cytokine protein levels, including IL-8 (A), IL-6 (B), TNF- α (C), and IL-1 β (D) were measured in the supernatants using ELISA. Data represent the mean $\pm$ SEM with statistical significance determined by two-way ANOVA (***P < 0.001 and **** P<0.00001).

4.4.17 TLR8 Inhibition by CU-CPT9a Attenuates SCV2-RNA2-Induced NF- κ B Activation in Primary Human PMNs

Next, we analysed the role of TLR8 in SCV2-RNA2-induced NF- κ B activation in primary human PMNs. Primary human PMNs were pre-incubated with the TLR8 inhibitor CU-CPT9a for 30 minutes and then stimulated with SCV2-RNA2 (10 $\mu\text{g/ml}$) for 2 hours. NF- κ B activation was determined by assessing the levels of I κ B α through western blot analysis, with β -actin serving as the loading control. The intensity of I κ B α bands was compared to β -actin, and the quantified data are represented in the bar graphs. The western blot shows that stimulation with SCV2-RNA2 for 2 hours results in a clear decrease in I κ B α levels compared to the unstimulated control (0 hours), suggesting that the NF- κ B signalling pathway is activated. Pre-incubation with CU-CPT9a led to a partial increase in the levels of I κ B α , indicating that blocking TLR8 reduces NF- κ B activation by SCV2-RNA2. While not statistically significant, densitometric analysis showed a 0.5 fold decrease in I κ B α levels upon stimulation with SCV2-RNA2, while I κ B α recovery was observed when CU-CPT9a was used (Fig. 4.20).

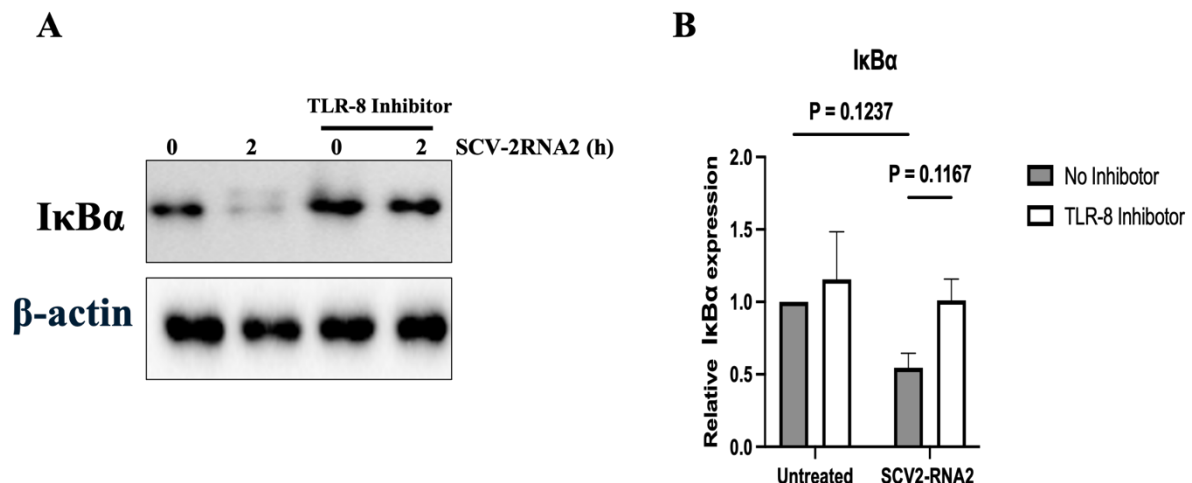


Figure 4.20 TLR8 Inhibition by CU-CPT9a Reduces SCV2-RNA2-Induced NF- κ B Activation in Primary Human Neutrophils

PMNs were obtained by density gradient isolation (n=3). PMNs (1×10^6 cells/mL) were pre-treated with the TLR8-specific inhibitor CU-CPT9a (5 μ M) for 30 minutes, followed by stimulation with SCV2-RNA2 (10 μ g/ml) for 4 hours. NF- κ B activation was assessed by measuring I κ B α protein levels through western blot analysis, using β -actin as a loading control. Densitometric analysis of I κ B α bands, normalized to β -actin, is presented as mean $\pm$ SEM with statistical significance determined by two-way ANOVA (*P < 0.05).

4.4.18 SCV2-RNA2 Induces Proinflammatory Cytokine Production in PMNs via a NF- κ B -Dependent Mechanism

Earlier findings, which emphasised the role of TLR8 in triggering pro-inflammatory cytokines in PMNs (**Fig 4.19**), led us to shift our focus to the downstream signalling pathways. In particular, we examined the role of NF- κ B in mediating this response. PMNs were pre-treated with the NF- κ B inhibitor Bay-11 (5 μ M) for 30 minutes and then stimulated with SCV2-RNA2 for 18 hours. The levels of cytokines in the collected supernatants were subsequently assessed by ELISA. The results indicated a significant increase in the levels of IL-8 (**Fig 4.21A**), IL-6 (**Fig 4.21B**), and TNF- α (**Fig 4.21C**) following stimulation with SCV2-RNA2. IL-1 β (**Fig 4.21D**) levels also increased, and this change approached significance. Pre-treatment with Bay-11 significantly decreased the levels of IL-6, IL-8, and TNF- α , while IL-1 β levels were reduced with a trend that nearly reached statistical significance.

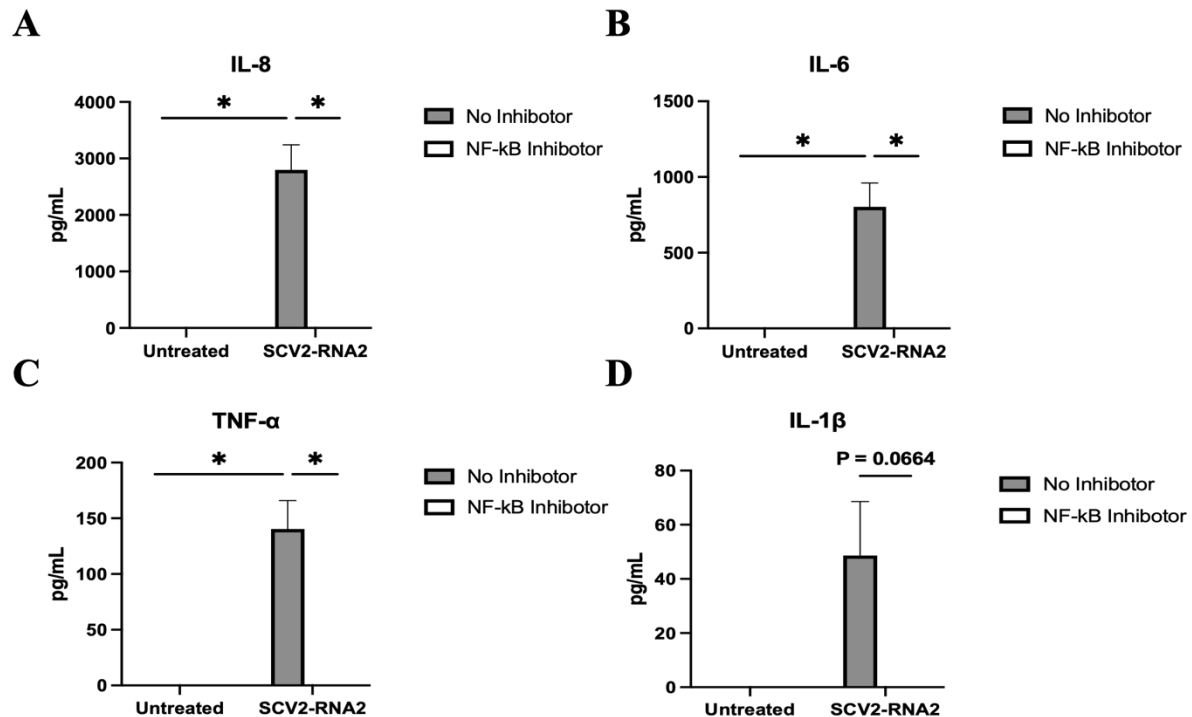


Figure 4.21 NF-κB Inhibition Blocks SCV2-RNA2-Induced IL-8, IL-6, TNF-α, and IL-1β Production in Human PMNs.

Human PMNs were obtained by density gradient isolation (n=3). PMNs (1×10^6) were pre-treated with the NF-κB inhibitor Bay-11 ($5 \mu\text{M}$) for 30 minutes before being stimulated with SCV2-RNA2 for 18 hours, and cytokine levels in the supernatants, including IL-8 (A), IL-6 (B), TNF-α (C), and IL-1β (D) were measured using ELISA. Data are presented as mean $\pm$ SEM, with statistical significance determined using a two-way ANOVA (* $p < 0.05$).

4.4.19 SCV2-RNA2 Induces Proinflammatory Cytokine Production in PMNs via a ERK -Dependent Mechanism

Having previously discovered that TLR8 is involved in regulating pro-inflammatory cytokines through PMNs (Fig. 4.19), we continued our analysis of the downstream signalling pathways involved. In particular, we next examined the role of ERK signalling in this response. PMNs were pre-treated with the ERK inhibitor PD98059 ($20 \mu\text{M}$) for 30 minutes before being stimulated with SCV2-RNA2 for 18 hours. The cytokine levels in the supernatants were then measured using ELISA. The results demonstrated a significant increase in the levels of IL-8 (Fig. 4.22A), IL-6 (Fig. 4.22B), and TNF-α (Fig. 4.22C) following SCV2-RNA2 stimulation compared to untreated controls. Levels of IL-1β (Fig. 4.22D) also rose, but as before (Fig. 4.21D), exhibited an almost significant change. The significant induction of IL-8, IL-6, and TNF-α, along with the near-significant induction of

IL-1 β , were lost upon pre-treatment with PD98059, indicating that SARS-CoV-2 ssRNA-induced cytokine production in neutrophils is ERK-dependent.

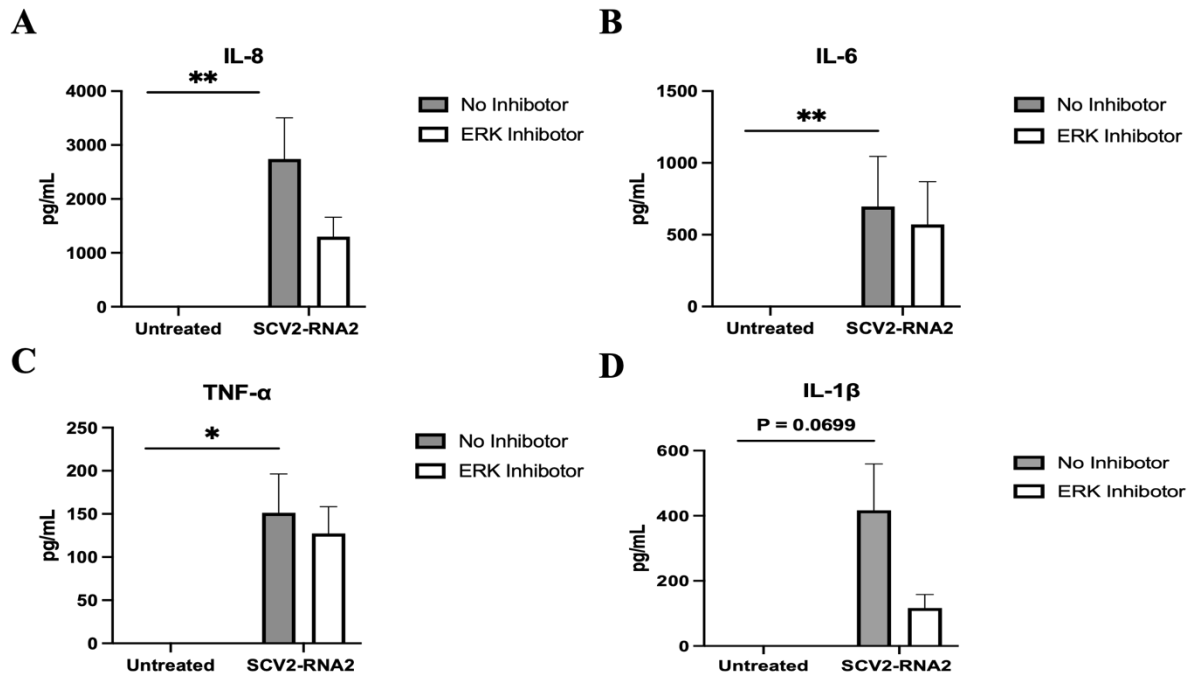


Figure 4.22 ERK Inhibition with PD98059 Reduces SCV2-RNA2-Induced Proinflammatory Cytokine Production in Primary Human PMNs.

Human PMNs were obtained by density gradient isolation (n=3). PMNs (1×10^6 cells/mL) were pre-treated with the ERK inhibitor PD98059 (20 μ M) for 30 minutes before stimulation with SCV2-RNA2 for 18 hours. Cytokine levels in the supernatants, including IL-8 (A), IL-6 (B), TNF- α (C), and IL-1 β (D) were measured using ELISA. Data are presented as mean $\pm$ SEM, and statistical significance was determined using a one-way ANOVA (* $p < 0.05$; and ** $p < 0.01$).

4.4.20 SCV2-RNA2 Induces Proinflammatory Cytokine Production in PMNs via a p38-Dependent Mechanism

After discovering that TLR8 is involved in the stimulation of pro-inflammatory cytokines in PMNs (Fig 4.19). Also NF- κ B inhibition (Bay-11) significantly reduced IL-6, IL-8, and TNF- α (Fig 4.21), while ERK inhibition (PD98059) showed non-significant reductions, suggesting partial ERK involvement (Fig 4.22). Next, we explored the role of p38 signalling in this response. To test this, PMNs were pre-incubated with a p38 inhibitor SB303580 (20 μ M) for 30 minutes before being activated with SCV2-RNA2 for 18 hours. Subsequently, an ELISA assessed cytokine levels in the supernatants. The results revealed a significant increase in the levels of IL-8 (Fig 4.23A), IL-6 (Fig 4.23B), and TNF- α (Fig 4.23C)

following SCV2-RNA2 stimulation compared to untreated controls. Levels of IL-1 β (Fig 4.23D) also increased but showed an almost significant change. The significant SCV2-RNA2-induced production of IL-8, IL-6, TNF- α , and IL-1 β was lost upon pre-treatment with SB303580, demonstrating that p38 MAPK signalling plays a critical role in mediating cytokine secretion in response to SARS-CoV-2 ssRNA.

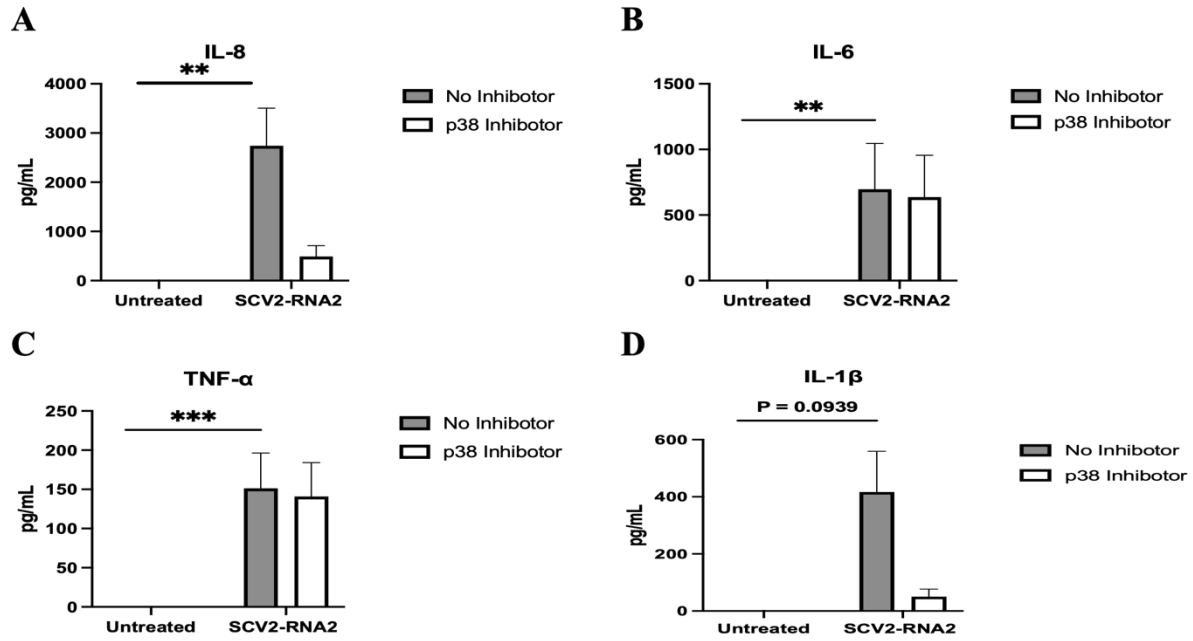


Figure 4.23 Effect of p38 Inhibition on SCV2-RNA2-Induced Proinflammatory Cytokine Production in Primary Human PMNs

Human PMNs were obtained by density gradient isolation (n=3). PMNs (1×10^6) were pre-treated with the p38 inhibitor SB303580 (20 μ M) for 30 minutes before stimulation with SCV2-RNA2 for 18 hours. Cytokine levels in the supernatants, including IL-8 (A), IL-6 (B), TNF- α (C), and IL-1 β (D) were measured using ELISA. Data are presented as mean $\pm$ SEM. Statistical significance was determined using one way ANOVA (**p < 0.01; and ***p < 0.001).

4.4.21 SCV2-RNA2 Induces ISG Induction in PMNs Following SCV2-RNA2 Stimulation

ISGs are key antiviral effectors that regulate viral restriction and immune defence. Since TLR7/8 signalling is known to induce ISG expression, this experiment aimed to assess whether SCV2-RNA2 treatment stimulates the mRNA expression of ISG15, PKR, and viperin in PMNs. mRNA levels were measured using qPCR, providing insight into the potential role of SCV2-RNA2 in TLR8-mediated antiviral immune responses. To further

understand the induction of these ISGs in response to viral RNA, primary human PMNs were stimulated with SCV2-RNA2 for 0, 2, and 4 hours. qPCR was performed to analyse gene expression levels. After 4 hours of stimulation, the levels of ISG15 (**Fig. 4.24A**), PKR (**Fig. 4.24B**), and viperin (**Fig. 4.24C**) were found to be significantly enhanced, thus confirming the gradual progression of the IFN signalling pathway activation in response to SCV2-RNA2. These results demonstrate that SCV2-RNA2 can lead to a strong ISG response in PMNs.

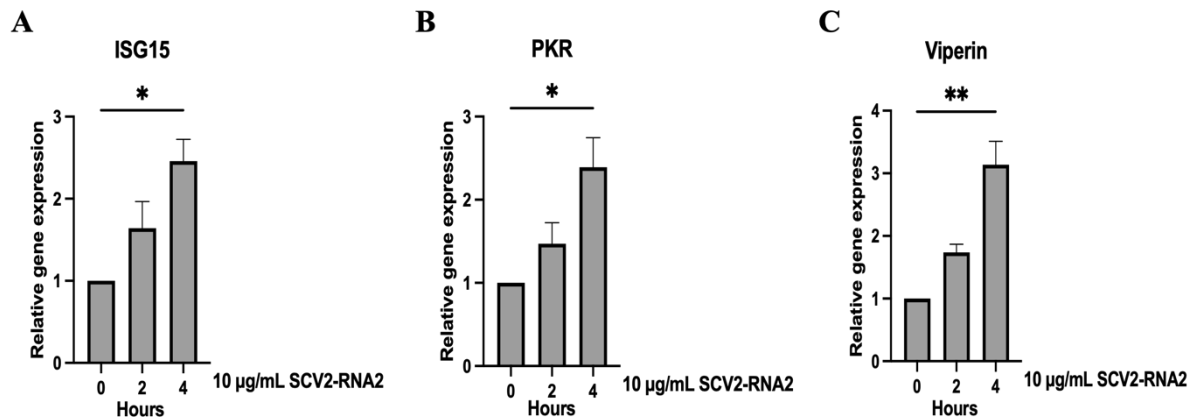


Figure 4.24 SCV2-RNA2 Stimulation Upregulates ISG15, PKR, and Viperin Expression in Human PMNs.

PMNs were isolated by density gradient centrifugation from healthy donors (n=3). PMNs (1×10^6) were stimulated with SCV2-RNA2 for 0, 2, and 4 hours. Total RNA was extracted and reverse transcribed to cDNA. Expression levels of ISGs ISG15 (A), PKR (B), and viperin (C) were analysed by qRT-PCR using gene-specific primers and normalized to the housekeeping gene β -actin. Data are presented as fold change relative to baseline (0h) calculated using the $2^{(-\Delta\Delta Ct)}$ method $\pm$ SEM. Statistical significance was determined using one-way ANOVA (* $p < 0.05$; ** $p < 0.01$).

4.4.22 dsRNA Does Not Upregulate Activation Marker Expression in Primary Human PMNs

During SARS-CoV-2 replication, dsRNA intermediates are formed and recognised as PAMPs by the innate immune system. Neutrophils, the first responders, recognise viral RNA through PRRs, potentially triggering immune activation and inflammation. Nevertheless, the effect of dsRNA on the activation of neutrophils remains unclear. To investigate this, we stimulated primary human PMNs with poly(I:C), a synthetic analogue of dsRNA, at 10 µg/mL for 2 hours, mimicking the presence of SARS-CoV-2 dsRNA intermediates. We then evaluated the surface expression of activation markers, CD66b (**Fig. 4.25A**), CD11b (**Fig.**

4.25B), CD16 (Fig. 4.25C), and CD62L (Fig. 4.25D) on PMNs by flow cytometry to determine whether there is a change in the level of PMN activation. Our analysis showed that poly(I:C) treatment did not result in significant alterations in the surface expression of any of the markers tested.

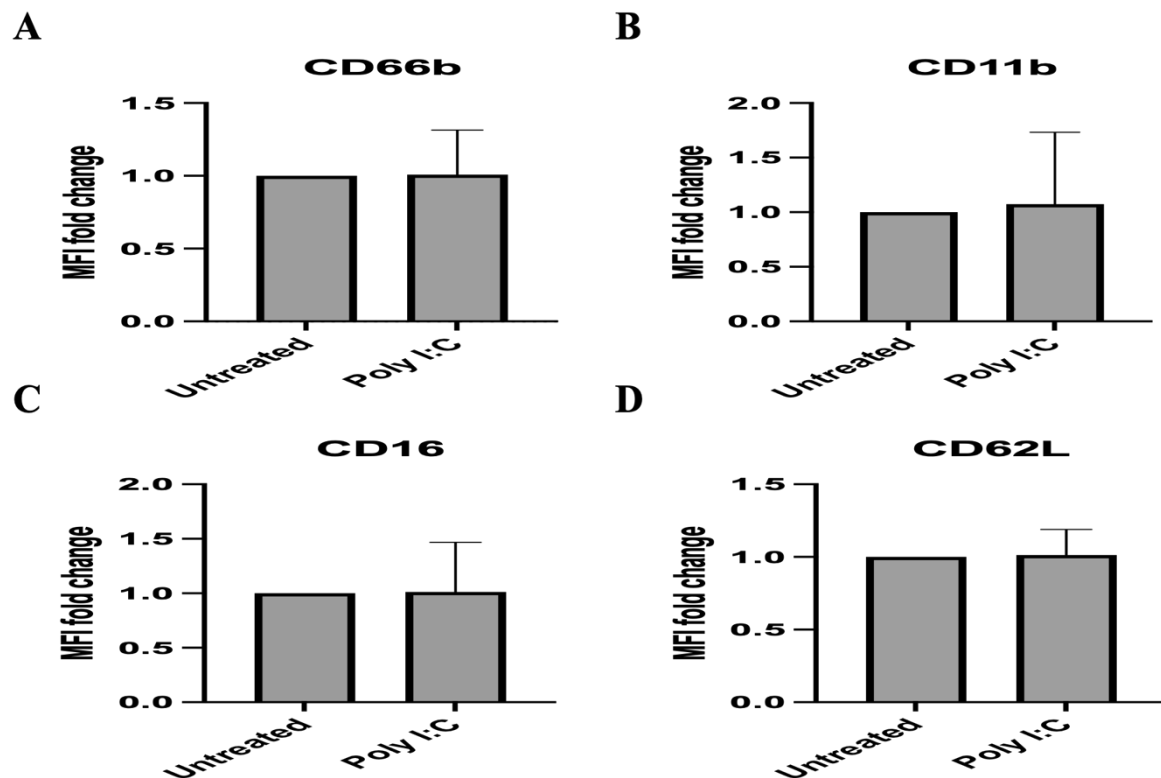


Figure 4.25 Effect of Poly(I:C) Treatment on PMNs Activation Markers

PMNs were isolated by density gradient centrifugation from healthy donors (n=3). PMNs (1×10^6) were stimulated with poly(I:C) (10 $\mu\text{g/mL}$) for 2 hours. Flow cytometric analysis was performed to assess PMN activation markers: CD66b (A), CD11b (B), CD16 (C), and CD62L (D). PMNs were identified by sequential gating on FSC and SSC properties, followed by singlet discrimination (SSC-A vs SSC-H) and live/dead staining to exclude doublets and non-viable cells. Surface marker expression was quantified on gated live neutrophil populations. Data represent MFI fold change relative to untreated controls $\pm$ SEM. Statistical comparisons were performed using one-way ANOVA.

4.4.23 dsRNA induces ROS production in Primary Human PMNs

To further explore the possible effects of dsRNA intermediates that are generated during the SARS-CoV-2 replication on PMNs, ROS production in PMNs was assessed following treatment with poly(I:C). PMNs isolated from healthy subjects were incubated with poly(I:C) (10 $\mu\text{g/mL}$) for 2 hours and the ROS production was assessed by flow cytometry.

The results showed a significant increase in ROS production by PMNs after poly(I:C) treatment compared to the untreated controls (Fig. 4.26).

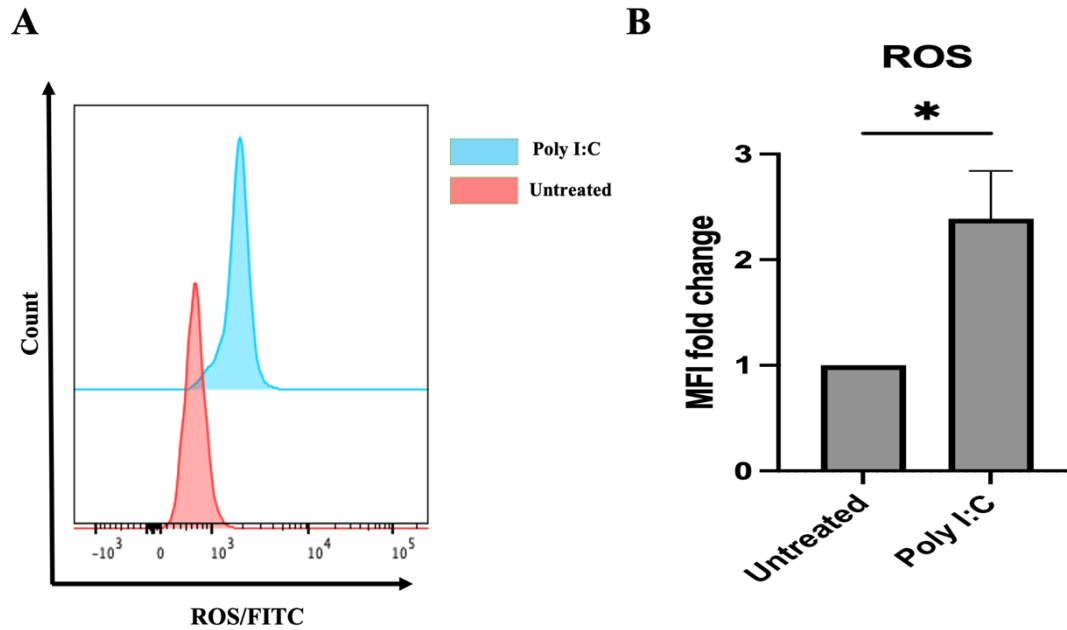


Figure 4.26 Effect of Poly(I:C) Treatment on ROS Production in Human PMNs

PMNs were isolated by density gradient centrifugation from healthy donors (n=3). PMNs (1×10^6) were stimulated with poly(I:C) (10 $\mu\text{g/mL}$) for 2 hours. ROS production was assessed by flow cytometry using CellROX Green Reagent. Cells were sequentially gated on FSC and SSC properties, singlet discrimination (SSC-A vs SSC-H), and live/dead staining to exclude doublets and non-viable cells before ROS analysis. (A) Representative histogram showing ROS fluorescence intensity distribution in untreated (red) versus poly(I:C)-treated (blue) PMNs from gated live cell populations. (B) Quantification of ROS production expressed MFI fold change relative to untreated controls $\pm$ SEM. Statistical significance was determined using unpaired t-test (* $p < 0.05$).

4.4.24: Poly(I:C) does not induce Proinflammatory Cytokine Production in Primary Human PMNs

Next, to explore the effects of dsRNA on cytokine production by PMNs, primary human PMNs were stimulated with poly(I:C) (10 $\mu\text{g/mL}$) for 18 hours. PMNs were also treated with CL075 (1 $\mu\text{g/mL}$) for 18 hours as a positive control. The production of IL-6 (Fig. 4.27A), IL-8 (Fig 4.27B), IL-1 β (Fig 4.27C), and TNF- α (Fig 4.27D) was assessed from the

culture supernatants using ELISA. The results revealed that poly(I:C) treatment led to no detectable induction of TNF- α or IL-1 β in PMNs. However, while not statistically significant, small amounts of IL-6 and IL-8 were produced in response to poly(I:C), indicating a mild activation of cytokine pathways. In contrast to poly(I:C), CL075 treatment induced robust production of IL-6, IL-8, and IL-1 β , and TNF- α .

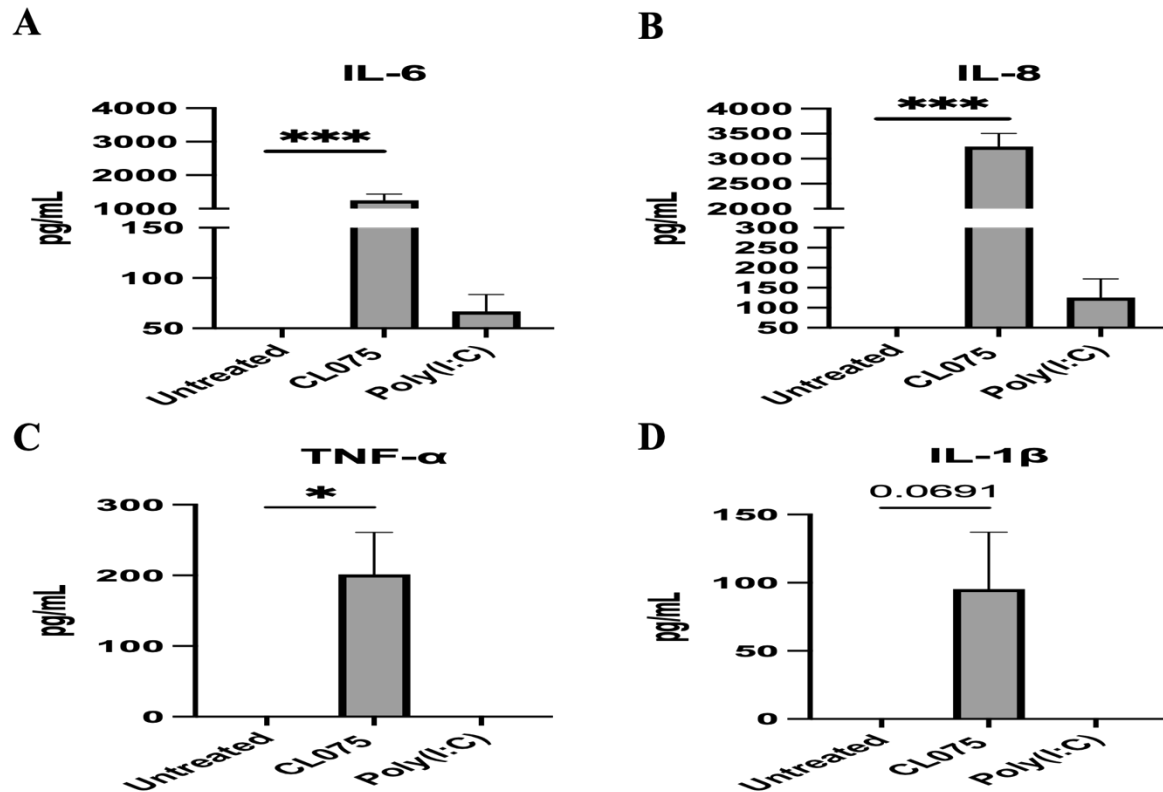


Figure 4.27 Effect of Poly(I:C) on Proinflammatory Cytokine Production in Primary Human PMNs

PMNs were isolated by density gradient centrifugation from healthy donors ($n=3$). PMNs (1×10^6) were stimulated with poly(I:C) (10 $\mu\text{g/mL}$) or CL075 (1 $\mu\text{g/mL}$) for 18 hours. Culture supernatants were collected and cytokine concentrations of IL-6 (A), IL-8 (B), IL-1 β (C), and TNF- α (D) were quantified by ELISA. Data are presented as mean cytokine concentration (pg/mL) $\pm$ SEM. Statistical significance was determined using one-way ANOVA (* $p < 0.05$; *** $p < 0.001$).

4.4.25: Poly(I:C) induces I κ B- α Degradation in primary human PMNs

Next, to understand the kinetics of I κ B- α degradation in response to dsRNA, primary human PMNs were treated with poly(I:C) (10 $\mu\text{g/mL}$) for 0, 5, 15, 30, 60, and 120 minutes. The expression of I κ B- α , a key regulator of the NF- κ B signalling pathway, was analysed by

Western blotting. Actin was also probed (as a loading control) to confirm similar protein loading across all time points. The results showed that I κ B- α degradation followed a clear time-dependent pattern. At 0 and 30 minutes, I κ B- α levels remained stable, indicating no immediate degradation. By 60 minutes, a noticeable reduction in I κ B- α was observed, which became further decreased at 120 minutes post-treatment. The actin blot confirmed consistent protein loading across all samples, supporting the validity of the observed changes. Densitometric analysis demonstrated a trend towards degradation at 60 minutes, becoming more evident by 120 minutes. The actin blot confirmed consistent protein loading across all samples, supporting the validity of the observed changes. Densitometric analysis, while not statistically significant, indicates a trend of I κ B- α degradation at 60 minutes, becoming more evident by 120 minutes. These findings indicate that poly(I:C) may trigger degradation of I κ B- α , leading to the activation of the NF- κ B signalling pathway in neutrophils.

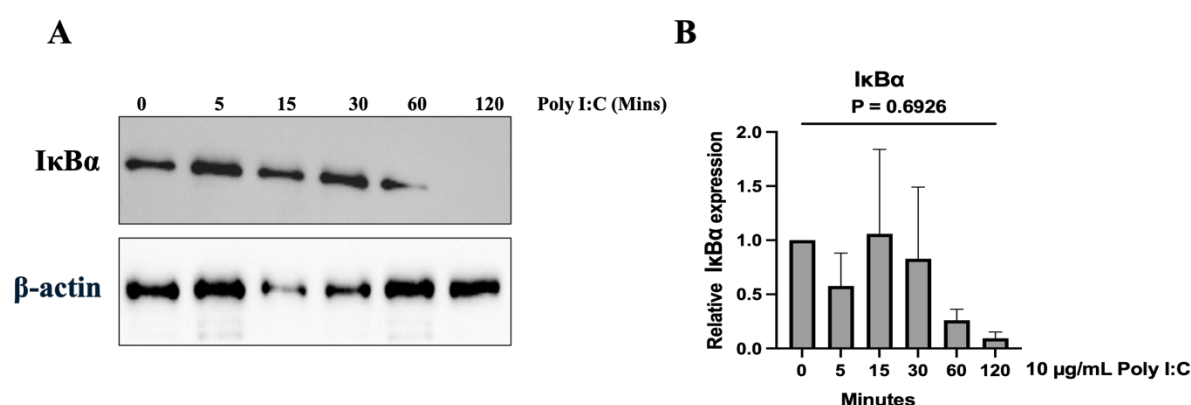


Figure 4. 28 Time-Dependent Degradation of I κ B- α in PMNs Treated with Poly(I:C)
Human PMNs were isolated by density gradient centrifugation (n=3) and treated with 10 μ g/mL poly(I:C) for 0, 5, 15, 30, 60, and 120 minutes. (A) Representative western blot showing I κ B α protein degradation over time, with β -actin as a loading control. (B) Densitometric quantification of I κ B α expression normalized to β -actin, presented as mean $\pm$ SEM. Statistical analysis was performed using one-way ANOVA

4.4.26 Poly(I:C) induces ISGs in primary human PMNs

Finally, to assess the effect of dsRNA upon ISGs in PMNs, primary human PMNs were stimulated with poly(I:C) (10 μ g/mL) for 0, 2, and 4 hours. The expression levels of PKR, viperin, and OAS were measured by qPCR. The results indicated a time-dependent trend in the expression of all three ISGs. While PKR, viperin, and OAS levels appeared to increase over the 4-hour period, these changes did not reach statistical significance, indicating that dsRNA does not induce a strong effect upon ISGs.

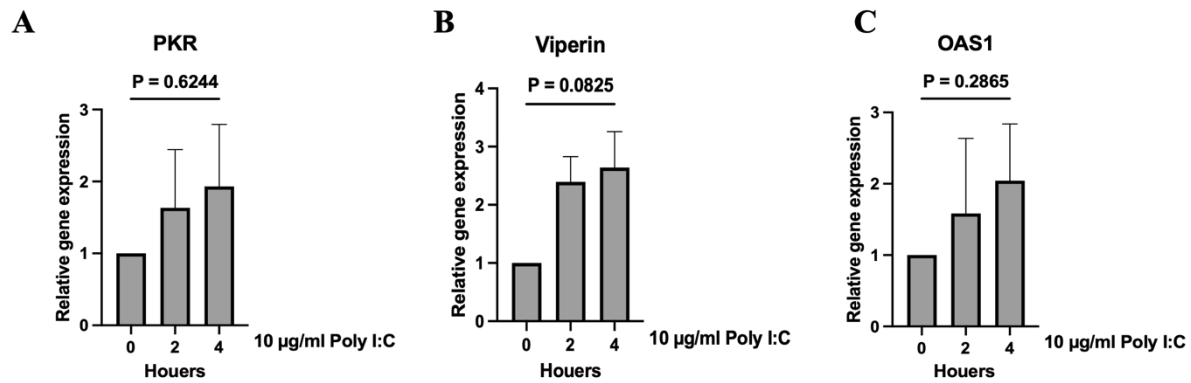


Figure 4. 29 Time-Course Analysis of ISGs Expression in Poly(I:C)-Treated PMNs

PMNs were isolated by density gradient centrifugation from healthy donors (n=3). PMNs (1×10^6) were stimulated with poly(I:C) (10 µg/mL) for 0, 2, and 4 hours. Total RNA was extracted, reverse transcribed to cDNA, and expression levels of ISGs PKR (A), viperin (B), and OAS (C) were analyzed by qRT-PCR using gene-specific primers. Gene expression was normalized to the housekeeping gene β -actin and calculated using the $2^{(-\Delta\Delta Ct)}$ method relative to untreated controls (0h). Data are presented as mean fold change $\pm$ SEM. Statistical significance was determined using one-way ANOVA.

4.5 Discussion

PRRs play a crucial role in recognising viral RNA and initiating innate immune responses. TLR7 and TLR8, well-characterised PRRs, specifically detect GU-rich ssRNAs and have been shown to identify various RNA viruses, including the influenza virus, HIV, and SARS-CoV-2. The recognition of viral RNA by TLR7 and TLR8 enables neutrophils and other immune cells to mount an appropriate inflammatory response to viral infections [533, 540, 542, 573]. Our findings reveal that PMNs robustly respond to SARS-CoV-2–derived GU-rich ssRNA (SCV2-RNA) through TLR8-dependent pathways. We observed that both the prototypic TLR7/8 agonist CL075 and the SARS-CoV-2 ssRNA SCV2-RNA2 significantly upregulated surface TLR8 on primary PMNs, consistent with reports that human neutrophils inherently express high levels of TLR8 compared to TLR7 [574]. Inhibition of TLR8 with CU-CPT9a markedly attenuated all SCV2-RNA2–induced responses (cytokine release, NF- κ B activation, adhesion molecule upregulation and NET formation), demonstrating that these effects are strictly TLR8-mediated [575]. Supporting this, studies by Salvi *et al.*, further demonstrated that viral ssRNA engages endosomal TLR7/8, activating MyD88-dependent IFN and inflammatory responses. These ssRNAs also stimulated human DCs, resulting in the production of cytokines, Th1 polarization, lung inflammation, immune cell

infiltration, and splenic DC maturation *in vivo* [533]. Similarly, Wang *et al.*, demonstrated that the influenza virus induces the migration of neutrophils and the secretion of cytokines, including IL-8. Their results also indicated that cytokine production depends on the viral entry into the neutrophil, rather than on viral replication, and that this process is influenced by endosomal acidification and viral uncoating. By employing knockout mice, their study identified TLR7 as a critical receptor for recognising the influenza virus and stimulating cytokine production in neutrophils [77].

4.5.1 TLR7/8 Expression in COVID-19 and Neutrophils

In our analysis of COVID-19 patient blood transcriptomes, TLR7 expression reached its highest point in moderate cases, while TLR8 expression continued to rise as disease severity worsened (**Fig 4.4**). The observed pro-inflammatory bias contradicts certain studies, which have found that TLR7/8 levels are decreased in patients with severe COVID-19 [576]. The observed difference could stem from variations between cell types (e.g. whole blood versus sorted cells) or disease progression stages. Viral infections are known to upregulate TLR8 rapidly; for example, TLR8 mRNA levels rise 2–6 hours after infections with influenza or parechovirus [577]. Our results showed that SARS-CoV-2 GU-rich ssRNAs treatment consistently elevated TLR8 surface expression on neutrophils (Sec. 4.3.6). The results indicate that TLR8 ligands (viral RNAs) enhance their sensing pathway through a feed-forward loop. Our results support the idea that GU-rich viral RNA sequences activate TLR8 and lead to its expression [577], even if some patient studies report overall TLR8 downregulation with severe illness.

4.5.2 PMN Activation Markers

The assessment of neutrophil activation involved measuring the expression of CD11b, CD66b, CD16, and CD62L. The SARS-CoV-2 ssRNAs (SCV2-RNA2) increased CD11b expression to a similar extent as the TLR7/8 agonist CL075 (Figure 4.4). This is consistent with previous studies that have shown TLR8 ligands increase CD11b on PMNs [575]. The levels of CD66b remained stable when exposed to viral RNA, as shown in (**Fig. 4.5**), consistent with Gardiman *et al.*, who found that SARS-CoV-2 ssRNAs did not alter CD66b levels [575]. These results indicate partial degranulation signalling: CD11b (a $\beta 2$ integrin) is mobilized, but specific granule markers like CD66b are not appreciably externalized by ssRNA alone. We also examined CD16 (Fc γ RIIIb) levels. Neither SARS-CoV-2 RNA nor CL075 caused classical CD16 shedding; in fact, CD16 surface MFI increased slightly in all treatments (**Fig. 4.8**). This contrasts with situations of strong activation (e.g. LPS) where CD16 is rapidly shed. Notably, Lood *et al.*, reported that TLR7/8 activation in immune

complex–stimulated neutrophils causes cleavage of FcγRIIA and a shift from phagocytosis to NETosis [553]. The key difference between our study and the findings of Lood *et al.*, likely arises from the stimuli used. R848 is a potent synthetic TLR7/8 agonist, designed to robustly stimulate these receptors. The absence of significant neutrophil activation in our study suggests that SCV2-RNA2 does not stimulate TLR7/8 as effectively as R848, or that other co-stimulatory factors are required for full activation. Additionally, a significant difference between our study and that of Lood *et al.*, is the phagocytic target. We assessed bacterial uptake (GFP-Staphylococcus aureus), whereas their study concentrated on immune complex phagocytosis, a process that heavily relies on Fcγ receptor function. Since R848 caused FcγRIIA shedding, it hindered neutrophil-mediated IC uptake, while our data indicated that SCV2-RNA2 does not affect neutrophil-mediated bacterial phagocytosis. This implies that the effects of TLR7/8 signalling may depend on the type of target and the immune environment. The CD16 marker remained intact, indicating that our stimuli activated PMNs in a specific manner. Overall, the SARS-CoV-2 ssRNAs triggered CD11b upregulation but did not affect CD66b or CD16 expression levels, thus indicating a moderate activation state that matches TLR8 engagement.

4.5.3 Pro-inflammatory cytokine production

PMNs treated with SCV2-RNA2 secreted high levels of IL-8 and IL-6 and modest increases in TNF-α and IL-1β. Pre-treatment with the TLR8 antagonist CU-CPT9a significantly reduced SCV2-RNA2–induced IL-6, IL-8, IL-1β and TNF-α, confirming that cytokine induction is largely TLR8-dependent. These findings are consistent with prior work: Gardiman *et al.*, similarly reported that SCV2 GU-rich RNAs strongly stimulate neutrophils to release TNF-α and IL-8 via TLR8, and that blockade of TLR8 abrogates these cytokine responses [575]. We similarly observed IL-8 release, and, interestingly, a significant IL-6 increase in response to our CL075 positive control (and to a lesser extent to SCV2-RNA2). Gardiman *et al.* reported no IL-6 release with viral RNA. The differences may be due to the stronger agonism of CL075 (our R848 analog) and donor variability. Importantly, NF-κB inhibition (Bay-11) almost completely abrogated IL-6, IL-8, and TNFα induction by SCV2-RNA2, confirming that these cytokines depend on the MyD88–NF-κB axis. ERK and p38 MAPK played milder roles: ERK inhibition partially reduced IL-6/IL-8, and p38 inhibition mainly limited IL-8 (**Fig. 4.3.19–21**). This is consistent with poly(I:C) studies showing that MAPKs contribute to TLR-mediated cytokine expression. In summary, SARS-CoV-2 GU-rich RNA drives neutrophils to secrete IL-8 and IL-6 (and modest TNFα), via a canonical TLR8–MyD88–NF-κB pathway, in agreement with TLR7/8 biology. Importantly, the

cytokines we observed (IL-6, IL-8, TNF- α) are key mediators of the “cytokine storm” in COVID-19; severe patients show elevated plasma IL-6, IL-8 and TNF- α alongside neutrophilia [575, 578]. Our data therefore suggest that viral ssRNA motifs can directly contribute to this hyperinflammatory milieu by driving neutrophil-derived cytokines via TLR8.

4.5.4 ROS and Degranulation

We next assessed classical antimicrobial responses. Remarkably, neither SARS-CoV-2 ssRNAs nor CL075 stimulated significant ROS production in neutrophils (**Fig. 4.10**). This finding is corroborated by Gardiman *et al.*, who also reported that SARS-CoV-2 ssRNAs and R848 were “very poor stimuli” for superoxide release [575]. Thus, viral GU-rich RNA does not efficiently trigger the oxidative burst, unlike strong activators such as PMA. Similarly, SARS-CoV-2 RNA did not induce MPO degranulation; in fact, all treatments (SCV2-RNA, SCV2-RNA-A, CL075) caused a slight decline in released MPO relative to control (Figure 4.10). This contrasts with Gardiman’s report that SCV2-RNA2 promoted rapid release of azurophilic granule enzymes (elastase and lactoferrin) at levels comparable to R848 [575]. The discrepancy may be methodological: we assessed degranulation by measuring MPO release, which reflects azurophilic granule exocytosis, whereas their study focused on elastase and lactoferrin, representing both azurophilic and specific granules. These differences in granule markers and detection methods could explain the contrasting outcomes. In any case, our data suggest that SARS-CoV-2 GU-rich RNA does not strongly activate oxidative or degranulation pathways in neutrophils. These “null” results are novel and noteworthy: they indicate that neutrophil microbicidal functions remain muted upon single TLR8 stimulation by viral RNA, highlighting that additional signals (e.g. cytokines or immune complexes) may be required for a full oxidative/degranulation response.

4.5.5 NETosis

Given reports of elevated NETs in COVID-19, we evaluated extracellular trap formation. Surprisingly, we observed no NETosis in response to SARS-CoV-2 ssRNAs within our 6-hour assay (**Fig. 4.12**). This negative result contrasts sharply with Gardiman *et al.*, who showed that a GU-rich SARS-CoV-2 ssRNA (SCV2-RNA2) potently induced NET release – more effectively than R848 [575]. Their data indicated approximately a two-fold increase in DNA-associated elastase release with SCV2-RNA2. The lack of NET induction in our experiments could stem from differences in conditions. For instance, in the study by Gardiman *et al.*, NETosis was assessed after 4 hours of stimulation using two complementary methods: (1) a DNA–elastase sandwich ELISA, where culture supernatants

were analyzed using NE-coated plates and a peroxidase-labeled anti-DNA antibody to quantify NET-derived complexes; and (2) fluorescence microscopy, where fixed neutrophils were stained with anti-elastase and DAPI to visualize NET structures and confirm extracellular DNA release. Importantly, their methodology included serum-free conditions and high-purity PMNs stimulated in HBSS buffer, which is known to preserve NETotic activity. The absence of serum is critical because serum components, particularly albumin, can scavenge ROS and inhibit histone citrullination, both of which are essential for NET formation [579]. Therefore, serum-free conditions likely facilitated a stronger NETotic response in their assays compared to our serum-containing setup. In contrast, our experimental setup involved RPMI 1640 supplemented with 10% FBS and the use of SYTOX Orange fluorescence in combination with flow cytometry to quantify extracellular DNA. While SYTOX Orange is highly specific for detecting membrane-impermeable DNA, this method may not capture early or subtle chromatin decondensation events, potentially underestimating the extent of NET formation compared to microscopy-based approaches. Furthermore, the presence of serum albumin is recognized to inhibit NETosis by scavenging cationic histones and interfering with ROS production pathways necessary for trap formation.

4.5.6 Anti-inflammatory Signalling and Neutrophil Lifespan

We further examined anti-inflammatory outputs and neutrophil fate. The SARS-CoV-2 ssRNAs did not affect the production of TGF- β by PMNs (**Fig. 4.13**). This suggests that TLR8 activation does not lead to TGF- β secretion in human neutrophils and is therefore unlikely to be responsible for the elevated TGF- β levels seen in severe COVID-19 [290]. The spontaneous apoptosis assays revealed a minimal non-statistically significant delay in cell death following ssRNA treatment (Figure 4.13). In contrast, the study by Tamassia *et al.*, demonstrated that viral RNA extended neutrophil survival time, but to a lesser extent than R848 [575]. Our failure to detect a strong anti-apoptotic effect may reflect the milder activation by SCV2-RNA or technical differences in assay timing. Finally, phagocytosis of GFP-Staphylococcus aureus was unchanged by SARS-CoV-2 RNA (**Fig. 4.15**). While Lood *et al.*, showed that potent TLR7/8 activation leads to Fc γ receptor cleavage and a shift away from phagocytosis, our stimuli did not induce this effect [553]. In other words, neutrophils maintained normal phagocytic function despite ssRNA exposure. Taken together, SARS-CoV-2 GU-rich RNA did not enhance anti-inflammatory (TGF- β) or clearance (phagocytosis) functions in neutrophils; rather, it selectively triggered inflammatory cytokines without extending lifespan appreciably.

4.5.7 ISG induction by SARS-CoV-2 RNA in PMNs

Another crucial aspect of our study is the activation of ISGs, which were significantly upregulated by SCV2-RNA2. Levels of ISG15, PKR, and viperin were markedly elevated in a time-dependent manner, peaking at 4 hours post-stimulation, indicating that neutrophils can mount antiviral signalling alongside their traditional role in inflammation. Interestingly, these findings contrast with those of Gardiman *et al.*, who reported no induction of ISGs (IFNA, IFNB1, IFIT1, and ISG15) in neutrophils treated with SARS-CoV-2 ssRNAs at 6 hours [575]. Several key differences may explain this discrepancy. First, our study examined earlier time points (0, 2, and 4 hours), detecting significant ISG induction at 4 hours. This suggests that ISG expression in neutrophils may peak early and decline before the 6-hour time point examined by Gardiman *et al.*, Second, we specifically measured ISG15, PKR (EIF2AK2), and viperin (RSAD2), which are antiviral effector proteins that can be induced independently of type I interferon production. While Gardiman *et al.*, confirmed the absence of type I interferon induction in their study, our detection of ISG15, PKR, and viperin indicates that SARS-CoV-2 ssRNA triggers a distinct antiviral gene expression program in neutrophils that does not require interferon production. Third, our targeted RT-qPCR approach may provide greater sensitivity for detecting moderate changes in specific ISG transcripts compared to RNA-seq at later time points. Thus, our results reveal a novel early and transient antiviral gene expression response in neutrophils to SARS-CoV-2 ssRNA that was not captured in previous studies.

4.5.8 Double-Stranded RNA (Poly I:C) Responses

Poly I:C stimulates neutrophils and induces antiviral responses through cytoplasmic RNA helicases, including MDA5 and RIG-I. Tamassia *et al.*, (2008) demonstrated that poly(I:C) activates human neutrophils, resulting in the production of type I IFN, immunoregulatory cytokines, and ISGs. Key signalling pathways identified include MAPK, PKR, NF- κ B, and TBK1/IRF3. It appears that the cytoplasmic RNA helicases MDA5 and RIG-I, rather than endosomal TLR3, are essential for neutrophil antiviral responses. Furthermore, MDA5-deficient neutrophils exhibited reduced production of IFN- β and TNF- α in response to viral stimulation, thus highlighting the importance of helicase-dependent antiviral signalling [580]. In our experiment, treating PMNs with poly(I:C) resulted in a two-fold increase in ISGs (PKR, viperin, OAS) (**Fig. 4.29**), indicating a relatively moderate activation of the antiviral response. Moreover, we observed a trend towards I κ B α degradation; however, this effect was not statistically significant, suggesting that the NF- κ B pathway was partially activated. These findings indicate that poly(I:C) can act as an activator of antiviral signalling

in neutrophils, but the response was not as robust as anticipated, potentially due to inadequate intracellular uptake or the need for additional co-factors for full activation. Additionally, we noted a slight increase in IL-6 and IL-8 production following poly(I:C) treatment, suggesting some modulation of proinflammatory cytokine signalling. We did not observe any significant changes in CD66b, CD11b, CD16, or CD62L expression; therefore, it appears that poly(I:C) does not enhance the level of neutrophil activation or adhesion properties under the conditions used. Furthermore, ROS production was significantly elevated following poly(I:C) stimulation, indicating activation of the oxidative machinery.

4.6 Future Work

Targeting TLR8 for Therapeutic Intervention: The role of TLR8 in neutrophil activation by SARS-CoV-2 ssRNAs suggests it is a potential therapeutic target. Small molecule inhibitors, such as CU-CPT9a, may provide a means to suppress excessive inflammation while preserving essential antiviral immune responses. Combining TLR8 inhibitors with other anti-inflammatory therapies may assist in managing the hyperinflammation observed in COVID-19.

Comparing Viral ssRNAs Across Pathogens: SARS-CoV-2 ssRNAs possess unique GU-rich motifs that strongly activate TLR8. By comparing these ssRNAs with those from other RNA viruses, such as the influenza virus, RSV, and HIV, we can identify both shared and distinct RNA characteristics that influence immune activation. Such comparative studies may deepen our understanding of ssRNA-TLR8 interactions and guide the development of new antiviral therapies or vaccines.

Understanding Co-stimulatory Pathways: SARS-CoV-2 ssRNAs likely function alongside other immune pathways to promote neutrophil activation. TLR8-mediated responses may be enhanced by co-stimulatory signals such as type I IFN, DAMPs, or other PRRs. Investigating these relationships could reveal further regulatory mechanisms and provide additional targets for therapeutic intervention regarding the dysfunctional immune response in severe COVID-19.

Mapping the Timeline of Neutrophil Activation: Temporal studies are essential for understanding how neutrophils respond to SARS-CoV-2 ssRNAs over time. Identifying when TLR8 signalling peaks and then resolves could establish therapeutic windows for intervention and the prevention of prolonged inflammation. Such insights may be critical for minimising tissue damage while ensuring adequate immune activation to combat the virus.

Investigating Alternative Pathways: Although TLR8 plays a vital role in the response to SARS-CoV-2 ssRNAs, several other signalling mechanisms may also contribute to neutrophil responses. Cytosolic RNA sensing, including RIG-I-like receptors and other nucleic acid detection mechanisms, could play an additional role. Investigating these pathways may enhance our understanding of how neutrophils are activated and how they can be therapeutically targeted.

4.7 Conclusion

The findings presented in this chapter illuminate the role of TLR8 in recognising GU-rich SARS-CoV-2 ssRNAs and regulating neutrophil-driven inflammation. Neutrophil stimulation with SCV2-RNA2 induced cytokine secretion and NF- κ B activation, while TLR8 signalling was identified as a crucial initial trigger. Additionally, these findings suggest that SCV2-RNA2 selectively activates neutrophils by upregulating adhesion markers CD11b and TLR8 expression but does not induce classical neutrophil effector functions, such as ROS production, degranulation, NETosis, or phagocytosis. This selective activation may contribute to SARS-CoV-2-induced immune dysregulation, where neutrophils are recruited and primed but do not exhibit full antimicrobial functions, potentially leading to chronic inflammation without effective pathogen clearance. Further studies are required to determine whether additional factors, such as secondary infections or cytokine signalling, influence neutrophil activation in the context of SARS-CoV-2 infection. The TLR8 signalling pathway represents a potential target for therapeutic intervention, and additional studies are needed to explore this in both preclinical and clinical settings to address neutrophil-mediated injury in SARS-CoV-2 infection. This work also enhances our understanding of the inflammatory mechanisms underlying COVID-19 by identifying TLR8-mediated pathways and establishes a foundation for immunomodulatory targeted therapies.

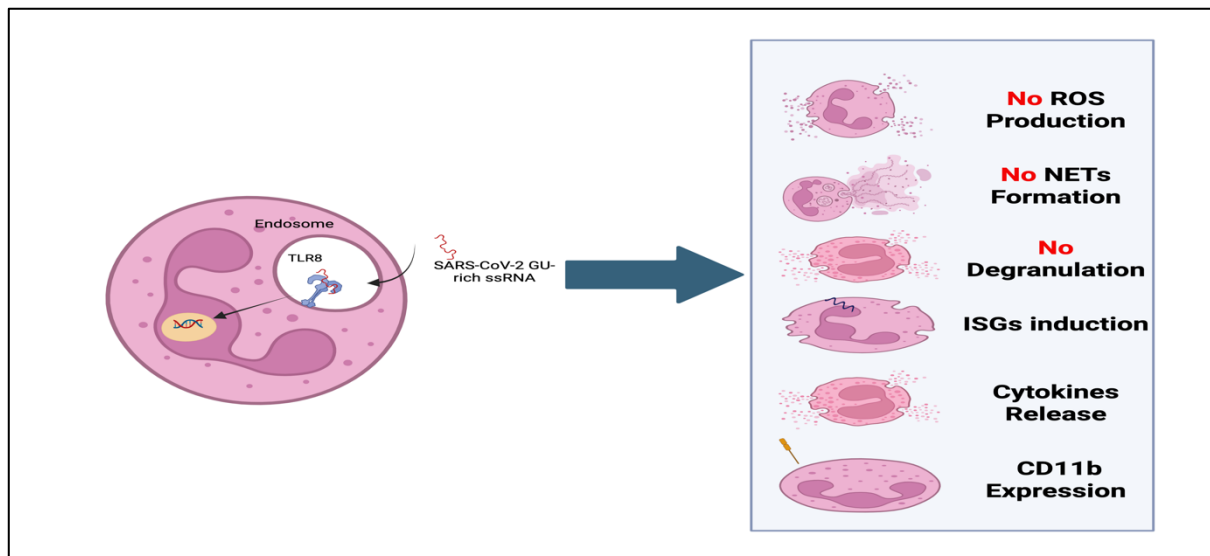


Figure 4.26. Summary of TLR8-Mediated Neutrophil Responses to SARS-CoV-2 GU-Rich ssRNA

This schematic summarizes the functional responses of human PMNs to SARS-CoV-2–associated GU-rich ssRNA via endosomal TLR8 signalling. Upon internalization, viral ssRNA engages TLR8, initiating selective intracellular signalling pathways. Our findings demonstrate that this stimulation leads to:

- **No ROS production.**
- **No NET formation.**
- **No granule degranulation (MPO release).**
- **Significant upregulation of ISGs** including viperin, PKR, and ISG15.
- **Proinflammatory cytokine release**, notably IL-6 and IL-8.
- **Increased surface expression of CD11b**, indicating partial neutrophil activation.

Chapter 5:

PMNs and Their Role in SARS-CoV-2 Infection: Direct and Indirect Interactions

5.1 Introduction

SARS-CoV-2 primarily enters host cells through SP's attachment to the ACE2 receptor, often with the assistance of cellular proteases like TMPRSS2 [581]. Neutrophils are not considered a primary target of SARS-CoV-2 because of the low expression of ACE2 [582]. However, research has found that SARS-CoV-2 variants of concern (VOCs) can activate human neutrophils *in vitro*, suggesting different mechanisms of viral entry [385]. These findings raise important questions about the role of direct neutrophil infection in the systemic inflammatory response seen in COVID-19 and the more general implications for neutrophil biology in viral infections. Besides the possible direct infection, the secretory profile of airway epithelial cells in SARS-CoV-2 infection significantly impacts neutrophil activation and behaviour. SARS-CoV-2 primarily attacks airway epithelial cells and produces cytokines, chemokines, and DAMPs. These soluble factors have vigorous activating activities and act as moderators of neutrophil function [583]. However, the composition and functions of supernatants derived from epithelial cells are diverse and depend on the strain of SARS-CoV-2. If factors like pathogenicity and the ability to trigger an inflammatory response are anything to go by, they should differ. Differences in cytokine and chemokine production can occur between strains, affecting neutrophil recruitment, activation, and effector functions and, therefore, the severity of the immune response [584]. This chapter has two primary objectives to address these critical gaps. The first is to explore whether neutrophils can be directly infected by SARS-CoV-2 and examine how viral presence affects neutrophil phenotype, ROS production, NETs formation, degranulation, and cytokine secretion. The second is to analyse the effects of supernatants from airway epithelial cells infected with various SARS-CoV-2 strains on neutrophil behaviour. Thus, by defining the strain-specific differences in mediators produced by epithelial cells and their impact on neutrophils, this study aims to decipher the interplay between SARS-CoV-2 strains and the host immune system. These investigations will result in novel insights into the direct and indirect interactions between neutrophils and SARS-CoV-2. This work will help advance current knowledge of the systemic immune response during COVID-19 and may inform the development of future therapeutic strategies aimed at reducing neutrophil-mediated inflammation or lessening the strain-specific effects of SARS-CoV-2.

5.2 Neutrophil Responses to SARS-CoV-2 Variants: Implications for COVID-19

Severity

Neutrophils exhibit an array of responses to SARS-CoV-2 VOCs, such as Delta and Omicron, according to the different structures of SPs, which trigger different immune activation pathways and inflammatory responses. In this *in vitro* study of human neutrophils, the S1 protein of the SARS-CoV-2 Omicron variant induced significant reactive oxygen species (ROS) production, whereas the ancestral Wuhan S1 protein did not. Immune complexes formed with Wuhan S1 and anti-spike IgG1 strongly triggered ROS production and degranulation (MPO release), while Omicron S1 immune complexes failed to elicit the same response [585]. Additionally, the neutrophils obtained from COVID-19 patients are highly heterogeneous, with large variation in activation states depending on disease severity. In severe cases, neutrophils are hyperactive and become a part of a pathologically exaggerated immune response. This hyperactivation is characterized by excessive degranulation of neutrophils, which releases toxic granule contents, such as MPO, neutrophil elastase, and defensins. These molecules destroy pathogens and, but through their overactivation, lead to worsening inflammation and thus ARDS. Furthermore, these hyperactivated neutrophils produce higher levels of proinflammatory cytokines and chemokines, including IL-6, TNF- α and IL-8, further escalating the inflammatory response and attracting other immune cells to the infected area [586]. This heightened neutrophil activity also leads to the formation of neutrophil NETs. While NETs serve to trap and neutralize pathogens, their overproduction in severe COVID-19 cases has been linked to widespread tissue damage, thrombosis, and multiorgan dysfunction. Additional research has shown that the most severe forms of the disease are associated with an increased number of immature neutrophils in the blood which are ineffective in controlling pathogens, but are highly inflammatory because of their increased reactivity [586]. Moreover, in severe COVID-19, NETs play a dual role, playing a defensive role and also contributing to the development of severe complications. These web-like structures, which are made of DNA, histones and proteins, are released to trap pathogens but can worsen inflammation, tissue damage and thrombosis. Excessive NET formation is responsible for inducing ARDS in the lungs, by promoting local inflammation and impairing oxygen exchange. Clot formation, a typical feature of severe cases, is also fuelled in part by NET components like DNA and histones, which promote clot formation through microthrombosis and vascular damage. Moreover, by activating immune cells and increasing cytokine release, NETs amplify inflammation, which leads to a vicious cycle of tissue injury and systemic complications

[587]. Furthermore, SARS-CoV-2 interferes with neutrophil degranulation, which results in the uncontrolled release of toxic enzymes such as MPO and NE. While these proteins are helpful in the destruction of pathogens, their overproduction is detrimental to tissues, compromises epithelial and endothelial barriers, and worsens inflammation. This inflammatory state is also marked by lymphopenia, a feature of severe COVID-19, through suppression of lymphocyte survival and function, adaptive immunity. The degranulation also enhances the formation of NETs, which are detrimental to the tissues and increase the chances of complications such as thrombosis [392]. Another study found that variants of concern of SARS-CoV-2, such as Delta and Omicron, activate human neutrophils differently, which could affect the severity of COVID-19. Human neutrophils exposed to infectious SARS-CoV-2 particles were directly activated through TLR7/8 and IRF3/STING pathways, resulting in rapid IL-8 secretion, ROS generation, and NET formation. Activation was variant-dependent among the six tested variants: ancestral B.1 (Wuhan), Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), Delta (B.1.617.2), and Omicron (B.1.1.529). The Delta variant triggered the strongest neutrophil responses, showing the highest proportion of CD11b^{high} CD62L^{low} cells, maximal IL-8 release, intense ROS production, and extensive NET formation. The ancestral, Alpha, Beta, and Gamma strains produced intermediate activation, whereas Omicron consistently elicited the weakest responses across all parameters [385].

5.3 Specific Aims

Aim 1:

To examine how the SARS-CoV-2 Delta variant affects PMN activation markers (CD11b, CD66b, CD16, CD62L, and CD63) by flow cytometry.

Hypothesis:

We hypothesise that the Delta variant interacts with surface receptors on PMNs, altering the expression of activation markers (CD11b, CD66b, CD16, CD62L, and CD63) in a concentration-dependent manner. We further hypothesise that the viral load increases the levels of PMN activation and modulates these markers.

Approach:

PMNs from healthy donor blood were isolated and treated with different concentrations of the Delta variant. Flow cytometry analysed activation markers CD11b, CD66b, CD16, CD62L, and CD63. Marker expression was compared across varying viral concentrations to determine the effect of the Delta variant on neutrophil activation and response.

Aim 2:

To determine the effect of the SARS-CoV-2 Delta variant on proinflammatory cytokine production and NF- κ B pathway activation in PMNs.

Hypothesis:

We hypothesise that the Delta variant triggers neutrophil-mediated inflammation by enhancing the production of IL-6, IL-8, IL-1 β and TNF- α , through the NF- κ B signalling pathway.

Approach:

PMNs isolated from healthy donors were exposed to the different concentrations of the Delta variant. Quantitative measurement of proinflammatory cytokines IL-6, IL-8, IL-1 β and TNF- α were performed by ELISA and RT-PCR for both secreted and transcriptional responses. Western blot analysis were used to determine I κ B degradation and thereby NF- κ B activation. The data will be analysed to determine the relationship between viral concentration, cytokine production, and activation of NF- κ B pathway.

Aim 3:

To investigate how the SARS-CoV-2 Delta variant affects ISG expression and STAT1 phosphorylation in PMNs.

Hypothesis:

We hypothesise that the Delta variant alters the IFN response in PMNs by enhancing ISGs in a dose-dependent fashion. This was accompanied by an increase in phosphorylated STAT1 (pSTAT1), suggesting that the JAK-STAT signalling pathway is activated.

Approach:

To assess the impact of the Delta variant on IFN signalling in PMNs, PMNs from healthy donors were stimulated with different concentrations of the Delta variant. Quantification of ISG transcription were carried out by qPCR to determine transcriptional responses. To determine whether STAT1 was activated, western blot analysis was performed to detect pSTAT1 to help determine the extent to which the Delta variant influenced the IFN response in neutrophils.

Aim 4:

To measure MPO and NE release in assessing the effect of the SARS-CoV-2 Delta variant on PMN degranulation.

Hypothesis:

We hypothesise that the Delta variant increases the rate of PMN degranulation, which in turn increases the secretion of MPO and NE in a concentration-dependent fashion.

Approach:

To quantify MPO and NE release into the supernatant, PMNs isolated from healthy donors were exposed to different concentrations of the Delta variant. The results were quantified using ELISA to determine whether the Delta variant directly induces PMN degranulation and contributes to inflammatory tissue damage.

Aim5:

To determine if the SARS-CoV-2 Delta variant infects PMNs, by detecting viral N protein expression.

Hypothesis:

We hypothesise that the Delta variant can directly infect PMNs, as indicated by the presence of SARS-CoV-2 N protein at both the transcriptional and protein levels.

Approach:

PMNs from healthy donors were exposed to the SARS-CoV-2 Delta variant. The presence of SARS-CoV-2 N protein mRNA were assessed by qPCR, and Western blot analysis was used to determine N protein expression at the protein level. These analyses helped to determine whether PMNs are a potential host cell type for the virus and thereby contribute to viral persistence or immune modulation.

Aim 6:

To examine if soluble factors secreted by epithelial cells infected by SARS-CoV-2 affect PMN responses by measuring cytokine production.

Hypothesis:

We hypothesise that epithelial cells are infected by SARS-CoV-2 and release soluble factors that modify PMN inflammatory responses. The cytokine profiles are different depending on the viral strain.

Approach:

A549-ACE2 cells which have the ACE2 receptor were infected at an MOI of 0.1 with SARS-CoV-2 strains (Alpha, Delta, Omicron, and reference isolate IC-19) and uninfected controls. After infection, culture supernatants were collected and analysed for cytokine levels (IL-6, IL-8, IL-1 β , TNF- α) by ELISA.

Aim7:

To determine the effect of soluble factors from SARS-CoV-2-infected epithelial cells on PMN activation and ROS production.

Hypothesis:

We hypothesise that soluble factors secreted by infected epithelial cells modulate PMN activation marker expression and ROS production in a strain-dependent manner.

Approach:

PMNs from healthy donors were treated with culture supernatants collected from A549-ACE2 cells infected with different SARS-CoV-2 strains (Alpha, Delta, Omicron, IC-19, and mock-infected controls). PMN activation were assessed by flow cytometry for CD66b, CD11b, CD16, and CD62L expression. ROS production was measured using a fluorescence-based assay. These experiments aimed to determine how epithelial-derived factors influence PMN activation and oxidative responses.

Aim8:

To evaluate the effect of soluble factors from SARS-CoV-2-infected epithelial cells on PMN NETs formation.

Hypothesis:

We hypothesise that supernatants from infected epithelial cells promote NETosis in PMNs in a strain-dependent manner, with certain SARS-CoV-2 variants inducing stronger responses.

Approach:

PMNs from healthy donors were treated with culture supernatants from A549-ACE2 cells infected with different SARS-CoV-2 strains (Alpha, Delta, Omicron, IC-19, and mock-infected controls). NETosis was assessed using flow cytometry with Sytox Orange staining, which detects extracellular DNA released during NET formation. This analysis aimed to determine whether epithelial-derived factors modulated NETosis and whether different viral strains influenced neutrophil extracellular trap responses differently.

Aim 9:

To assess the effect of soluble factors from SARS-CoV-2-infected epithelial cells on PMN degranulation by measuring MPO and NE release.

Hypothesis:

We hypothesise that supernatants from infected epithelial cells enhance PMN degranulation in a strain-dependent manner, leading to increased secretion of MPO and NE.

Approach:

PMNs from healthy donors were treated with culture supernatants from A549-ACE2 cells infected with different SARS-CoV-2 strains (Alpha, Delta, Omicron, IC-19, and mock-infected controls). The release of MPO and NE into the supernatant were quantified using

ELISA. These results aimed to determine whether epithelial-derived factors drive neutrophil degranulation and how different SARS-CoV-2 strains influence this response.

5.4 Experiments & Discoveries

5.4.1 SARS-CoV-2 Delta Variant infection does not induce the expression of CD66b in Primary Human PMNs

Neutrophils are involved in the immune response to viral infections, and the level of neutrophil activation determines the extent and severity of the disease. Neutrophil activation and degranulation are marked by CD66b, a neutrophil activation marker that is known to increase during infection with different viruses, including DENV and rhinovirus [313, 318]. Research on severe COVID-19 has revealed that although neutrophils are activated, CD66b is not increased, which points to a specific immune modulation by SARS-CoV-2 [500]. Since the immune responses to SARS-CoV-2 are different from those of other viral infections, it is essential to establish whether SARS-CoV-2 activates neutrophils and alter CD66b expression.

In this experiment, we aimed to determine whether the SARS-CoV-2 Delta variant could directly upregulate CD66b expression in primary human PMNs. To investigate this, we infected the cells at varying MOIs (0.1, 0.5, 1, and 2) for 2 hours, including a mock-infected control, and assessed CD66b surface expression using flow cytometry.

The results showed no increase in the level of CD66b in the different MOI conditions, suggesting that infection alone does not lead to neutrophil degranulation.

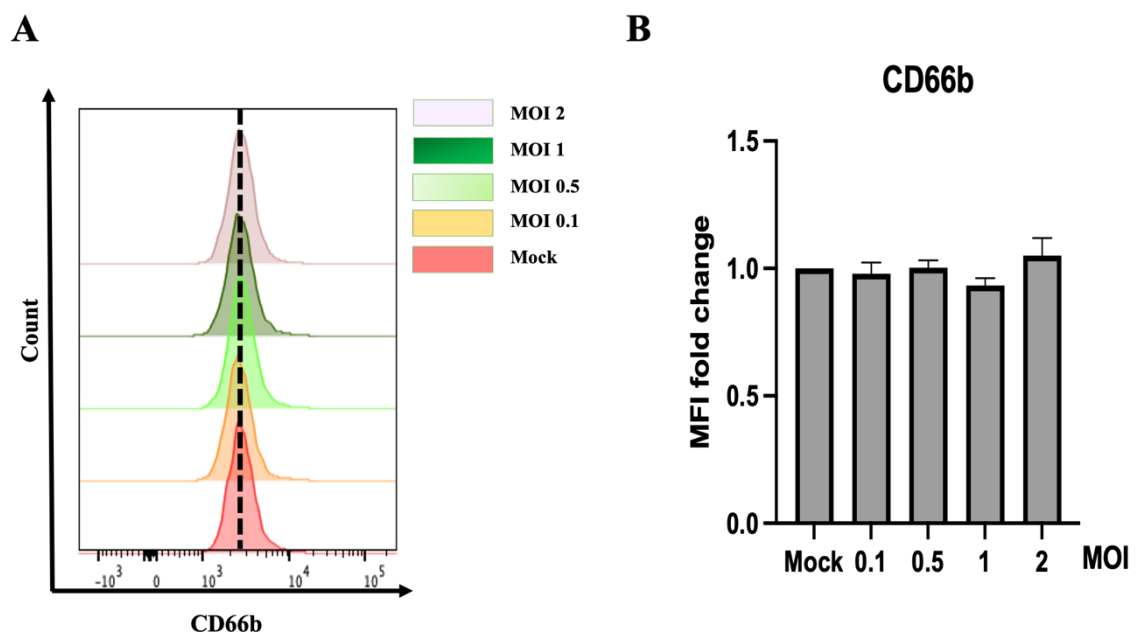


Figure 5. 1: CD66b Expression in PMNs Following SARS-CoV-2 Delta Variant Infection.

Human PMNs were obtained by density gradient isolation (n=3). PMNs (1×10^6) were infected with the SARS-CoV-2 Delta variant at multiplicities of infection (MOI) of 0.1, 0.5, 1, and 2, alongside a mock-infected control. After a 2-hour incubation, cells were stained with a fluorescently conjugated anti-CD66b antibody and analysed using flow cytometry. (A) Representative flow cytometry histograms showing CD66b expression intensity distributions for each condition. The vertical dashed line indicates the gating threshold. (B) Quantification of CD66b expression MFI fold change relative to mock-infected control across different MOI conditions. Data represent mean $\pm$. Statistical analysis was performed using one-way ANOVA.

5.4.2 SARS-CoV-2 Delta Variant infection decreased the expression of CD11b in Primary Human PMNs

CD11b⁺ cells, including neutrophils, are protective in peripheral viral infections by producing type 1 IFNs and ROS [588]. The expression of CD11b on neutrophils is essential in regulating immune responses during infection. For instance, during RSV bronchiolitis, neutrophils exhibit increased CD11b expression, while shedding CD31 and CD62L, facilitating their migration from blood to airways [589]. Interestingly, the regulation of CD11b levels on neutrophils remains a topic of debate. While some studies have reported CD11b to be down-regulated [500, 501], others have found no significant changes [590].

To investigate the effect of SARS-CoV-2 Delta variant infection on PMN activation, CD11b expression was measured following viral exposure. PMNs were isolated from healthy subjects and infected with the Delta variant at different MOIs (0.1, 0.5, 1, and 2) or left uninfected (mock). After two hours of incubation, the cells were stained with a fluorescently conjugated anti-CD11b antibody and flow cytometry was used to analyse the samples. While not statistically significant, the results showed some decrease in CD11b expression upon SARS-CoV-2 infection, compared to mock control, with MOI 0.1 showing the greatest reduction (mean fold change ~ 0.4),.

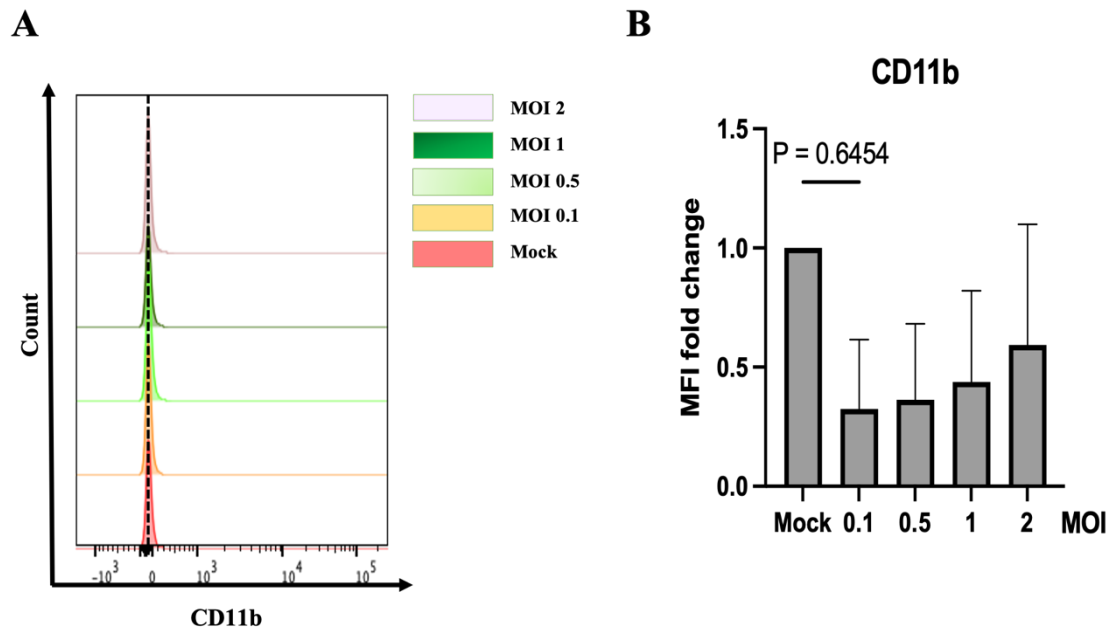


Figure 5. 2: CD11b Expression in PMNs Following SARS-CoV-2 Delta Variant Infection.

Human PMNs were obtained by density gradient isolation (n=3). PMNs (1×10^6) were infected with the SARS-CoV-2 Delta variant at MOI of 0.1, 0.5, 1, and 2, alongside a mock-infected control. After a 2-hour incubation, cells were stained with a fluorescently conjugated anti-CD66b antibody and analysed using flow cytometry. (A) Representative flow cytometry histograms showing CD11b expression intensity distributions for each condition. The vertical dashed line indicates the gating threshold. (B) Quantification of CD11b expression MFI fold change relative to mock-infected control across different MOI conditions. Data represent mean $\pm$. Statistical analysis was performed using one-way ANOVA.

5.4.3 SARS-CoV-2 Delta Variant infection does not induce shedding of CD16 in Primary Human PMNs

Neutrophils are involved in immune defence through antibody dependent cellular cytotoxicity (ADCC) a process carried out through CD16 (Fc γ RIII), playing a key role in antiviral immunity. Similar to how EBOV GP interacts with CD16b to regulate neutrophil function, viral proteins can modulate neutrophil-mediated immune responses by targeting key surface receptors [439]. Furthermore, the severity of COVID-19 is associated with dysregulated neutrophil activation, and reduced CD16 expression is indicative of worsening disease [591]. This experiment is designed to determine if infection by the SARS-CoV-2

Delta variant modifies the expression of CD16 on primary human PMNs, which in turn can modulate ADCC. To test this PMNs were isolated from healthy subjects and infected with the Delta variant at different MOIs (0.1, 0.5, 1, and 2) with a mock-infected control. After a 2 hour incubation, the cells were stained with a fluorescently conjugated anti-CD16 antibody and then analysed by flow cytometry. The results showed that there were no statistical changes in the CD16 expression at any MOI when compared to the mock-infected control.

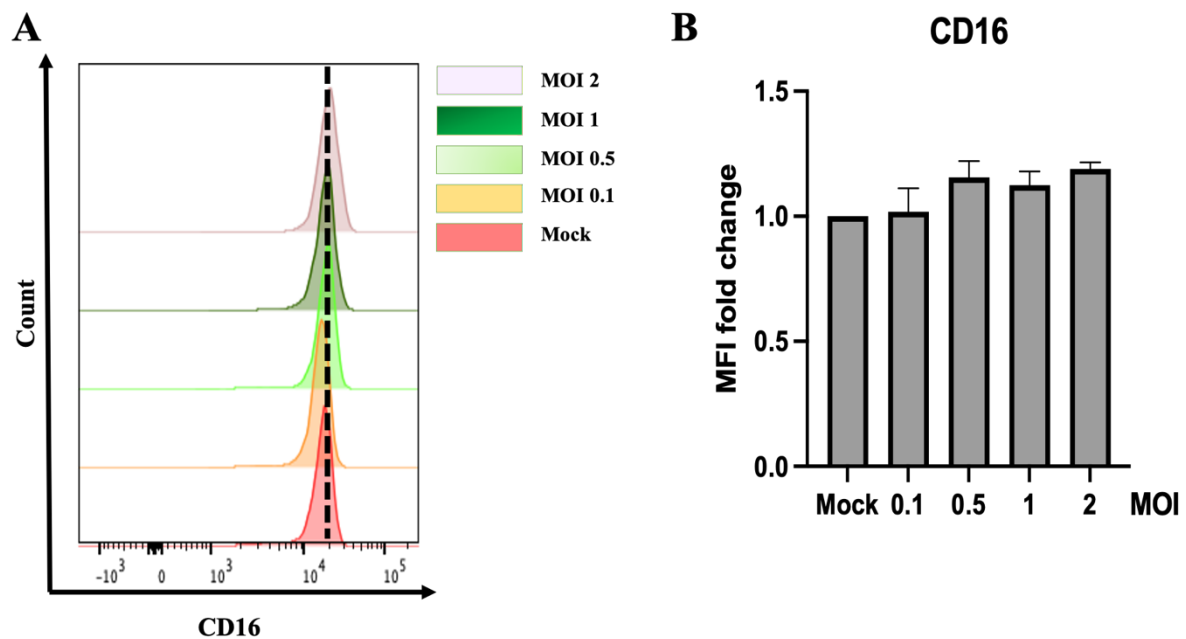


Figure 5.3: CD16 Expression in PMNs Following SARS-CoV-2 Delta variant infection.

Human PMNs were obtained by density gradient isolation (n=3). PMNs (1×10^6) were infected with the SARS-CoV-2 Delta variant at MOI of 0.1, 0.5, 1, and 2, alongside a mock-infected control. After a 2 hour incubation, cells were stained with a fluorescently conjugated anti-CD16b antibody and analysed using flow cytometry. (A) Representative flow cytometry histograms showing CD66b expression intensity distributions for each condition. The vertical dashed line indicates the gating threshold. (B) Quantification of CD16 expression MFI fold change relative to mock-infected control across different MOI conditions. Data represent mean $\pm$. Statistical analysis was performed using one-way ANOVA.

5.4.4 SARS-CoV-2 Delta Variant infection does not induce the shedding of CD62L in Primary Human PMNs

Next, we investigated whether infection with the SARS-CoV-2 Delta variant affects PMN activation, analysing the level of CD62L expression following viral exposure. The primary

human PMNs were infected with the Delta variant at the MOIs of 0.1, 0.5, 1, and 2, and then a mock-infected control was added. After a 2 hour incubation, the cells were stained with a fluorescently conjugated anti-CD62L antibody and analysed by flow cytometry. CD62L (L-selectin) is a neutrophil adhesion molecule that is critical for their trafficking and immune responses [318]. While previous research found that there were no significant differences in the level of neutrophils CD62L between COVID-19 patients and those with other infections [590], others reported decreased CD62L expression on neutrophils in severe COVID-19 cases [500]. However, Our results showed no changes in the CD62L expression at any MOI compared to the mock-infected control.

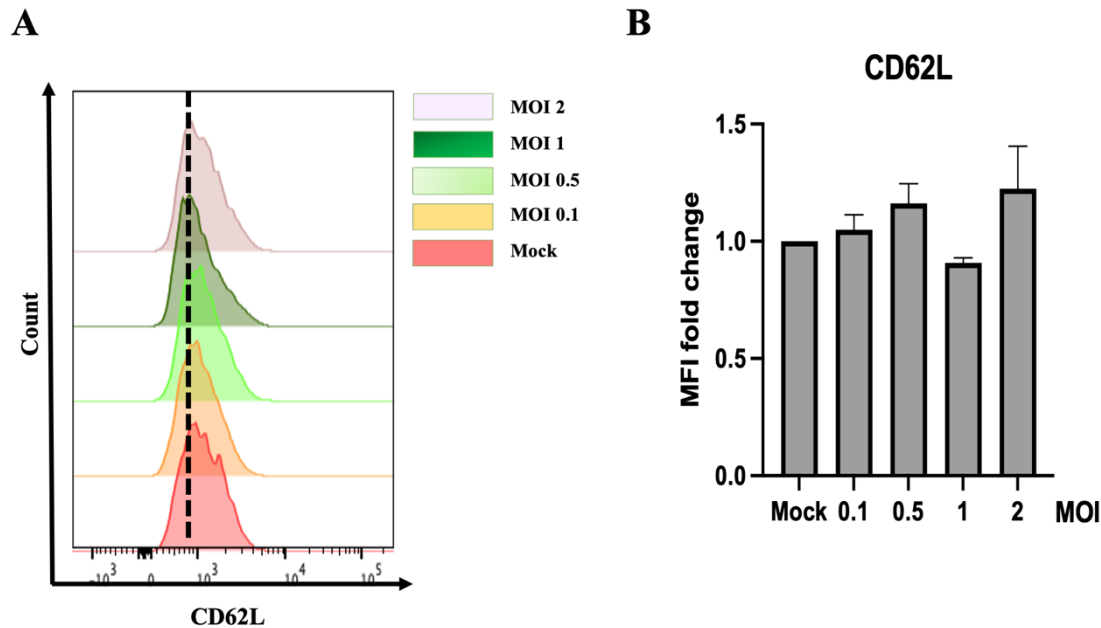


Figure 5. 4: CD62L Expression in PMNs Following SARS-CoV-2 Delta Variant Infection.

Human PMNs were obtained by density gradient isolation (n=3). PMNs (1×10^6) were infected with the SARS-CoV-2 Delta variant at MOI of 0.1, 0.5, 1, and 2, alongside a mock-infected control. After a 2-hour incubation, cells were stained with a fluorescently conjugated anti-CD66b antibody and analysed using flow cytometry. (A) Representative flow cytometry histograms showing CD62L expression intensity distributions for each condition. The vertical dashed line indicates the gating threshold. (B) Quantification of CD62L expression MFI fold change relative to mock-infected control across different MOI conditions. Data represent mean $\pm$. Statistical analysis was performed using one-way ANOVA.

5.4.5 SARS-CoV-2 Delta Variant infection induce the expression of CD63 in Primary Human PMNs

Next, we evaluated whether SARS-CoV-2 Delta variant infection affects PMN degranulation by examining CD63 expression after viral exposure. Primary human PMNs were stained with an anti-CD63 antibody and analysed using flow cytometry following infection with the Delta variant for 2 hours at MOIs: 0.1, 0.5, 1, and 2, alongside a mock-infected control. CD63 is a marker for primary granule exocytosis in neutrophils, indicating degranulation, which is a critical immune defence mechanism against pathogens [592]. CD63 has been reported in apoptotic human neutrophils [593]. Our results showed a nearly significant increase in CD63 expression ($p=0.0795$) at an MOI of 0.5, compared to the mock-infected control; the other MOIs (0.1, 1, and 2) did not show significant changes.

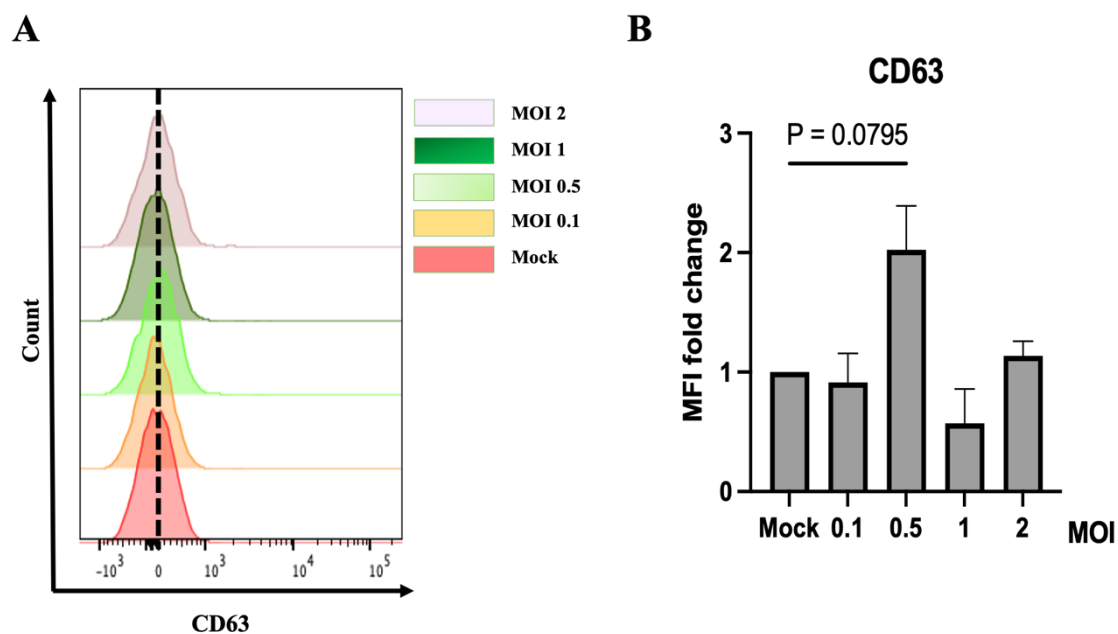


Figure 5. 5: CD63 Expression in PMNs Following SARS-CoV-2 Delta Variant Infection.

Human PMNs were obtained by density gradient isolation ($n=3$). PMNs (1×10^6) were infected with the SARS-CoV-2 Delta variant at MOI of 0.1, 0.5, 1, and 2, alongside a mock-infected control. After a 2-hour incubation, cells were stained with a fluorescently conjugated anti-CD63 antibody and analysed using flow cytometry. (A) Representative flow cytometry histograms showing CD63 expression intensity distributions for each condition. The vertical dashed line indicates the gating threshold. (B) Quantification of CD63 expression MFI fold change relative to mock-infected control across different MOI

conditions. Data represent mean $\pm$. Statistical analysis was performed using one-way ANOVA.

5.4.6 SARS-CoV-2 Delta Variant infection does not induce the expression of CD64 in Primary Human PMNs

CD64 (Fc γ RI) is a high affinity receptor for immunoglobulin G (IgG), and is usually increased on neutrophils during infection and inflammation and acts as a marker of immune activation. Neutrophil CD64 expression levels help distinguish bacterial infections from influenza A. A study showed that in 22 patients with influenza A with or without complications compared with 29 patients with acute bacterial infections and 29 healthy subjects, neutrophil expression of CD64 was increased in influenza A with a raised proportion expressing CD64 in complicated compared to uncomplicated influenza. The expression was significantly higher in bacterial infections compared to both influenza groups [315]. Another study has shown that CD64 expression on neutrophils and monocytes is significantly upregulated in severe COVID-19 patients compared to healthy controls [500]. To assess whether SARS-CoV-2 Delta variant infection impacts PMN activation, CD64 expression was analysed following viral exposure. PMNs were infected with the Delta variant at MOIs of 0.1, 0.5, 1, and 2, and a mock-infected control was also included. After a 2-hour incubation, the cells were stained with a fluorescently conjugated anti-CD64 antibody and analysed by flow cytometry. The results showed no change in CD64 expression in any of the MOIs when compared to the mock-infected control.

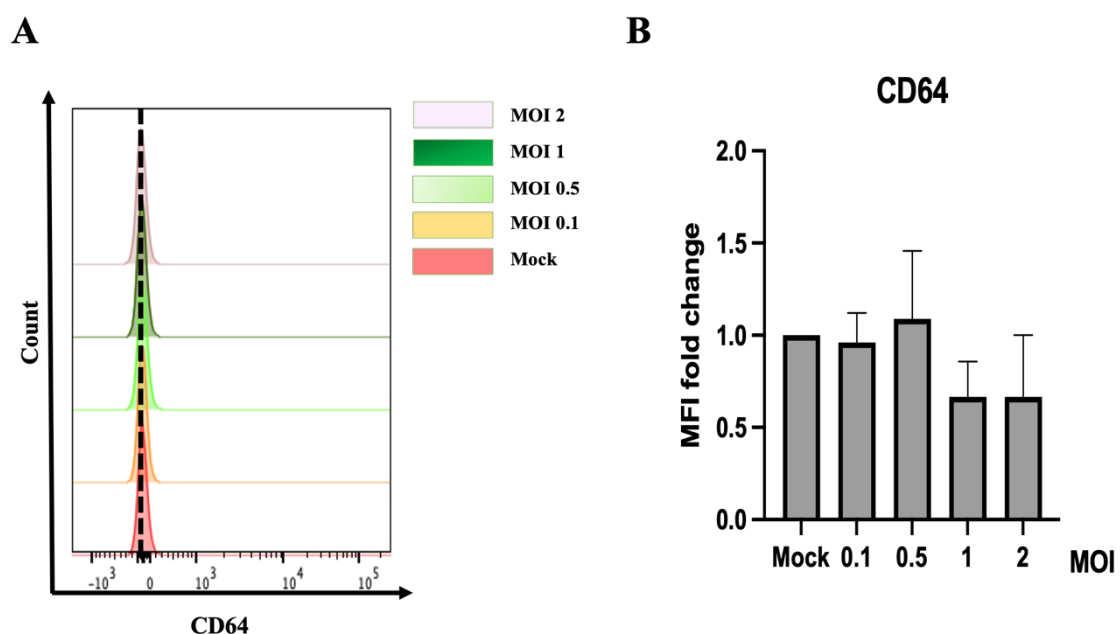


Figure 5. 6: CD64 Expression in PMNs Following SARS-CoV-2 Delta Variant Infection.

Human PMNs were obtained by density gradient isolation (n=3). PMNs (1×10^6) were infected with the SARS-CoV-2 Delta variant at MOI of 0.1, 0.5, 1, and 2, alongside a mock-infected control. After a 2-hour incubation, cells were stained with a fluorescently conjugated anti-CD66b antibody and analysed using flow cytometry. (A) Representative flow cytometry histograms showing CD64 expression intensity distributions for each condition. The vertical dashed line indicates the gating threshold. (B) Quantification of CD64 expression MFI fold change relative to mock-infected control across different MOI conditions. Data represent mean $\pm$. Statistical analysis was performed using one-way ANOVA.

5.4.7: Gene Expression Analysis of Proinflammatory Cytokines in PMNs Increased IL-1 β mRNA Following SARS-CoV-2 Delta Variant Infection

Neutrophils are major producers of proinflammatory cytokines that determine the nature of the immune response to infections. The SARS-CoV-2 Delta variant has been linked to increased inflammation and worse disease outcomes, but the impact of the variant on neutrophil cytokine expression is still unknown. To investigate if SARS-CoV-2 Delta variant infection alters the expression of proinflammatory cytokine genes, human primary PMNs and a mock-infected control were infected with the virus at MOIs of 0.1, 0.5, 1, and 2. After a 2 hour incubation, RNA was extracted, and the expression levels of IL-6 (**Fig. 5.7A**), IL-8 (**Fig. 5.7B**), IL-1 β (**Fig. 5.7C**), and TNF- α (**Fig. 5.7D**) were quantified by qPCR. All gene expression levels were normalized to the housekeeping gene β -actin, and relative fold changes were calculated compared to the mock-infected control. The results showed that there were no changes in IL-6, IL-8 or TNF- α expression when comparing any of the MOI to the mock-infected control. At MOI 1, IL-1 β appeared to be increased 9-fold, but this was not statistically significant.

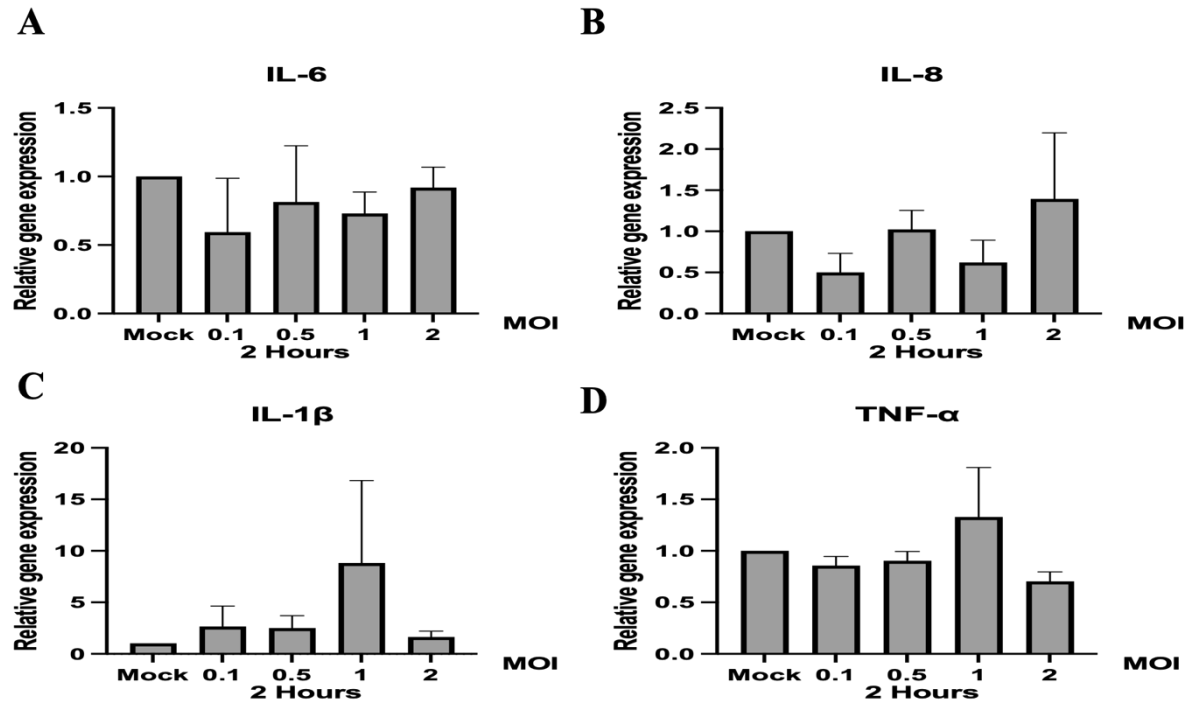


Figure 5. 7: qPCR Analysis of IL-6, IL-8, TNF- α , and IL-1 β Expression in PMNs Following SARS-CoV-2 Delta Variant Infection.

Human PMNs were obtained by density gradient isolation (n=3). PMNs (1×10^6) were infected with the SARS-CoV-2 Delta variant at MOI of 0.1, 0.5, 1, and 2, alongside a mock-infected control. After a 2-hour incubation, RNA was extracted, and gene expression levels of IL-6 (A), IL-8 (B), IL-1 β (C), and TNF- α (D) were measured by qPCR and normalized to β -actin. Relative fold changes were calculated compared to the mock-infected control. The bar graph represents the relative fold change in gene expression compared to the mock-infected control. Data represent mean $\pm$. Statistical analysis was performed using one-way ANOVA.

5.4.8: SARS-CoV-2 Delta Variant Infection does not induce I κ B α Degradation in PMNs

Because our previous work showed that SARS-CoV-2 Delta variant infection slightly enhanced IL-1 β mRNA expression in PMNs (**Fig. 5.7C**), we then proceeded to examine if this increase is associated with NF- κ B activation, a vital pathway in proinflammatory signalling.

To explore this, we exposed primary human PMNs to the Delta variant at different MOIs, 0.1, 0.5, 1, and 2, while the control was mock-infected. After 2 hours of incubation, whole cell lysates were collected, I κ B α was analysed by Western blot, and β -actin was used as a

loading control. We used PMA to provide a positive control for I κ B α degradation. The results showed that PMA treatment resulted in a marked decrease of I κ B α . The SARS-CoV-2 Delta variant did not trigger I κ B α degradation. More experiments are required to confirm the findings, as the experiment was only conducted once. However, this experiment showed that levels of I κ B α were slightly enhanced, especially at MOI 0.5

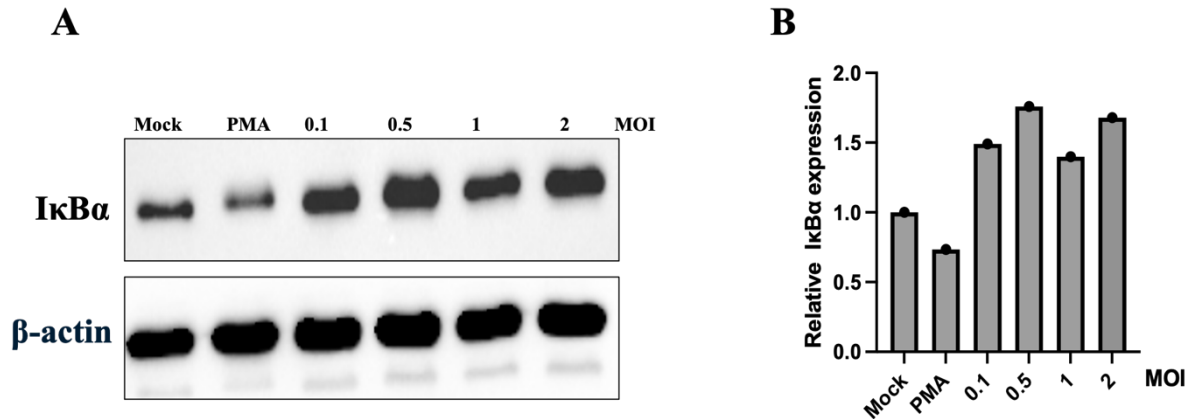


Figure 5. 8: I κ B α Levels in PMNs Following SARS-CoV-2 Delta Variant Infection.

Human PMNs were obtained by density gradient isolation (n=1). PMNs (1×10^6) were infected with the SARS-CoV-2 Delta variant at MOI of 0.1, 0.5, 1, and 2, alongside a mock-infected control. After 2-hour incubation, cells were lysed and analyzed by Western blot. (A) Representative Western blot showing I κ B α protein expression across different conditions, with β -actin serving as a loading control. PMA treatment was included as a positive control for I κ B α degradation. (B) Densitometric quantification of I κ B α expression normalized to β -actin and presented as fold change relative to mock control. Data represent a single experiment; further validation with additional replicates is required.

5.4.9 Protein Expression Analysis of Proinflammatory Cytokines in PMNs Reveals Increased IL-1 β Levels Following SARS-CoV-2 Delta Variant Infection

Having observed that the mRNA levels of IL-1 β were slightly increased after the exposed to the SARS-CoV-2 Delta variant infection (**Fig. 5.7C**), we investigated whether this increase was mirrored at the protein level. To assess this, we exposed primary human PMNs to the Delta variant at different MOIs, 0.1, 0.5, 1, and 2, while the control was mock-infected. Cytokine levels (IL-6, IL-8, TNF- α , IL-1 β , and TGF- β) were measured from the culture supernatant after a 24-h incubation using ELISA. The results showed that IL-1 β was induced at MOIs 0.5 and 1.0 significantly (**Fig. 5.9**), while no significant change was seen in IL-6,

IL-8, TNF- α , or TGF- β at any MOI (data not shown). These findings indicate that SARS-CoV-2 infection may contribute to inflammation via the induction and secretion of IL-1 β .

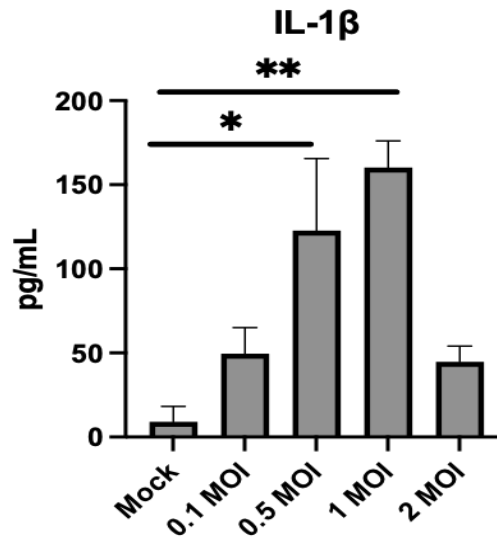


Figure 5. 9: SARS-CoV-2 Delta Variant Induces IL-1 β Production in Human PMNs

Human PMNs were obtained by density gradient isolation (n=3). PMNs (1×10^6) were infected with the SARS-CoV-2 Delta variant at MOI of 0.1, 0.5, 1, and 2, alongside a mock-infected control for 24 hours. IL-1 β levels were measured in the supernatant using ELISA, Data are presented as mean $\pm$ SEM, with statistical significance determined by one-way ANOVA (*p < 0.05; **p < 0.01).

5.4.10: TLR8 Signalling Contributes to IL-1 β Induction in PMNs During SARS-CoV-2 Delta Variant Infection

TLR8 has been reported to activate IL-1 β signalling via the STING-IRF3 pathway during viral infections [385]. However, its role in SARS-CoV-2-induced neutrophil activation is still elusive. Our previous findings have established that SARS-CoV-2 Delta variant infection enhanced the expression of IL-1 β mRNA in PMNs (Fig 5.7), which suggests that PRR signalling may be involved in this response. To identify whether TLR8 signalling is involved in IL-1 β stimulation, we first treat PMNs with the TLR8 inhibitor CU-CPT9a, then challenged the cells with the Delta variant, (MOI=1) and then quantified IL-1 β mRNA levels

by qPCR. The results showed that IL-1 β expression was significantly reduced in PMNs pre-treated with CU-CPT9a compared to untreated infected cells, suggesting that TLR8 signalling contributes to SARS-CoV-2-induced IL-1 β upregulation.

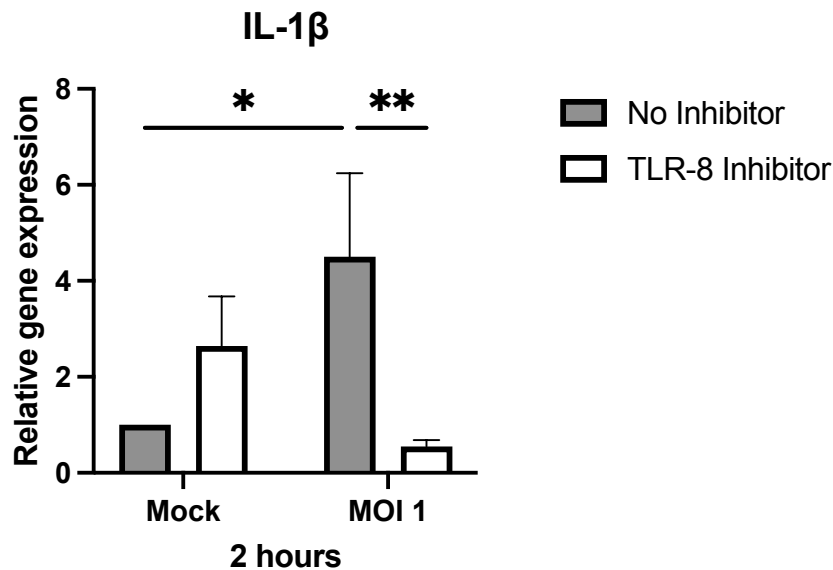


Figure 5. 10 SARS-CoV-2 Delta Variant Induces IL-1 β Expression in Neutrophils via TLR8-Dependent Signaling

Human PMNs were obtained by density gradient isolation (n=3). PMNs (1×10^6) were pre-treated with the TLR8 inhibitor CU-CPT9a (5 μ M) for 30 minutes, followed by SARS-CoV-2 Delta variant infection at MOI 1 or mock-infected for 2 hours. IL-1 β mRNA expression was quantified using qPCR, normalized to β -actin expression, and then expressed relative to the untreated mock-infected control. Data are presented as mean $\pm$ SEM, with statistical significance determined by two-way ANOVA (*p < 0.05; **p < 0.01).

5.4.11: SARS-CoV-2 Delta Variant Modulates ISGs Expression in PMNs

ISGs such as PKR, MxA, OAS1, and viperin are pivotal in limiting viral replication and shaping immune responses [594]. However, how SARS-CoV-2 Delta variant infection affects their expression in primary human PMNs remains unclear. To address this, we exposed primary human PMNs to the Delta variant at different MOIs, 0.1, 0.5, 1, and 2, while the control was mock-infected, and qPCR was used to measure the expression of PKR (Fig. 5.11A), MxA (Fig. 5.11B), viperin (Fig. 5.11C), and OAS1 (Fig. 5.11D). The results showed a significant increase in PKR expression at MOIs 0.5 and 1, indicating an antiviral

response through the activation of PKR, a viral RNA sensor. However, OAS1, MxA, and viperin did not show significant changes.

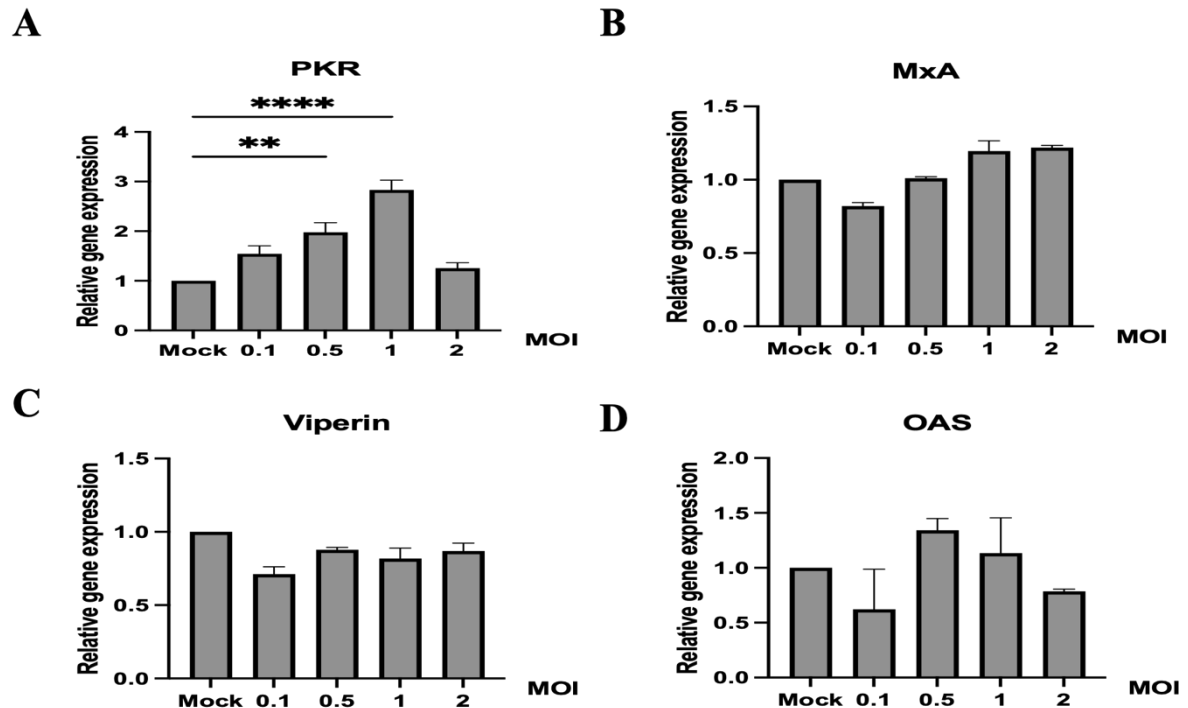


Figure 5.11 Induction of PKR in PMNs Following SARS-CoV-2 Delta Variant Infection.

Human PMNs were obtained by density gradient isolation (n=3). PMNs (1×10^6) were infected with the SARS-CoV-2 Delta variant at MOIs of 0.1, 0.5, 1, and 2 or mock-infected for 2 hours. PKR (A), MxA (B), viperin (C), and OAS1 (D) mRNA expression were measured by qPCR, normalized to β -actin expression, and expressed relative to the untreated mock-infected control. Data are presented as mean $\pm$ SEM, with statistical significance determined by one-way ANOVA (**P < 0.01; and ***P < 0.001).

5.4.12: SARS-CoV-2 Delta Variant Modulates STAT1 Phosphorylation in PMNs in a Viral Dose-Dependent Manner

Having observed PKR induction (Fig 5.11), we next turned to whether SARS-CoV-2 Delta variant infection affects upstream pSTAT1, a critical IFN signalling and antiviral immune response regulator. Primary human PMNs were exposed to the Delta variant at different MOIs, 0.1, 0.5, 1, and 2, while the control was mock-infected, and pSTAT1 levels were

assessed. The results indicated an increase in pSTAT1 at MOI 0.5, suggesting that an IFN-mediated response is activated at this viral concentration. However, there is less induction pSTAT1 upon exposure to SARS-CoV-2 at lower (0.1) and higher MOIs (1 and 2) (**Fig 5.12**). These results demonstrate that live SARS-CoV-2 infection triggers interferon signalling at specific viral loads, consistent with the PKR induction by live Delta variant. More experiments are required to confirm the findings, as the experiment was only conducted once.

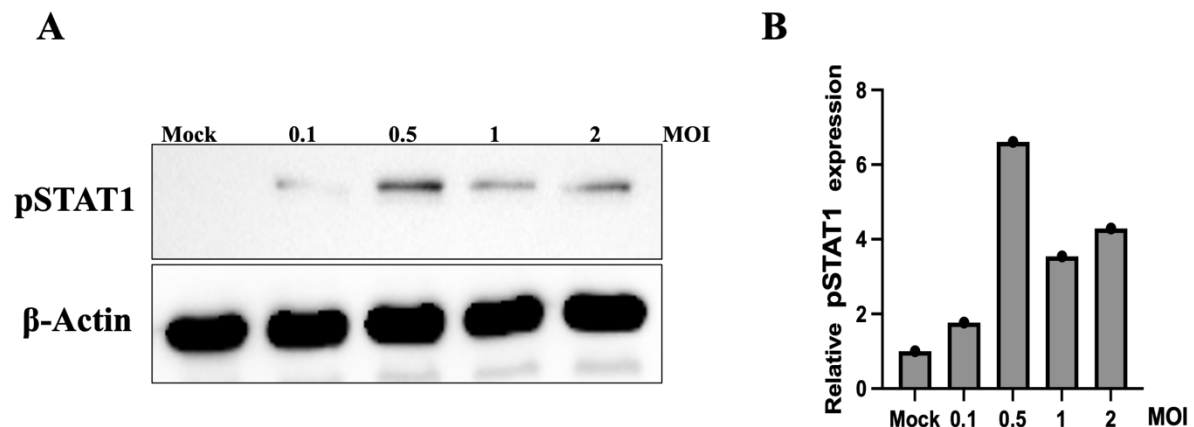


Figure 5. 12: Phosphorylation of STAT1 in PMNs Following SARS-CoV-2 Delta Variant.

Human PMNs were obtained by density gradient isolation (n=1). PMNs (1×10^6) were infected with the SARS-CoV-2 Delta variant at multiplicities of infection (MOI) of 0.1, 0.5, 1, and 2, or mock-infected, for 2 hours. Cells were lysed and analyzed by Western blot. (A) Representative Western blot showing pSTAT1 protein expression across different infection conditions, with β -actin serving as a loading control. (B) Densitometric quantification of pSTAT1 expression normalized to β -actin and presented as fold change relative to mock control. Data represent a single experiment; further validation with additional replicates is required.

5.4.13: SARS-CoV-2 Delta Variant Does Not Significantly Induce PMN Degranulation

Neutrophils are involved in the immune response through the secretion of granule proteins, MPO and NE, which are essential for antimicrobial activity and inflammation [332]. To determine if the SARS-CoV-2 Delta variant affects PMNs degranulation, primary human PMNs were infected at MOIs of 0.1, 0.5, 1, and 2, or left uninfected for 2 hours, and MPO

and NE levels were determined by ELISA. PMA is a known inducer of neutrophil degranulation and was used as a positive control. The results showed that PMA induced strongly MPO (Fig. 5.13A) and NE (Fig. 5.13B) release, thus confirming its role as an activator of neutrophil degranulation. SARS-CoV-2 infection did not induce significantly MPO or NE release.

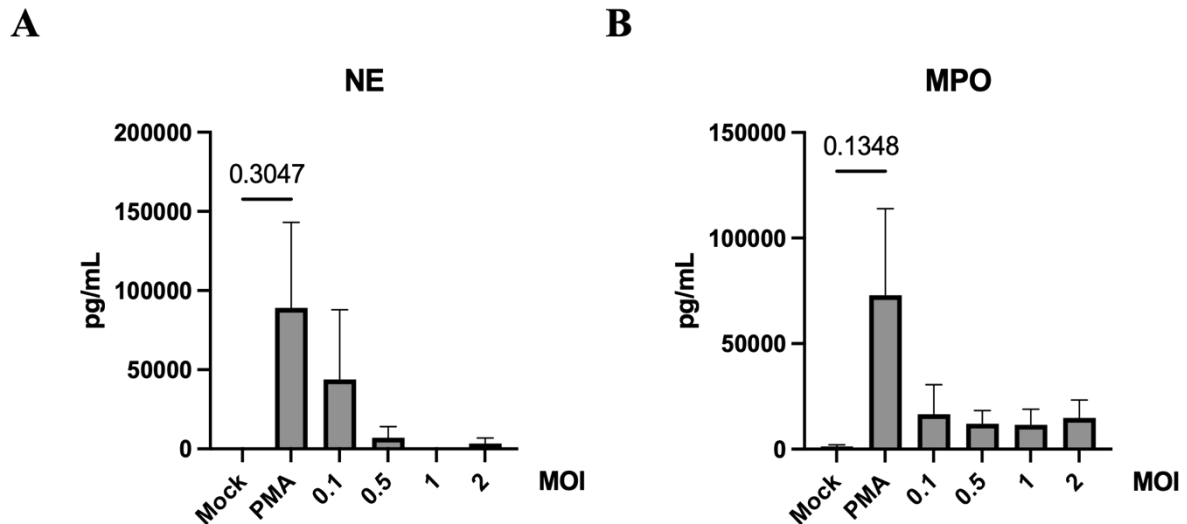


Figure 5. 13: MPO and NE Release in PMNs Following SARS-CoV-2 Delta Variant Infection.

Human PMNs were obtained by density gradient isolation (n=3). PMNs (1×10^6) were exposed to the SARS-CoV-2 Delta variant at MOI of 0.1, 0.5, 1, and 2, or mock-infected, for 2 hours. Supernatants were collected and analysed by ELISA to measure neutrophil degranulation markers. (A) MPO and NE. PMA treatment served as a positive control for neutrophil degranulation in both assays. Data are presented as mean $\pm$ SEM with statistical significance determined using one-way ANOVA.

5.4.14: Detection of SARS-CoV-2 Nucleocapsid Protein and RNA in PMNs Following SARS-CoV-2 Delta Variant Infection

Neutrophils contribute to inflammation and antiviral defence; thus, direct exposure by the SARS-CoV-2 Delta variant may change their function and change the disease pathology. To establish whether PMNs are infected by SARS-CoV-2, we analysed the expression of viral N protein by Western blot and qPCR after infection at MOIs of 0.1, 0.5, 1, and 2, or mock-infected for 2 hours. Infection was assessed by measuring the expression of SARS-CoV-2N protein using western blot (Fig. 5.14A) and qPCR (Fig. 5.14B). The results showed that N protein expression increased starting from MOI 0.5, with higher levels detected at MOIs 1

and 2. The presence of viral RNA and protein are present within PMNs after contact with SARS-CoV-2, indicates infection of the cells.

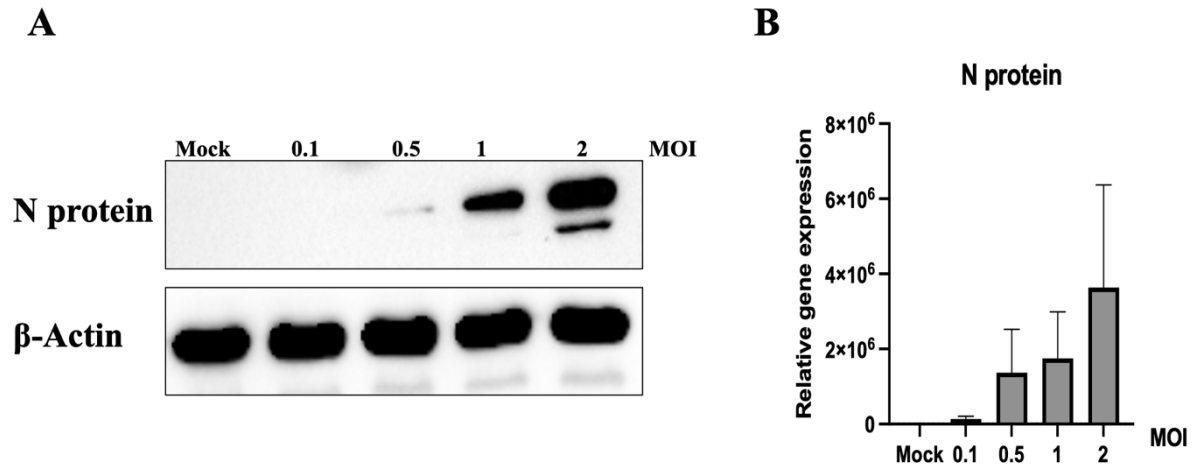


Figure 5. 14 Detection of SARS-CoV-2 Nucleocapsid Protein in PMNs by Western Blot and qPCR.

Human PMNs were obtained by density gradient isolation. PMNs (1×10^6) were infected with SARS-CoV-2 Delta variant at multiplicities of infection (MOI) of 0.1, 0.5, 1, and 2, or mock-infected, for 2 hours. SARS-CoV-2 nucleocapsid (N) protein detection was assessed by Western blot and qPCR analysis. (A) Representative Western blot showing N protein expression across different MOI conditions, with β -actin serving as a loading control ($n=1$). (B) q PCR analysis of N protein mRNA levels normalized to β -actin and expressed as relative gene expression compared to mock-infected control ($n=3$). Data are presented as mean $\pm$ SEM.

5.4.15: SARS-CoV-2 Strain- and Time-Dependent Induction of IL-8 in A549-ACE2 Cells and Its Potential Role in PMNs Recruitment

Next, we investigated whether factors secreted by infected cells might affect neutrophil activity. A549-ACE2 cell line was infected with SARS-CoV-2 strains (Alpha, Delta, Omicron, and IC-19 reference isolate) at MOI 0.1 for 24, 48, and 72 hours, with supernatants analysed for IL-8 by ELISA. The results showed time- and strain-dependent IL-8 production. At 24 hours (**Fig. 5.15A**), all strains produced similar IL-8 levels (338-461 pg/mL) compared to mock controls (361.7 pg/mL). However, the Delta strain showed decreased IL-8 secretion at 48 hours (**Fig. 5.15B**) (900.5 pg/mL vs 1097.8 pg/mL mock) and 72 hours (**Fig. 5.15C**) (1862 pg/mL vs 2427.3 pg/mL mock), while other strains maintained

or increased levels across all time points. Analysis of IL-6, IL-1 β , TNF- α , TGF- β , and IL-10 revealed no induction for any of these cytokines (data not shown).

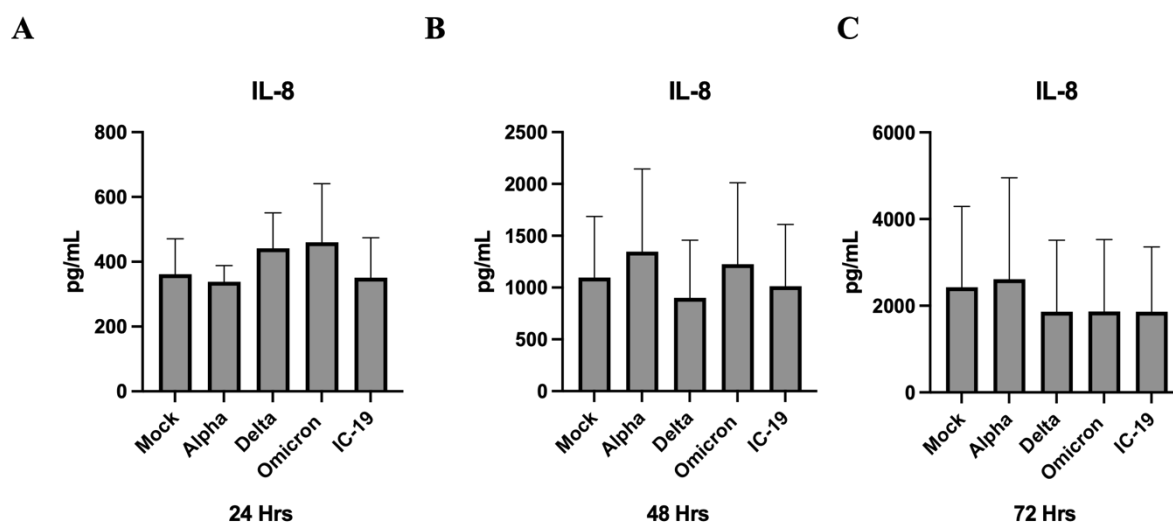


Figure 5. 15: IL-8 Production in A549-ACE2 Cells Following Infection with SARS-CoV-2 Strains at Different Time Points.

A549-ACE2 cells were infected with SARS-CoV-2 (Mock, Alpha, Delta, Omicron, and IC-19) and supernatants were collected at 24, 48, and 72 hours post-infection (n=4). ELISA measured IL-8 levels. (A) represent IL-8 levels at 24 hours, (B) at 48 hours, and (C) at 72 hours. Data are presented as mean (pg/mL) $\pm$ SEM, with statistical significance determined using one-way ANOVA.

5.4.16: Soluble Factors from SARS-CoV-2-Infected Epithelial Cells Modulate PMNs CD66b Expression in a Strain-Dependent Manner

To determine whether soluble factors (such as IL-8) from SARS-CoV-2-infected epithelial cells affect PMN activation, PMNs were treated with supernatants from A549-ACE2 cells infected with different SARS-CoV-2 strains (Mock, Alpha, Delta, Omicron, and IC-19). PMNs were incubated for 2 hours with supernatants collected at 24, 48, and 72 hours post-infection, and CD66b expression was measured by flow cytometry as a marker of activation. At 24 hours no significant changes in CD66b expression were observed across conditions (**Fig. 5.16A**). At 48 hours Delta supernatants induced a marked increase (1.64-fold), while Alpha, Omicron, and IC-19 remained close to baseline (**Fig. 5.16B**). At 72 hours Delta maintained elevated CD66b expression (1.40-fold), whereas Alpha, Omicron, and IC-19 did not differ from Mock (**Fig. 5.16C**).

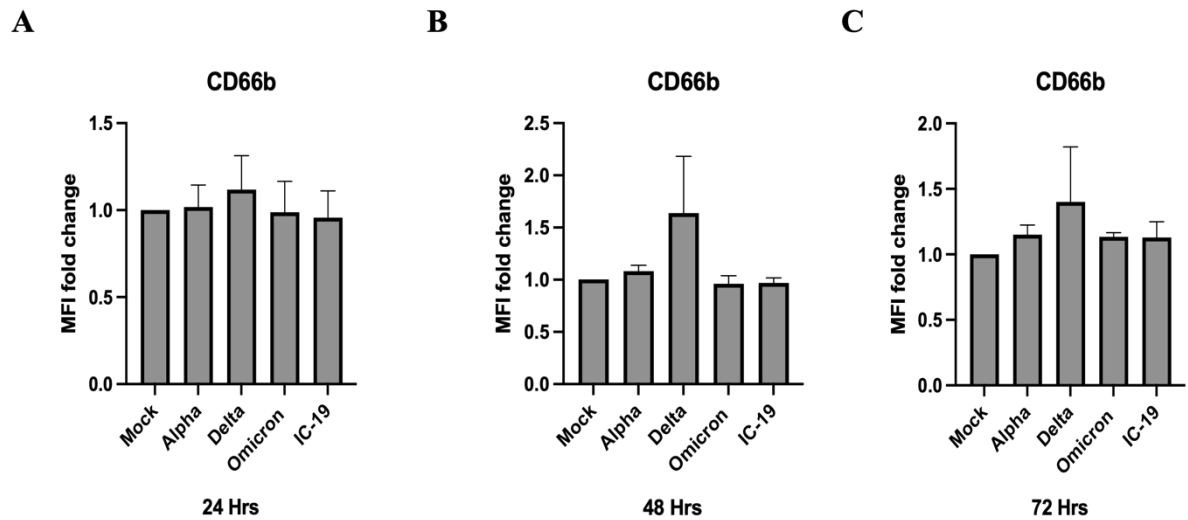


Figure 5.16: CD66b Expression in PMNs Treated with Supernatants from SARS-CoV-2-Infected A549-ACE2 Cells.

Human PMNs were obtained by density gradient isolation (n=3). PMNs (1×10^6) were treated for 2 hours with supernatants from A549-ACE2 cells infected with different SARS-CoV-2 strains (Mock, Alpha, Delta, Omicron, IC-19) at MOI 0.1. Supernatants were collected at 24, 48, and 72 hours post-infection of A549-ACE2 cells for PMNs treatment. CD66b surface expression on PMNs was measured by flow cytometry as a marker of PMN activation. (A) CD66b expression on PMNs after treatment with 24-hour supernatants. (B) CD66b expression on PMNs after treatment with 48-hour supernatants. (C) CD66b expression on PMNs after treatment with 72-hour supernatants. Data are presented as MFI fold change relative to mock control $\pm$ SEM. Statistical significance was determined by one-way ANOVA.

5.4.17: PMNs Activation in Response to Soluble Factors from SARS-CoV-2-Infected Epithelial Cells: Time- and Strain-Dependent Effects on CD11b Expression

To investigate whether soluble factors from SARS-CoV-2-infected epithelial cells affect PMN activation, primary human PMNs were incubated with supernatants from A549-ACE2 cells infected with Mock, Alpha, Delta, Omicron, and IC-19 strains. Cells were treated with supernatants collected at 24, 48, and 72 hours post-infection, and adhesion was assessed by measuring CD11b surface expression via flow cytometry. At all-time points (24, 48, and 72 hours), no significant changes in CD11b expression were observed with any of the tested supernatants.

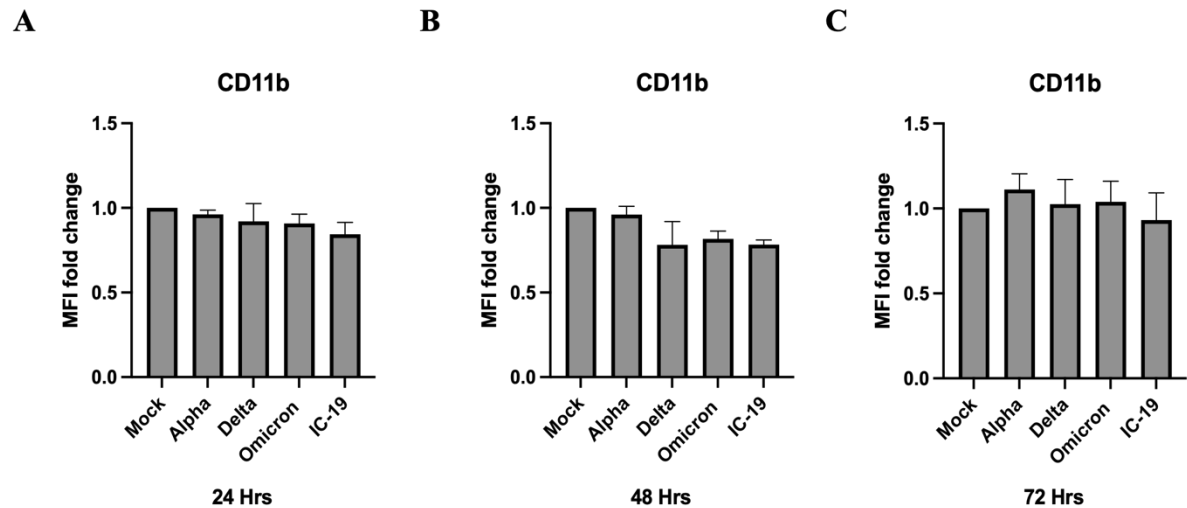


Figure 5.17: CD11b Expression in PMNs Treated with Supernatants from SARS-CoV-2-Infected A549-ACE2 Cells.

Human PMNs were obtained by density gradient isolation (n=3). PMNs (1×10^6) were treated for 2 hours with supernatants from A549-ACE2 cells infected with different SARS-CoV-2 strains (Mock, Alpha, Delta, Omicron, IC-19) at MOI 0.1. Supernatants were collected at 24, 48, and 72 hours post-infection of A549-ACE2 cells for PMNs treatment. CD11b surface expression on PMNs was measured by flow cytometry as a marker of PMN activation. (A) CD11b expression on PMNs after treatment with 24-hour supernatants. (B) CD11b expression on PMNs after treatment with 48-hour supernatants. (C) CD11b expression on PMNs after treatment with 72-hour supernatants. Data are presented as MFI fold change relative to mock control $\pm$ SEM. Statistical significance was determined by one-way ANOVA.

5.4.18: CD16 Expression and Shedding in PMNs Treated with Supernatants from SARS-CoV-2-Infected Epithelial Cells

To investigate whether soluble factors from SARS-CoV-2-infected epithelial cells influence CD16 shedding in PMNs, primary human PMNs were treated with supernatants collected at 24, 48, and 72 hours post-infection from A549-ACE2 cells infected with Alpha, Delta, Omicron, and IC-19, or from uninfected cells (Mock). Surface levels of CD16 were measured by flow cytometry, and the mean fold change relative to Mock was calculated. At 24 hours, PMNs exposed to Omicron and IC-19 supernatants showed increased CD16 expression, 1.38-fold and 1.35-fold, respectively (**Fig. 5.18A**); whereas Alpha and Delta remained close to baseline. At 48 hours, Delta supernatants markedly reduced CD16 (0.63-fold), while there were modest increases in Alpha (1.24-fold), Omicron (1.19-fold), and IC-

19 (1.33-fold) (**Fig. 5.18B**). At 72 hours, Delta continued to reduce CD16 (0.89-fold), whereas Alpha, Omicron, and IC-19 remained close to baseline (**Fig. 5.18C**).

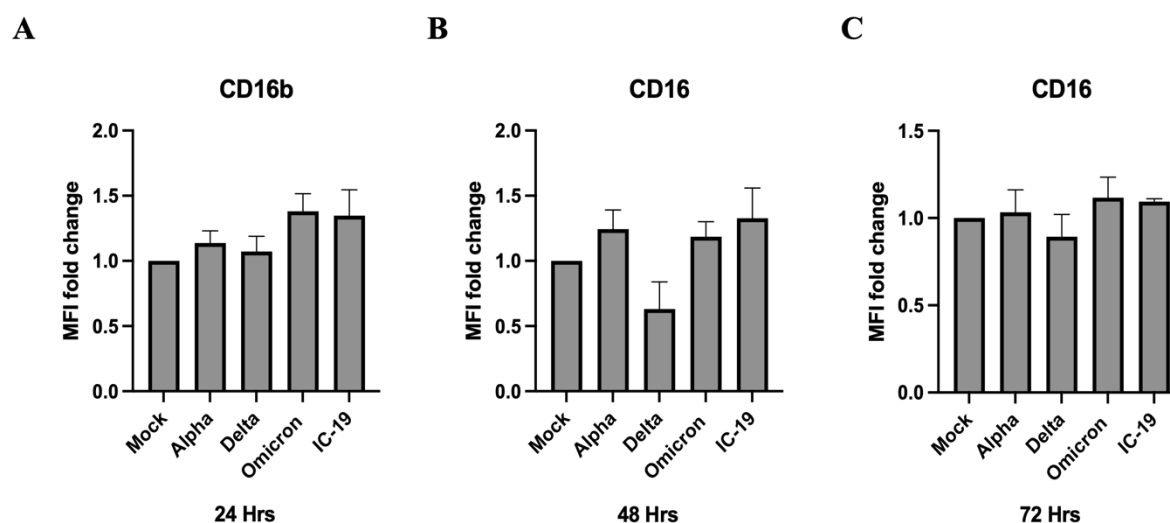


Figure 5. 18: CD16 Expression in Neutrophils Treated with Supernatants from SARS-CoV-2-Infected A549-ACE2 Cells.

Human PMNs were obtained by density gradient isolation (n=3). PMNs (1×10^6) were treated for 2 hours with supernatants from A549-ACE2 cells infected with different SARS-CoV-2 strains (Mock, Alpha, Delta, Omicron, IC-19) at MOI 0.1. Supernatants were collected at 24, 48, and 72 hours post-infection of A549-ACE2 cells for PMNs treatment. CD16 surface expression on PMNs was measured by flow cytometry as a marker of PMN activation. (A) CD16 expression on PMNs after treatment with 24-hour supernatants. (B) CD16 expression on PMNs after treatment with 48-hour supernatants. (C) CD16 expression on PMNs after treatment with 72-hour supernatants. Data are presented as MFI fold change relative to mock control $\pm$ SEM. Statistical significance was determined by one-way ANOVA.

5.4.19: Soluble Factors from SARS-CoV-2-Infected Epithelial Cells Modulate CD62L Shedding in PMNs in a Strain- and Time-Dependent Manner

To further explore how soluble factors secreted by epithelial cells infected with SARS-CoV-2 affect CD62L expression in PMNs, primary human PMNs were incubated with supernatants collected at 24, 48, and 72 hours post-infection from A549-ACE2 cells infected with Alpha, Delta, Omicron, and IC-19, or from uninfected cells (Mock). CD62L expression was analysed by flow cytometry, and the mean fold change relative to Mock was calculated. At 24 hours, supernatants from all strains increased CD62L expression, with Alpha (1.68-fold), Delta (1.70-fold), Omicron (1.91-fold), and IC-19 (1.74-fold) all elevated compared

to Mock (**Fig. 5.19A**). At 48 hours, expression remained essentially unchanged, with Alpha and Delta, Omicron (1.20-fold), and IC-19 (1.20-fold) showing only slight increases (**Fig. 5.19B**). At 72 hours, Delta (1.38-fold) and IC-19 (1.38-fold) maintained increased expression, while Alpha and Omicron returned to baseline (**Fig. 5.19C**).

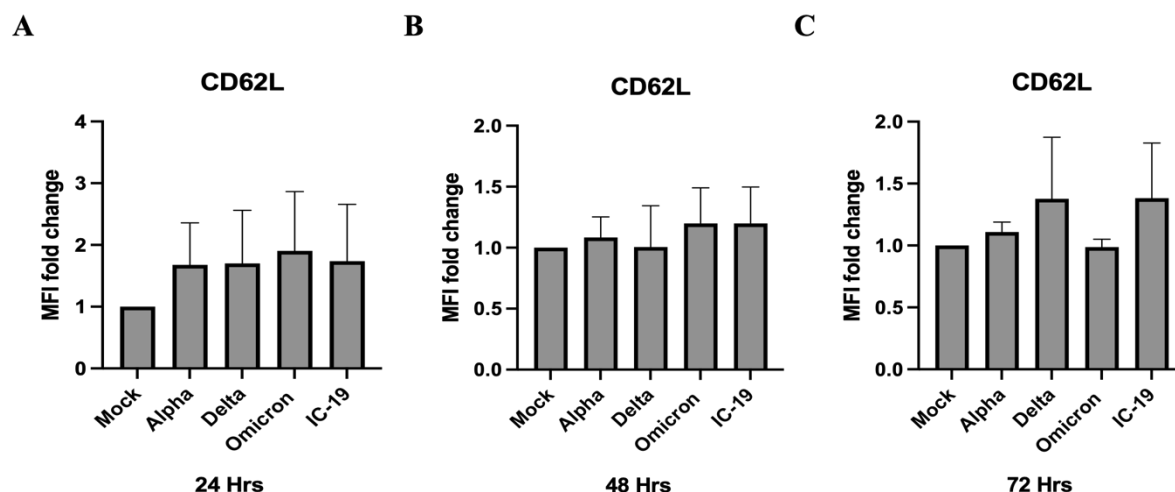


Figure 5. 19: CD62L Shedding in Neutrophils Treated with Supernatants from SARS-CoV-2-Infected A549-ACE2 Cells.

Human PMNs were obtained by density gradient isolation (n=3). PMNs (1×10^6) were treated for 2 hours with supernatants from A549-ACE2 cells infected with different SARS-CoV-2 strains (Mock, Alpha, Delta, Omicron, IC-19) at MOI 0.1. Supernatants were collected at 24, 48, and 72 hours post-infection of A549-ACE2 cells for PMNs treatment. CD62L surface expression on PMNs was measured by flow cytometry as a marker of PMN activation. (A) CD62L expression on PMNs after treatment with 24-hour supernatants. (B) CD62L expression on PMNs after treatment with 48-hour supernatants. (C) CD62L expression on PMNs after treatment with 72-hour supernatants. Data are presented as MFI fold change relative to mock control $\pm$ SEM. Statistical significance was determined by one-way ANOVA.

5.4.20: Effect of SARS-CoV-2-Infected Epithelial Cell Supernatants on Neutrophil ROS Production

To determine whether soluble factors secreted by epithelial cells after SARS-CoV-2 infection affect PMN ROS production, primary human PMNs were incubated with supernatants collected at 24, 48, and 72 hours post-infection from A549-ACE2 cells infected with Alpha, Delta, Omicron, and IC-19, or from uninfected cells (Mock). ROS production was analysed by flow cytometry, and the mean fold change relative to Mock was calculated.

At 24 hours, Delta and IC-19 supernatants reduced ROS levels, 0.72-fold and (0.71-fold), respectively; while Alpha and Omicron remained close to baseline (**Fig. 5.20A**). At 48 hours, all viral strains suppressed ROS production, with Alpha (0.67-fold), Delta (0.57-fold), Omicron (0.53-fold), and IC-19 (0.63-fold) reduced compared to Mock (**Fig. 5.20B**). At 72 hours, Alpha supernatants increased ROS (1.39-fold), Delta remained reduced (0.74-fold), Omicron was close to baseline, and IC-19 also returned to baseline (**Fig. 5.20C**).

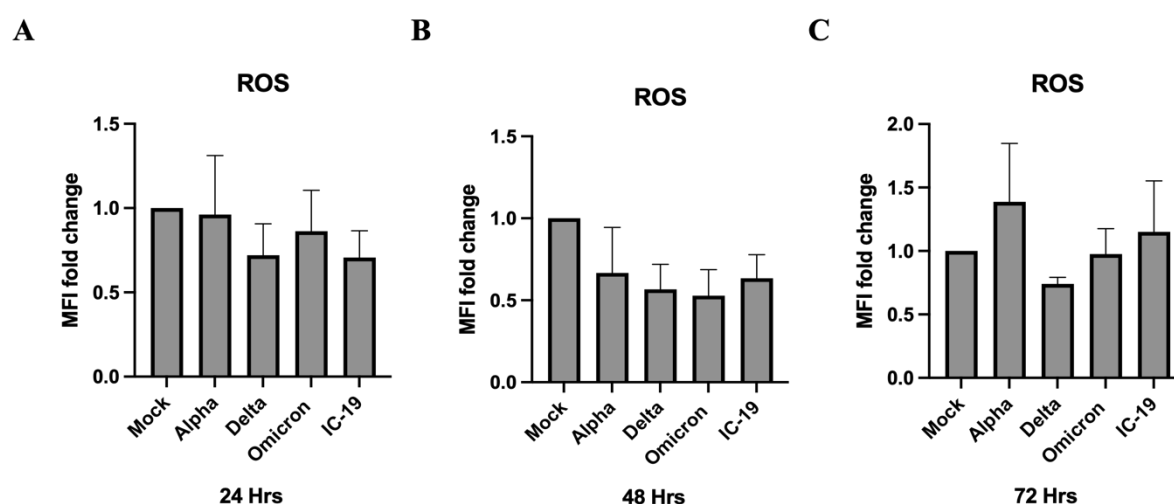


Figure 5. 20: ROS Production in Neutrophils Treated with Supernatants from SARS-CoV-2-Infected A549-ACE2 Cells.

Human PMNs were obtained by density gradient isolation (n=3). PMNs (1×10^6) were treated for 2 hours with supernatants from A549-ACE2 cells infected with different SARS-CoV-2 strains (Mock, Alpha, Delta, Omicron, IC-19) at MOI 0.1. Supernatants were collected at 24, 48, and 72 hours post-infection of A549-ACE2 cells for PMNs treatment. ROS production in PMNs was measured by flow cytometry as a marker of PMN activation. (A) ROS expression on PMNs after treatment with 24-hour supernatants. (B) ROS expression on PMNs after treatment with 48-hour supernatants. (C) ROS expression on PMNs after treatment with 72-hour supernatants. Data are presented as MFI fold change relative to mock control $\pm$ SEM. Statistical significance was determined by one-way ANOVA.

5.4.21: Effect of SARS-CoV-2-Infected Epithelial Cell Supernatants on PMNs NETosis

To assess whether soluble factors released by SARS-CoV-2-infected epithelial cells induce PMN NETosis, primary human PMNs were incubated with supernatants collected at 24, 48, and 72 hours post-infection from A549-ACE2 cells infected with Alpha, Delta, Omicron, and IC-19, or from uninfected cells (Mock). NETosis was analysed by flow cytometry using Sytox Orange staining, and the mean fold change relative to Mock was calculated. At 24

hours (**Fig. 5.21A**), IC-19 supernatants increased NET formation (3.62-fold), while Alpha and Omicron showed no change, and Delta was reduced (0.74-fold). At 48 hours (**Fig. 5.21B**), Delta induced an increase (1.64-fold), Alpha (1.31-fold) also showed a rise, Omicron remained near baseline, and IC-19 was reduced (0.60-fold). At 72 hours (**Fig. 5.21C**), both Alpha (2.01-fold) and IC-19 (2.52-fold) markedly enhanced NETosis, Omicron induced an increase (1.36-fold), while Delta remained at baseline.

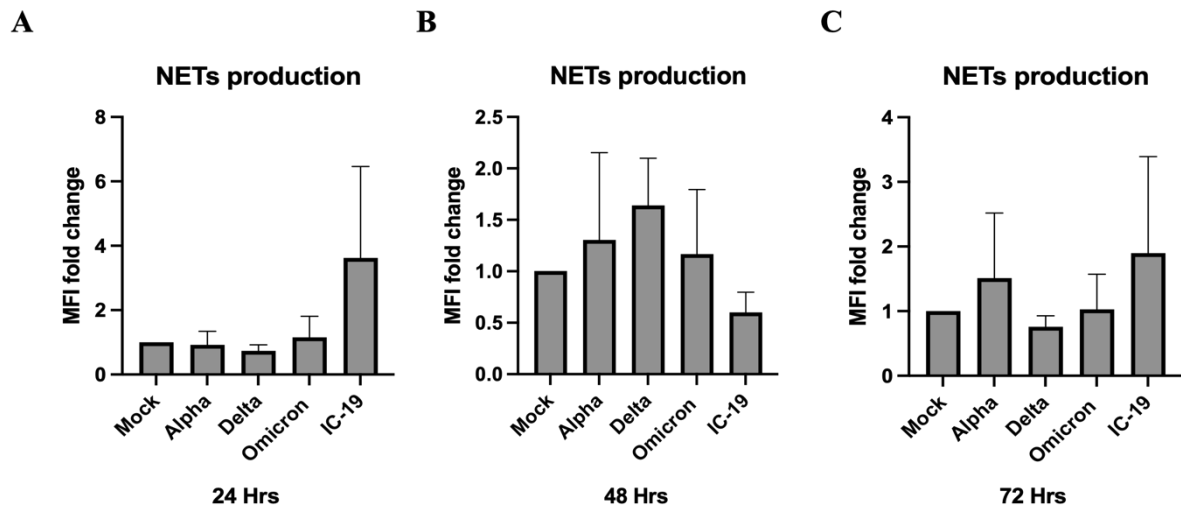


Figure 5. 21 Effect of SARS-CoV-2-Infected Epithelial Cell Supernatants on Neutrophil NETosis.

Human PMNs were obtained by density gradient isolation (n=3). PMNs (1×10^6) were incubated for 6 hours with supernatants from A549-ACE2 cells infected with SARS-CoV-2 strains (Mock, Alpha, Delta, Omicron, IC-19) at MOI 0.1 for 24, 48, and 72 hours. NETosis was measured using SYTOX Orange staining and flow cytometry. Data are presented as MFI $\pm$ SEM. Statistical significance was determined by one-way ANOVA.

5.4.22: Effect of SARS-CoV-2-Infected Epithelial Cell Supernatants on PMNs MPO Release

To determine whether soluble factors secreted by SARS-CoV-2-infected epithelial cells modulate PMN MPO release, primary human PMNs were exposed to culture supernatants from A549-ACE2 cells infected with various SARS-CoV-2 variants (Alpha, Delta, Omicron, and IC-19) or mock-infected controls at a multiplicity of infection (MOI) of 0.1. Culture supernatants were collected at 24, 48, and 72 hours post-infection and co-incubated with neutrophils for 6 hours. ELISA quantified MPO release. No significant alterations in MPO release were detected across any time point or viral variant compared to mock-infected controls.

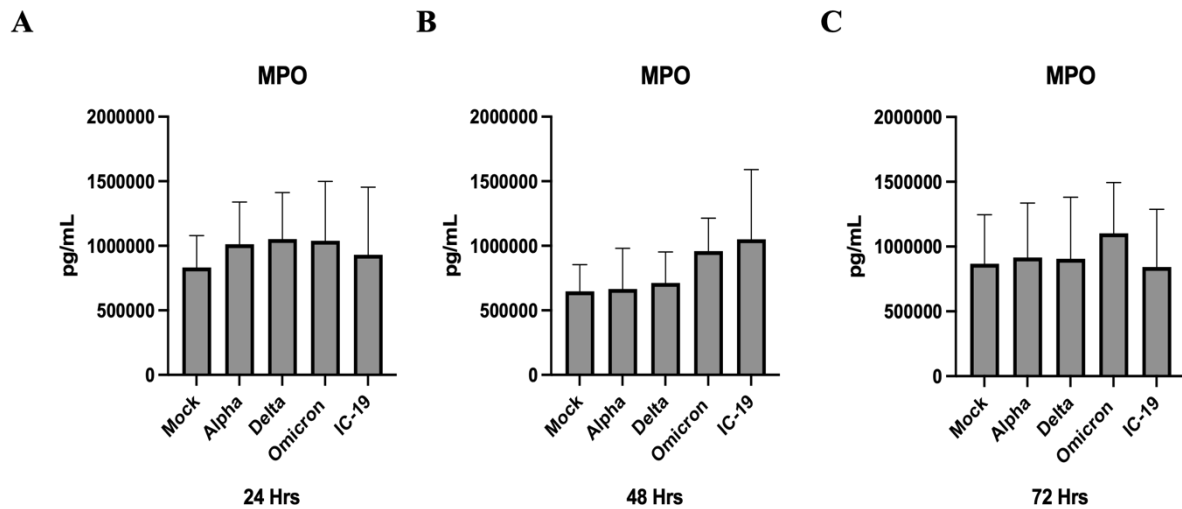


Figure 5.22 MPO Release in PMNs Treated with Supernatants from SARS-CoV-2-Infected A549-ACE2 Cells.

Primary human PMNs were isolated by density gradient isolation (n=3) from donors and treated with culture supernatants from A549-ACE2 cells infected with SARS-CoV-2 variants (Alpha, Delta, Omicron, IC-19) or mock-infected controls at MOI 0.1. Supernatants were collected at (A) 24 hours, (B) 48 hours, and (C) 72 hours post-infection and incubated with PMNs (1×10^6) for 6 hours. ELISA quantified MPO release. Data represent mean $\pm$ SEM (pg/mL). Statistical analysis was performed using one-way ANOVA.

5.4.23: Effect of SARS-CoV-2-Infected Epithelial Cell Supernatants on PMN NE Release

To determine whether soluble factors from SARS-CoV-2-infected epithelial cells influence PMN elastase release, primary human PMNs were treated for 6 hours with supernatants collected from A549-ACE2 cells infected with Mock, Alpha, Delta, Omicron and IC-19 strains at MOI 0.1. ELISA measured neutrophil elastase release after A549-ACE2 cells were infected with Mock, Alpha, Delta, Omicron, and IC-19 at MOI 0.1 for 24, 48, and 72 hours, and supernatants were harvested. At all-time points and across all variants, no significant changes in PMNs elastase release were observed, except for a trend toward significance ($p=0.5$) with IC-19 at 24, 48, and 72 hours.

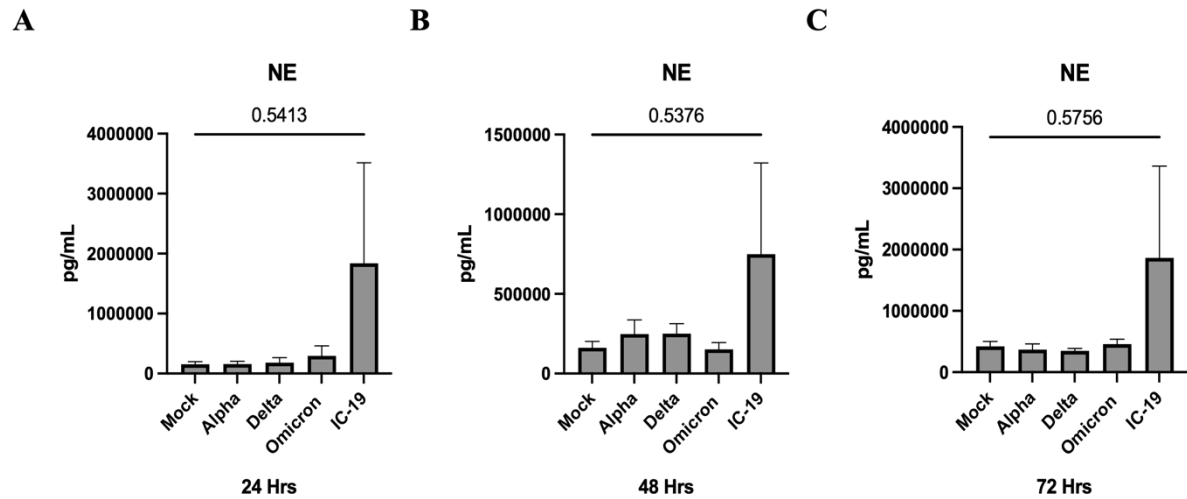


Figure 5. 23 NE Release in Response to Supernatants from SARS-CoV-2-Infected A549-ACE2 Cells.

Primary human PMNs were isolated by density gradient isolation (n=3) from donors and treated with culture supernatants from A549-ACE2 cells infected with SARS-CoV-2 variants (Alpha, Delta, Omicron, IC-19) or mock-infected controls at MOI 0.1. Supernatants were collected at (A) 24 hours, (B) 48 hours, and (C) 72 hours post-infection and incubated with PMNs (1×10^6) for 6 hours. ELISA quantified NE release. Data represent mean $\pm$ SEM (pg/mL). Statistical analysis was performed using one-way ANOVA.

5.5 Discussion

In this chapter, we investigated how PMNs respond to SARS-CoV-2 through both direct infection and indirect stimulation. Direct exposure to the Delta variant induced only partial changes, with increased CD63 expression, reduced CD11b expression, increased IL-1 β production, and activation of antiviral pathways via PKR-dependent STAT1 phosphorylation. IL-1 β induction was reduced when TLR8 signalling was blocked, underscoring its role in viral RNA recognition, and these effects occurred in the absence of PMN degranulation. By contrast, supernatants from infected epithelial cells did not elicit strong PMN responses, and only IC-19-derived supernatant induced NE release, while other strains failed to produce marked effects.

5.5.1 Direct Effects of SARS-CoV-2 on PMNs

A- Surface Marker Expression and Degranulation

In our direct infection studies, we observed that the expression of activation markers such as CD66b, CD16, CD62L and CD64 did not change significantly during 2 h of exposure to the Delta variant at different MOIs (0.1–2). CD11b was the only molecule that showed a decrease at the lowest MOI (0.1), and CD63's expression increased ($p=0.07$) at an MOI of 0.5. This finding shows the specific effect of SARS-CoV-2 on surface neutrophil markers during different levels of viral exposure. Neutrophil CD11b/CD18 (also known as Mac-1) is an integrin receptor critical to adhesion, migration, and phagocytosis processes [595]. It is also crucial for establishing neutrophil polarity and directing their migration towards the infection or inflammation site [596]. Interestingly, while stimulation usually increases the surface expression of the adhesion molecule CD11b/CD18, this upregulation is not strictly required for neutrophils to adhere to the endothelium [597]. CD11b/CD18 is also involved in regulating neutrophil functional plasticity and affects their fate during inflammation resolution [598]. However, the regulation of CD11b levels during SARS-CoV-2 infection remains controversial. Some studies have indicated a reduction in CD11b on neutrophils in COVID-19 patients [500, 501], others have shown no significant change [590]. Our observation of a reduction in CD11b expression at low MOI is consistent with the earlier studies. It implies that even low levels of viral exposure can affect the surface receptor expression of neutrophils, which may affect their function during infection. Previous work on the Wuhan strain showed an acute neutrophil activation response with increasing viral load, characterized by the expansion of CD11b^{hi}/CD62L^{low} neutrophil populations, increased IL-8 secretion, and enhanced ROS production, but this was only apparent at higher MOIs (100–500) [385]. In contrast, our data shows a decrease in CD11b expression at a

much lower MOI (0.1). This suggests that the Delta variant may have a different or even reduced neutrophil activation signature during the short-term exposure. This contrast suggests strain-specific variations in the manner of SARS-CoV-2 in regulating neutrophil responses, with Delta possibly suppressing some of the activation markers at low viral exposure, unlike the Wuhan strain, which needed higher viral doses for activation. At an MOI of 0.5, almost a significant increase in CD63 expression was observed, indicating a possible alteration in the neutrophil activation or degranulation dynamics upon SARS-CoV-2 exposure. CD63 is a marker mainly linked to primary granule exocytosis and greatly enhanced during neutrophil degranulation events, especially under inflammatory conditions. Enhanced surface accumulation is a marker of the release of azurophilic granules, which have the important function of pathogen clearance; however, when dysregulated, it may result in adverse tissue damage [599]. Neutrophil activation and degranulation are also characteristic of severe COVID-19 cases, with prolonged upregulation of CD63 expression, which may contribute to tissue injury and reflect sustained, adverse signalling [600]. However, the substantial decrease in CD11b at low MOI could be seen as an initial inhibitory modulation or a receptor shedding that limits adhesion to the substrate. At the same time, the increase in CD63 suggests that, to some extent, the degranulation processes are initiated, but they do not develop fully within 2 hours. These subtle differences demonstrate that the rate of activation and the extent of activation of the neutrophils may depend on the viral load and the variant-specific properties of the virus.

B- Cytokine Gene Expression and NF- κ B Signalling

Gene expression analysis revealed that in the early stages of infection, key proinflammatory cytokines (IL-6, IL-8, TNF- α) were not significantly upregulated at the transcriptional level, although IL-1 β was slightly upregulated at MOI 1. In contrast, a 24-hour exposure induced a substantial increase in IL-1 β protein secretion, which suggests that although early transcriptional responses were modest, effective protein production can occur with time. This delayed yet robust IL-1 β response has also been reported by other studies. For instance, Rodrigues *et al.*, showed that SARS-CoV-2 targets monocytes through the NLRP3 inflammasome, which triggers the processing and release of IL-1 β . This cytokine is closely linked to the hyperinflammatory response characteristic of severe COVID-19 [601]. Other studies also examined antigen-presenting cells like monocyte-derived macrophages and DCs, and the results have revealed that SARS-CoV-2 infection induces a robust production of proinflammatory cytokines such as IL-6, TNF- α and IL-1 β [602]. The selective upregulation of IL-1 β in neutrophils upon SARS-CoV-2 exposure, especially at 24 h, might

be related to the specific mechanisms of this cytokine regulation as opposed to others, such as IL-6, IL-8, and TNF- α . IL-1 β is produced differently from many cytokines; it is a two-step process: first, transcriptional priming by pathogen recognition receptors induces pro-IL-1 β mRNA and protein production; second, processing to the active form, which requires post-translational processing, including the NLRP3 inflammasome and caspase-1-mediated cleavage to its active form. This dual-level control could result in greater sensitivity and faster or more targeted release during specific signals [603]. It is known that neutrophils contain preformed components of the inflammasome and can quickly secrete mature IL-1 β upon stimulation [604]. The lack of significant transcriptional induction of IL-6, IL-8 or TNF- α indicates that SARS-CoV-2 might not permanently activate classical NF- κ B-driven proinflammatory responses in neutrophils, particularly over the short term or at low MOI. Instead, the virus may have a selective ability to activate inflammasome-dependent pathways that lead to a disproportionate IL-1 β response that may contribute to early inflammation and immune priming without leading to generalized cytokine release. This may also be accompanied by a distinct cytokine pattern, which further suggests that SARS-CoV-2 has evolved to evade strong immune activation while still being able to trigger appropriate signals that facilitate viral propagation or the modification of host conditions. Interestingly, our Western blot analysis of I κ B α , a negative regulator of NF- κ B, showed increased levels rather than the expected degradation, suggesting that the Delta variant may interfere with or delay NF- κ B activation in neutrophils which aligns with findings that the SARS-CoV-2 nsp3 protein suppresses the NF- κ B pathway by promoting the degradation of NEMO/IKK γ , a key immune regulator, ultimately weakening the antiviral response and facilitating viral replication [605].

C- ISGs Induction and STAT1 Phosphorylation

Our data also showed a selective induction of the ISGs PKR at moderate viral loads (MOIs 0.5 and 1), while other ISGs like OAS1, MxA, and viperin remained unaltered. Furthermore, STAT1 phosphorylation was enhanced at MOI 0.5 and reduced at higher viral MOI. These findings align with the broader role of ISGs in antiviral defence against SARS-CoV-2, as highlighted in recent studies [606]. The IFN system is involved in producing an antiviral response through the activation of ISGs, which limit viral replication by several mechanisms [607]. ISGs such as OAS1, ISG15, MxA and PKR have been found to have direct antiviral activity through the cleavage of viral RNA, inhibition of viral replication and interference with viral protein synthesis against SARS-CoV-2 [606]. For instance, OAS1 triggers RNase L to degrade viral RNA, and ISG15 tags viral and host proteins by ISGylation, which

disrupts SARS-CoV-2 protein function [608, 609]. The role of MxA in preventing the formation of viral replication complexes and thus restricting virus spread has also been documented [610]. An essential function of PKR is to recognize viral RNA and translate this into a stop signal by phosphorylating eIF2 α , thereby arresting protein synthesis and viral propagation [611]. Nonetheless, SARS-CoV-2 subverts the ISG anti-viral responses, including by suppressing STAT1 phosphorylation and inhibiting PKR activation, as we have found in the present study [606].

D- Viral Protein Detection in PMNs

Because other viruses have been seen to replicate in neutrophils, we wanted to determine if SARS-CoV-2 can infect and alter these critical immune cells. Neutrophils are traditionally considered as 'bystanders' in viral infections; however, there is evidence that these cells can also be the targets for viral entry and modulation. For example, murine neutrophils' migratory and phagocytic behaviour changes when they interact *in vitro* with CMV [612]; activated neutrophils have been observed harboring vacuoles containing varicella-zoster virions with extended cytoplasmic projections directed toward these virions [613]; and studies by Blackmon and Ginsberg have documented that multiple viruses are capable of attaching to and penetrating neutrophils [614]. Also, the infection of neutrophils by influenza virus has been well investigated. It was found that exposure results in the inhibition of endocytosis, acceleration of apoptosis, and activation of type I IFN signalling pathways [615], findings that are further supported by this *in vivo* observations with H5N1 virus [612]. Because of this background, our study aims to determine whether SARS-CoV-2 can infect PMNs. We found that SARS-CoV-2 has the potential to target neutrophils in our experiments, where the SARS-CoV-2 N protein was found inside neutrophils with an MOI of 0.5, indicating that viral components can be taken up by these cells. However, our assays do not distinguish between absolute viral replication and the internalisation of viral particles, and therefore, further investigations are required to resolve this question. These should include time-course experiments to track viral RNA transcription and the formation of replication forks, plaque assays or TCID50 to determine the formation of infectious virions, and high-resolution imaging, including confocal and electron microscopy, to detect intracellular viral replication complexes. Furthermore, functional assays should be performed to analyse the effects of viral uptake on neutrophil functions, such as chemotaxis, phagocytosis, degranulation, the generation of ROS, and NET formation, to understand how SARS-CoV-2 affects neutrophil function. All these aspects will be investigated not only to

understand whether neutrophils are productively infected but also to elucidate the immunopathology of COVID-19.

E- Role of TLR8 Signalling

Pre-treatment of neutrophils with the TLR8 inhibitor CU-CPT9a significantly reduced IL-1 β expression following SARS-CoV-2 infection. This demonstrates that neutrophils recognize viral single-stranded RNA and mediate early IL-1 β responses through a TLR8-dependent mechanism. Other studies have underscored the importance of TLR pathways in COVID-19 pathogenesis [533, 572], and our findings support targeting TLR8 to modulate excessive inflammatory responses.

5.5.2 Indirect Effects via Epithelial Cell-Derived Soluble Mediators

Muralidharan *et al.*, (2022) showed that Calu-3 lung epithelial cells infected with the SARS-CoV-2 wild-type strain USA-WA1/2020 at MOIs of 0.01 and 0.1 for 48 hours produced conditioned media that when added to neutrophils for 4 hours, significantly reduced MPO release compared to uninfected controls. Although a clear dose response relationship was not observed, neutrophil elastase secretion slightly increased at the higher MOI. Intracellular staining further demonstrated a reduction in elastase-positive cells, suggesting dysregulated azurophil granule degranulation. In parallel, direct exposure of neutrophils to live SARS-CoV-2 for 2 hours increased viral genome copies and induced significant CD16 shedding, but this was accompanied by reduced release of MPO and elastase. Furthermore, when peripheral blood lymphocytes were incubated with conditioned media from infected neutrophils or co-cultured with them, there was a substantial reduction in B cells, CD8⁺ T cells, and CD4⁺ T cells [392]. However, we examined whether soluble factors from infected epithelial cells could prime or modulate neutrophil responses. Using A549-ACE2 cells infected with various SARS-CoV-2 strains (Alpha, Delta, Omicron, and the IC-19 at low MOI (0.1), we found that:

A- IL-8 Secretion

The production of IL-8 was strain- and time-dependent. At 24 hours post-infection, all SARS-CoV-2 strains (Alpha, Delta, Omicron, and IC-19) produced similar IL-8 levels (338-461 pg/mL) that were comparable to mock-infected controls (361.7 pg/mL), indicating no early enhancement of IL-8 secretion by any strain. However, strain-specific differences emerged at later time points. The Delta strain uniquely showed reduced IL-8 secretion

compared to mock controls at both 48 hours (900.5 pg/mL vs 1097.8 pg/mL) and 72 hours (1862 pg/mL vs 2427.3 pg/mL), while Alpha, Omicron, and IC-19 strains maintained or increased IL-8 levels comparable to uninfected controls across all time points. No induction of other inflammatory cytokines (IL-6, IL-1 β , TNF- α , TGF- β , and IL-10) was observed during infection with any of the strains. These data indicate that IL-8 is the principal cytokine affected by SARS-CoV-2 infection in epithelial cells, with the Delta variant uniquely suppressing IL-8 production at extended time points, potentially affecting neutrophil recruitment dynamics. This finding aligns with evidence from Kaiser *et al.*, study [398], which demonstrated that severe COVID-19 is associated with heightened IL-8 levels, promoting neutrophil activation and NET formation. The study found that patients with severe COVID-19 had markedly higher IL-8 levels than those with mild disease, facilitating neutrophil migration and activation. These neutrophils also underwent degranulation and NET formation, which worsened inflammation and endothelial damage.

B- Modulation of PMN Activation Markers

Supernatants from SARS-CoV-2-infected A549-ACE2 cells showed marked differences in their ability to activate PMNs, with Delta standing out from other strains. Only Delta supernatants triggered substantial CD66b upregulation at 48h (1.64-fold) and 72h (1.40-fold), whereas Alpha, Omicron, and IC-19 had minimal effects. Interestingly, CD11b levels remained stable across all treatments, suggesting that soluble factors from infected cells primarily affect degranulation pathways without influencing adhesion mechanisms. The CD16 response varied considerably between strains. Delta supernatants reduced CD16 expression at later time points (0.63-0.89-fold at 48-72h), likely reflecting activation-induced shedding. In contrast, Omicron and IC-19 increased CD16 levels at 24h, pointing to different activation kinetics. CD62L showed an interesting temporal pattern. All strains initially boosted CD62L expression at 24h (1.68-1.91-fold), but this effect was short-lived for Alpha and Omicron. Delta and IC-19, however, maintained elevated CD62L at 72h (1.38-fold), which may impact neutrophil migration and tissue recruitment. Overall, Delta infection appears to trigger a more robust and sustained PMN activation compared to other variants. The combination of enhanced degranulation, CD16 shedding, and persistent CD62L modulation suggests that Delta may drive stronger inflammatory responses, potentially explaining some of the clinical differences observed between variants.

C- ROS Production and NETosis

The temporal and strain-specific effects of SARS-CoV-2-infected epithelial cell supernatants on PMN function revealed complex patterns of immune modulation. Regarding

ROS production, early exposure (24 h) to Delta and IC-19 supernatants reduced PMNs oxidative burst capacity (0.72-fold and 0.71-fold, respectively), while Alpha and Omicron supernatants had minimal impact. By 48 hours, a broader immunosuppressive effect emerged, with all viral strains suppressing ROS production, most notably Omicron (0.53-fold) and Delta (0.57-fold). However, at 72 hours, Alpha supernatants uniquely stimulated ROS production (1.39-fold), while Delta maintained suppression and Omicron and IC-19 returned to baseline levels.

NETosis induction showed distinct temporal and variant-specific patterns compared to ROS production. At 24 hours, IC-19 supernatants dramatically enhanced NET formation (3.62-fold), while Delta suppressed this response (0.74-fold). By 48 hours, the pattern shifted with Delta and Alpha supernatants promoting NETosis (1.64-fold and 1.31-fold, respectively), while IC-19 became suppressive (0.60-fold). At 72 hours, both Alpha and IC-19 supernatants markedly enhanced NETosis (2.01-fold and 2.52-fold, respectively), with Omicron also showing increased activity (1.36-fold). Together, these findings indicate that SARS-CoV-2 variants differentially regulate neutrophil effector functions over time, reflecting distinct immunomodulatory capacities. Early ROS suppression by Delta and IC-19 may impair antimicrobial activity and facilitate viral persistence, while delayed but pronounced NETosis with Alpha and IC-19 may exacerbate inflammation and tissue damage. Conversely, Omicron's minimal ROS production and modest NET induction suggest a dampened neutrophil response, potentially explaining its reduced clinical severity. These variant-specific activation patterns indicate that the innate immune response to SARS-CoV-2 varies by viral genotype, with implications for both viral clearance and immunopathology.

D- Granule Protein Release

Finally, we observed that supernatants from IC-19-infected cells seemed to elicit the strongest responses in PMN NE release. This aligns with findings that SARS-CoV-2 infection can dysregulate neutrophil degranulation. In this study, circulating neutrophils from healthy donors were isolated and treated with supernatants from infected epithelial cells, as well as directly infected with SARS-CoV-2 *in vitro*. Results showed that infected epithelial cells induced a dysregulated degranulation of primary granules, characterized by a decrease in MPO release but a slight increase in NE secretion [392]. Our results differed: we observed no significant change in MPO release, indicating that the IC-19 strain primarily enhanced NE secretion without affecting other granule proteins. This difference may reflect strain-specific characteristics or cell-type-dependent signalling, as A549-ACE2 and Calu-3

cells produce different cytokine and damage-associated molecule profiles. Both studies demonstrate that epithelial SARS-CoV-2 infection dysregulates neutrophil granule release, with patterns influenced by viral strain and epithelial cell type.

5.5.4 Limitations and Future Directions

While our work provides detailed insight into PMN responses, several limitations warrant discussion. The model used in this study is a short-term (2 h) exposure model, and it may not have captured the full range of the *in vivo* PMN activation with prolonged exposure and more cytokine signals. Moreover, the viral N protein in PMNs does not necessarily mean that the virus has productively infected them. It also would be helpful in future studies to extend the incubation time, include more activation markers, and employ other techniques (for example, immunofluorescence for viral replication complexes or plaque assays), to distinguish between virus uptake and replication. Furthermore, while our experiments with epithelial cell supernatants are informative concerning the potential for indirect activation, the question of which soluble mediators other than IL-8 control these responses remains unanswered. Broader cytokine/chemokine profiling and blockade studies may help to define the essential stimuli of neutrophil activation in COVID-19.

5.6 Conclusion

Our findings reveal that SARS-CoV-2 employs distinct strategies to modulate PMN function. Direct Delta variant exposure caused minimal surface marker changes - CD11b was downregulated while other markers remained unchanged, with only CD63 increasing. However, the virus triggered IL-1 β production via TLR8 signalling and PKR upregulation, indicating selective immune recognition without full activation. Indirect effects through infected epithelial cell supernatants showed strain-specific modulation. All viral strains suppressed ROS production at 48 hours, while NET formation varied dramatically - IC-19 strongly enhanced NETosis early then fluctuated. Notably, IC-19 supernatants consistently increased neutrophil elastase release across all time points.

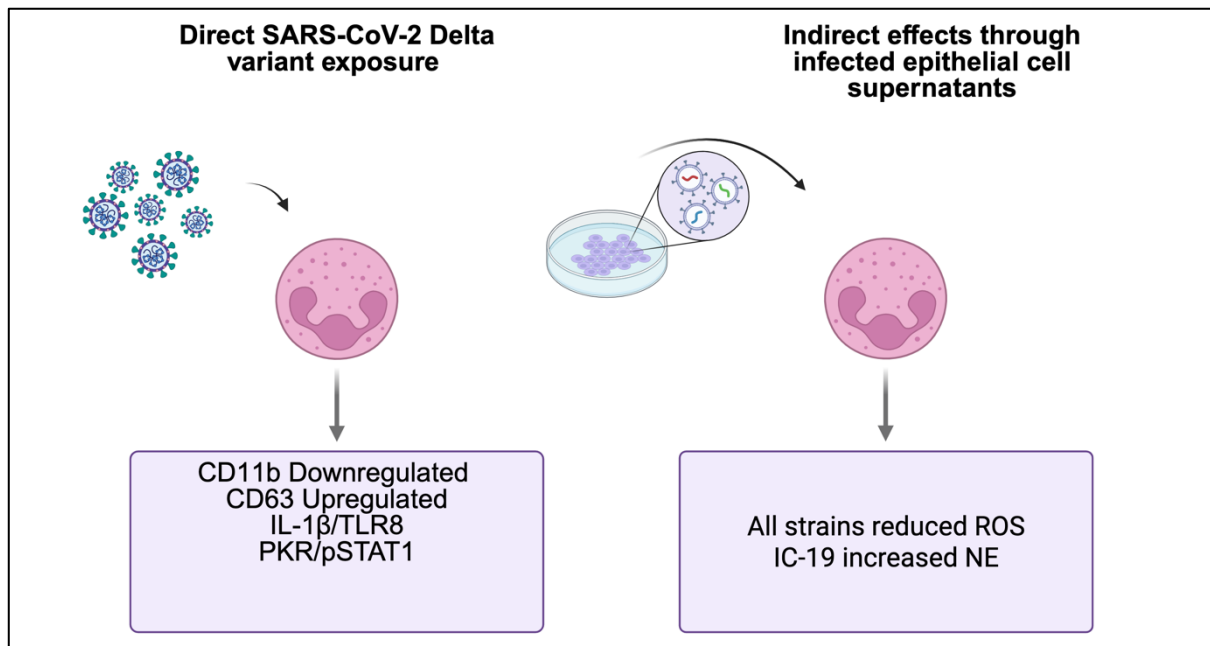


Figure 5. 24 Distinct Direct and Indirect Effects of SARS-CoV-2 Delta Variant on PMN Function

SARS-CoV-2 modulates PMN function through direct and indirect mechanisms. Direct Delta variant exposure minimally affects surface markers (CD11b downregulation, CD63 upregulation) but triggers IL-1 β production via TLR8 signalling and PKR upregulation. Epithelial cell supernatants from SARS-CoV-2-infected cells showed trending strain-specific PMN responses: while all tested strains consistently suppressed ROS production, IC-19 supernatants showed a trend toward increased NE.

Chapter 6

General Discussion

6. General Discussion

6.1 Introduction

PMNs play a significant role in COVID-19 pathology. SARS-CoV-2 causes abnormal neutrophil degranulation and affects MPO and NE release [392]. SARS-CoV-2 promotes NET formation, which is linked to increased ROS production and contributes to thrombosis [374, 616]. During severe SARS-CoV-2 infection, neutrophils contribute significantly to lung immunopathology, and elevated neutrophil counts are strongly associated with adverse outcomes [617]. SARS-CoV-2-induced neutrophil dysfunction includes the emergence of immature and immunosuppressive subsets, excessive NET formation, and enhanced lung infiltration [618]. These neutrophil dysfunctions drive ARDS, cytokine storm, and immunothrombosis [619, 620]. Targeting PMNs and NETs could represent a novel therapeutic strategy in severe COVID-19.

6.2 Overview of Key Findings

This thesis investigated how SARS-CoV-2 alters PMN function through viral components such as SP and ssRNA, direct viral infection, and indirect stimulation by supernatants from infected epithelial cells. The results showed that SARS-CoV-2 activates PMNs through various pathways, leading to PMN overactivation and resulting in the inflammation, tissue destruction, and immunosuppression observed in COVID-19. One of the main discoveries was that SARS-CoV-2 SP selectively activated PMNs, leading to the marked secretion of IL-8, an essential chemokine in the regulation of PMN recruitment. However, SARS-CoV-2 SP stimulation did not result in classical effector functions of PMNs, such as enhanced CD11b or CD66b surface expression, ROS production, degranulation or NETosis. Notably, SARS-CoV-2 SP induced TGF- β production, a cytokine with immunomodulatory, tissue remodelling, immunosuppressive, and fibrotic functions. This TGF- β production suggests that SARS-CoV-2 SP elicits a regulatory rather than cytotoxic neutrophil response, which may contribute to pulmonary complications in COVID-19. Additionally, SARS-CoV-2 SP enhances PMN survival by reducing apoptosis, promoting prolonged PMN viability. Prolonged survival of PMNs in infected tissues may exacerbate inflammation, prolong its resolution, and thereby exacerbate tissue damage. In contrast, SARS-CoV-2 ssRNA triggered a robust proinflammatory response through endosomal TLR8. Activation of this pathway led to the downstream engagement of MyD88, NF- κ B, and p38 MAPK signalling, resulting in elevated secretion of IL-6, IL-8, TNF- α , and IL-1 β . Importantly, TGF- β was not induced by SARS-CoV-2 ssRNA, indicating that SARS-CoV-2 SP and ssRNA elicit distinct

PMN responses. These results identify TLR8 as a key viral RNA sensor in PMNs, orchestrating the acute inflammation and cytokine storm features of severe COVID-19. This thesis also showed that PMNs are directly susceptible to infection by the SARS-CoV-2 Delta variant. PMNs may engulf live SARS-CoV-2, as indicated by detection of viral N protein and RNA within the cells. SARS-CoV-2-infected PMNs exhibited enhanced STAT1 phosphorylation, upregulation of ISGs such as PKR, and TLR8-dependent IL-1 β production. These results demonstrate PMNs as active effector cells and direct participants in viral sensing and innate immune response propagation. In addition to direct contact, the thesis also established the role of epithelial-derived factors in the activation of PMNs. The SARS-CoV-2-infected A549-ACE2 epithelial cell line produced factors that reduced ROS and enhanced NE production. These results indicate that paracrine signalling from infected epithelial cells may prime PMNs, even in the absence of direct viral contact, and contribute to the creation of the inflamed pulmonary environment characteristic of COVID-19.

6.3 SARS-CoV-2 SP selectively induced IL-8 production in PMNs

IL-8 is highly regulated at the transcriptional level and is often induced through PRRs, including TLRs, which signal downstream via NF- κ B and MAPK signalling cascades [621]. It is secreted by monocytes, epithelial cells and endothelial cells in response to microbial products, cytokines and cellular stress [622]. Once secreted, IL-8 binds to neutrophil CXCR1 and CXCR2 receptors, leading to chemotaxis, degranulation, respiratory burst, and increased cell survival, thereby enhancing the inflammatory response [623]. IL-8 is one of the key cytokines that directs the recruitment and activation of PMNs during viral infections, making it a central mediator in viral respiratory pathogenesis. Clinical studies demonstrate the pathological significance of IL-8 in viral diseases. In children with virus-induced asthma, neutrophilic inflammation and symptom severity were strongly correlated with elevated levels of IL-8 in nasal aspirates, highlighting its role in disease severity [624]. Studies have also shown that elevated IL-8 levels correlate with disease severity and prolonged illness in severe COVID-19 patients [625]. Furthermore, IL-8 can suppress neutrophil apoptosis upon engagement with immune complexes, prolonging neutrophil lifespans and worsening inflammation and tissue damage in acute lung injury [626]. IL-8 also stimulates neutrophil degranulation, leading to the release of proteolytic enzymes that can degrade tissue structures, as observed in joint damage models and potentially in viral infection-induced epithelial injury [627]. Our study demonstrated that stimulation of human primary PMNs with SARS-CoV-2 SP induced selective IL-8 production but not IL-1 β or IL-6. This selective IL-8 upregulation is consistent with prior evidence indicating that IL-8 plays a

crucial role in neutrophil-driven inflammation and may contribute to the immunopathology of COVID-19. Mechanistically, our inhibition experiments revealed that this response occurs through partial activation of TLR2 and TLR4. However, while blocking TLR2 and TLR4 decreased SP-induced IL-8 secretion, this decrease was not statistically significant, suggesting the involvement of alternative or redundant pathways. Downstream signalling analysis further supported the complexity of this response. While inhibition of NF- κ B reduced IL-8 levels to a limited extent, inhibition of p38 MAPK and ERK1/2 also had a similarly small modest effects, suggesting that SARS-CoV-2 SP can stimulate IL-8 production via multiple partially redundant signalling pathways. This pathway redundancy may represent an evolved viral strategy to ensure consistent IL-8 induction despite host defensive responses. This selective activation pattern may represent a virally-evolved mechanism that selectively activates PMNs without triggering a cytokine storm during early infection stages. Such a targeted IL-8 response may be beneficial in controlling viral infection at the initial stage of the disease. However, it may contribute to lung inflammation and injury during later stages by sustaining PMN recruitment, delaying apoptosis, and promoting tissue destruction. This, however, is not unique to SARS-CoV-2 SP. Other stimuli have been shown to selectively stimulate IL-8 without affecting other proinflammatory cytokines. For example, Intravenous immunoglobulin therapy (IVIg) induces monocytes to produce only IL-8 and IL-1ra without inducing IL-1 β , TNF- α or IL-6 [628]. Similarly, ultrashort synthetic lipopeptides have been observed to stimulate IL-8 production by macrophages without inducing the production of IL-1 β or TNF- α , indicating that specific structural or receptor-mediated mechanisms can lead to an IL-8 response without affecting the levels of IL-1 β or TNF- α [629]. In the context of viral infection, HCMV has also been shown to induce IL-8 production in endothelial cells independently of IL-1 or IL-6 signalling [630]. DENV has been reported to increase IL-8 production in monocytes, epithelial, and endothelial cells by activating NF- κ B and histone acetylation [631]. Notably, IL-8 enhances the replication of positive-strand RNA viruses, such as encephalomyocarditis virus and CMV, suggesting a potential pro-viral role in pathogenesis and viral persistence [632, 633]. Given that SARS-CoV-2 is also a positive-strand RNA virus, SP-induced IL-8 production may similarly facilitate viral replication and persistence, representing a potential therapeutic target for COVID-19 treatment.

6.4 SARS-CoV-2 SP Delays PMN Apoptosis and Promotes Cell Survival

Beyond cytokine production, SARS-CoV-2 SP also modulates PMN lifespan. Our data demonstrate that the SARS-CoV-2 SP significantly boosts the survival of human primary PMNs. Neutrophils are generally short-lived cells with constitutive apoptosis that occurs within a few hours [634]. During inflammatory conditions, various mediators, such as LPS, GM-CSF, and G-CSF, inhibit neutrophil apoptosis, thereby extending their functional lifespan and potentially exacerbating tissue damage [635, 636]. Our experiments revealed a significant reduction in apoptotic PMNs at SARS-CoV-2 SP concentrations of 50 and 100 µg/mL compared to untreated controls, indicating that SARS-CoV-2 SP delays PMN apoptosis in a dose-dependent manner. This survival enhancement is particularly significant in the context of IL-8, which is not only a powerful chemoattractant but also a potent survival factor that suppress PMN apoptosis. Previous studies have shown that IL-8, especially when bound to anti-IL-8 autoantibodies, can prolong the survival of neutrophils by inhibiting apoptosis through caspase inhibition [637]. Since we observed SP-induced IL-8 production under the same experimental conditions, it is likely that autocrine or paracrine IL-8 signalling significantly contributes to enhanced neutrophil survival. However, prolonged PMN lifespan can be detrimental, as activated PMNs continue to release ROS, proteolytic enzymes, and proinflammatory mediators that exacerbate tissue damage. In the context of COVID-19, this may lead to persistent neutrophilic inflammation in the lungs and other affected organs, thereby contributing to the development of ARDS. Our findings therefore suggest that SARS-CoV-2 SP promotes PMN activation and persistence in inflamed tissues by selectively inducing IL-8, thereby amplifying the inflammatory cascade characteristic of severe COVID-19. Importantly, these results align with a growing body of evidence that many pathogens suppress neutrophil apoptosis as an evolutionary strategy to enhance their survival and spread. For example, RSV delays neutrophil apoptosis via PI3K and NF-κB signalling pathways that are regulated by intracellular TLRs and IL-6 signalling [638]. Similarly, *Coxiella burnetii* prevents apoptosis by activating MAPK pathways and increasing the pro-survival protein Mcl-1 [639]. Additionally, HCMV has been shown to significantly extend neutrophil survival by suppressing apoptosis. This mechanism may contribute to viral persistence and tissue damage. Exposure of neutrophils to clinical HCMV strains such as Merlin activates ERK/MAPK and NF-κB pathways in a viral gene expression-independent manner, resulting in marked apoptosis delays [418]. These viral precedents, along with similar findings in other viral and bacterial infections, suggests that SARS-CoV-2 SP employs a conserved pathogenic mechanism to inhibit PMN apoptosis.

Future mechanistic studies should confirm the involvement of key survival pathways such as PI3K/Akt, ERK1/2, and NF- κ B in this process and determine whether similar cytokine-driven feedback loops prolong PMN lifespan during SARS-CoV-2 infection. Given the central role of dysregulated neutrophil survival in COVID-19 pathogenesis, these pathways represent promising targets for novel therapeutic approaches to mitigate neutrophil-mediated inflammation and tissue destruction in COVID-19.

6.5 SARS-CoV-2 SP Induces TGF- β Production in Human Primary PMNs via Integrin-Dependent Mechanisms (ATN-161)

The multifunctional cytokine TGF- β plays a dual role in immune regulation. While essential for controlling excessive inflammation and maintaining immune homeostasis, its overexpression promotes immune suppression and fibrosis [640]. In viral infections, TGF- β has been reported to dampen host antiviral responses and contribute to tissue remodelling. For instance, in influenza A infection, TGF- β functions as a viral promoter by suppressing early immune responses, including IFN β production [641]. Similarly, in hepatocytes, the HCV core protein increases TGF- β 1 transcription and expression and activates latent TGF- β signalling via thrombospondin-1, thereby facilitating stromal hepatocyte crosstalk [642, 643]. Importantly, our study demonstrated that stimulation of primary human PMNs with SARS-CoV-2 SP led to a marked increase in TGF- β production, a finding that was notably absent in SARS-CoV-2 ssRNA-stimulated PMNs, highlighting the specificity of this SP-mediated response. These results suggest that PMNs may represent an additional cellular source of TGF- β during SARS-CoV-2 infection, contributing to the immunosuppressive and tissue-remodelling environment characteristic of COVID-19. The selective induction of TGF- β by SP, but not ssRNA, suggests distinct pathogenic roles for different viral components in shaping the host immune landscape. The ability of PMNs to produce TGF- β in response to SP stimulation carries multiple critical implications for disease progression and pathogenesis. First, increased TGF- β levels can suppress the activity of effector T cells and NK cells, which may adversely affect the host's antiviral immune response, potentially facilitating viral persistence and immune evasion. Second, TGF- β induces Treg differentiation, which further sustains immunosuppression and creates a permissive environment for viral replication. Third, TGF- β is a key mediator of fibrosis and extracellular matrix accumulation, and its elevation has been implicated in lung fibrosis among patients with severe or prolonged COVID-19, particularly in cases of post-acute COVID-19 syndrome. Mechanistically, further investigation is needed to fully elucidate the

signalling pathways through which SARS-CoV-2 SP induces TGF- β production in PMNs. Promising candidates include Smad-dependent pathways, MAPKs, and PI3K/Akt, which are known to regulate TGF- β expression in various immune cells. Understanding how SARS-CoV-2 subverts TGF- β signalling may help elucidate COVID-19 immunopathogenesis and identify potential therapeutic strategies to counter immune suppression and tissue fibrosis. Our mechanistic studies revealed that SP-induced TGF- β production significantly depends on integrin signalling. Pre-treatment of PMNs with ATN-161, a selective inhibitor of RGD-binding integrins (primarily $\alpha 5\beta 1$ and $\alpha v\beta 3$), significantly reduced TGF- β secretion, suggesting that SARS-CoV-2 SP engages integrins to trigger downstream signalling cascades leading to TGF- β release. This integrin-mediated mechanism aligns with recent studies suggesting that SARS-CoV-2 may manipulate RGD motifs, integrin binding, and ACE2 to modulate host cell functions [644, 645], representing a convergent pathogenic strategy that exploits multiple cellular entry and signalling pathways to establish a favourable microenvironment for viral persistence and tissue damage.

6.6 TLR8 Recognizes GU-Rich ssRNA in SARS-CoV-2

While SARS-CoV-2 SP selectively activates PMNs through specific pathways, viral RNA components trigger broader inflammatory responses, representing a complementary mechanism of immune activation. TLR8 is a key endosomal sensor of ssRNA, particularly recognizing the GU-rich sequences abundant in the SARS-CoV-2 genome. Upon recognizing these sequences in immune cells such as neutrophils, TLR8 triggers a cascade of proinflammatory responses via MyD88-dependent NF- κ B and MAPK signalling pathways [646]. This mechanism is evolutionarily conserved across viruses, as GU-rich ssRNAs from both HIV-1 and SARS-CoV-2 exhibit similar immunostimulatory effects [647], suggesting that TLR8 serves as a broad-spectrum viral RNA sensor. Our study demonstrated that GU-rich ssRNAs derived from the SARS-CoV-2 genome are potent activators of TLR8 signalling in PMNs, leading to robust proinflammatory responses. Importantly, this contrasts sharply with the regulatory TGF- β response induced by SP, highlighting how different viral components drive distinct immunological outcomes. These effects likely contribute to viral pathogenesis by exacerbating inflammation and promoting the cytokine storm characteristic of severe COVID-19. Specifically, the SARS-CoV-2 ssRNA fragment SCV2-RNA1 activated TLR8 and upregulated CD11b expression in human primary PMNs, promoting cellular activation and robust release of key proinflammatory cytokines including TNF- α , IL-6, IL-1 β and IL-8. Additionally, SCV2-

RNA2 significantly modulated I κ B α protein levels and activated the NF- κ B pathway, a central regulator of inflammatory gene expression. Crucially, both NF- κ B activation and cytokine production were strictly dependent on TLR8, as treatment with CU-CPT9a, a specific TLR8 inhibitor, markedly reduced these responses, confirming the central role of TLR8 in mediating ssRNA-induced PMN activation. Furthermore, downstream analysis revealed activation of p38 MAPK signalling, indicating that TLR8 engagement triggers multiple convergent inflammatory pathways. Collectively, our findings highlight the pivotal role of TLR8–NF- κ B and MAPK signalling in mediating the proinflammatory effects of SARS-CoV-2 ssRNAs, positioning TLR8 as a critical mediator of viral RNA-induced cytokine storm and a potential therapeutic target for mitigating excessive inflammation in COVID-19.

6.7 SARS-CoV-2 Delta Variant Alters Neutrophil Activation Markers and Induces IL-1 β via TLR8-PKR-STAT1 Axis

Our findings demonstrate that the SARS-CoV-2 Delta variant infection of human primary PMNs significantly alters their phenotype and inflammatory signalling pathways. The Delta variant infection downregulated the surface expression of CD11b, a key integrin adhesion molecule involved in neutrophil migration, while simultaneously upregulating CD63, a marker of primary granule exocytosis and degranulation. This CD11b/CD63 expression shift represents critical functional reprogramming of neutrophils toward secretory activation rather than adhesion-mediated extravasation, potentially favouring local inflammatory mediator release over tissue infiltration. These phenotypic changes are consistent with previous data demonstrating that the Delta variant is more effective than other strains in activating neutrophils [648]. Furthermore, gene and protein expression analyses revealed enhanced IL-1 β levels in Delta-infected PMNs, suggesting robust proinflammatory signalling and inflammasome activation. Mechanistically, we found that this IL-1 β induction was mediated through TLR8-dependent signalling. This aligns with previous work establishing that SARS-CoV-2 variants can directly activate neutrophils through TLR7/8 and IRF3/STING-dependent mechanisms, with variant-specific differences in activation intensity that correlate with clinical severity [648], thus linking our viral component studies to direct infection outcomes. Importantly, we found that PKR was significantly upregulated at multiplicity of infections (MOIs) 0.5 and 1, indicating that a robust antiviral response was activated. PKR is a well-characterized ISG that mediates translational arrest and inflammasome priming [649], making it a critical node in the antiviral response network. In

addition, STAT1 phosphorylation increased with higher viral doses, suggesting that viral RNA sensing triggers inflammation and primes the IFN signalling pathway. However, our findings indicate that the Delta variant does not completely suppress innate sensing in neutrophils, contrasting with epithelial cells where it reportedly strongly inhibits IFN responses [650], revealing cell-type-specific differences in viral immune evasion strategies.

6.8 IC-19-Infected Epithelial Supernatants Show a Trend Toward NE Release Without Significant Activation

In addition to direct viral effects, infected epithelial cells release soluble factors that influence PMN. In this thesis, we investigated whether epithelial-derived factors from SARS-CoV-2-infected cells stimulate PMN degranulation by measuring NE levels after incubating PMNs with supernatants from A549-ACE2 cells infected with different SARS-CoV-2 variants. Of all variants evaluated (Alpha, Delta, Omicron, and IC-19), only IC-19 showed a consistent trending increase in NE release at 24, 48, and 72 hours post-infection, although not statistically significant ($p = 0.5$), this consistent trend suggests that the IC-19 variant may induce partial PMN activation through soluble factors released by infected epithelial cells. This NE release pattern reveals a potentially important aspect of IC-19 variant-mediated regulation of PMN granule release that warrants further investigation with larger sample sizes. Our findings are partially consistent with those of Muralidharan *et al.*, who demonstrated that supernatants from SARS-CoV-2-infected epithelial cells induced only modest increases in NE release but marked reductions in MPO release. In their study, this dysregulated degranulation was observed even in the absence of other immune cells and also occurred when neutrophils were directly infected with SARS-CoV-2. Although Muralidharan *et al.*, employed a different epithelial model (Calu-3 cells) and a distinct viral strain (USA-WA1/2020), their data similarly demonstrated that SARS-CoV-2 infection lead to selective and aberrant neutrophil degranulation, supporting the concept of variant-independent degranulation dysregulation. We observed a comparable trend with the IC-19 variant but not with Alpha, Delta, or Omicron variants, suggesting that variant-specific differences in epithelial responses may influence secreted factor profiles and consequently affect neutrophil behaviour [392].

6.9 Methodological Strengths and Innovations

In this thesis, a multifaceted and rigorous experimental strategy was employed to investigate how SARS-CoV-2 affects the function of PMNs. This work is significant for its experimental precision, physiological relevance, and methodological innovation, addressing

critical confounders noted in recent studies while refining immune cell analysis approaches and establishing new methodological standards for SARS-CoV-2 research. A major methodological strength was using highly purified, endotoxin-free, glycosylated SARS-CoV-2 SP produced in mammalian systems. This choice was based on the seminal study by Cinquegrani *et al.*, which revealed that many recombinant SARS-CoV-2 SP preparations used in previous studies were contaminated with endotoxins or were underglycosylated, leading to erroneous findings about SARS-CoV-2 SP-induced inflammation [511]. By rigorously controlling for these variables, this thesis demonstrates that the observed IL-8 and TGF- β responses represent genuine SARS-CoV-2 SP-specific effects rather than artifacts of contamination or protein misfolding, thereby establishing the validity of SP as a direct immune modulator. Another innovative approach involved the strategic use of synthetic SARS-CoV-2 ssRNA to selectively stimulate TLR8 and using specific pharmacological inhibitors to suppress downstream signalling, thereby definitively validating pathway specificity. Through this strategy, the thesis dissected the mechanistic basis of PMN inflammatory responses and demonstrated that cytokine production is primarily driven by the TLR8-NF- κ B/p38 MAPK signalling axis, providing unprecedented mechanistic clarity. A particularly notable finding of this study was the demonstration of direct infection of human PMNs by live SARS-CoV-2 Delta variant, a phenomenon that has not been fully characterized in primary human PMNs. Our results reveal a previously underappreciated role for PMNs as both permissive and virus-responsive cells that engage innate immune signalling through viral RNA and N protein detection, leading to STAT1 phosphorylation and induction of ISGs such as PKR. Furthermore, this thesis employed a novel approach using conditioned media from SARS-CoV-2-infected epithelial cells (A549-ACE2) to mimic paracrine signalling at the tissue level. This model significantly advanced physiological relevance by replicating the effects of soluble factors on PMN priming and activation, thereby bridging direct viral interactions with the infected tissue microenvironment and providing insights into indirect viral pathogenesis mechanisms. A cutting-edge methodological technique used in this thesis was the development of a flow cytometry-based NETosis assay, which provides key advantages over traditional microscopy approaches. Freshly isolated PMNs were stained with CD66b, a neutrophil-specific granulocyte marker, and SYTOX Orange, a membrane-impermeable dye that binds extracellular DNA, a hallmark of NETosis. Dual labelling enabled precise quantification of NET-forming cells by flow cytometry. This approach allowed rapid, high-sensitivity analysis of large cell populations with minimal subjectivity, unlike microscopy, which relies

on manual image analysis and is subject to observer bias. Incorporating a live/dead viability dye further increased specificity by excluding non-viable cells, while a standardised staining and gating strategy ensured reproducible and scalable results across donors and treatment conditions. This methodological advance represents a significant contribution to the field, offering researchers a more objective and high-throughput approach to NETosis analysis. This technique does have limitations. The primary drawback is the lack of spatial and morphological information, as flow cytometry cannot visualise NET structures or discriminate between vital and suicidal NETosis. Additionally, fixation steps required for biosafety and signal stabilization may alter cell morphology or fluorescence intensity if not carefully optimized. Despite these limitations, the method proved robust, straightforward to implement, and highly suitable for comparative NETosis analysis in large-scale studies, making it a valuable addition to the neutrophil research toolkit.

6.10 Limitations of the Study

This thesis comprehensively analyses SARS-CoV-2-induced PMN responses using a multi-layered approach. However, several significant limitations need to be acknowledged that provide important directions for future research. First, although SARS-CoV-2 N protein and RNA were detected in PMNs exposed to the Delta variant (confirming direct infection), we did not assess the extent of viral replication or the potential for viral transfer to other cells. It remains unclear whether PMNs function as true viral host cells or merely as carriers of viral material, a distinction with critical implications for viral dissemination and pathogenesis. Advanced techniques such as replication-competent virus tracking or single-cell transcriptomics will be required to definitively clarify this mechanistically important question. Second, although this work's use of highly purified, endotoxin-free recombinant SARS-CoV-2 SP represents a major methodological strength, the precise physiological concentrations and forms of SARS-CoV-2 SP that PMNs encounter during natural infection remain unclear. Circulating SARS-CoV-2 SP levels vary considerably among patients, influenced by viral load, tissue localisation, and host metabolic factors, as well as disease severity and temporal progression. Therefore, caution is warranted when extrapolating concentration-dependent *in vitro* effects to clinical settings, though our dose-response studies provide valuable mechanistic insights. Third, TLR8 signalling was studied using synthetic ssRNA mimics, which provide clean receptor engagement but may not fully recapitulate the structural complexity or cellular context of viral genomic RNA during infection. Additional studies using extracted viral RNA or virion-associated RNA could

provide a more accurate representation of TLR engagement *in vivo*, potentially revealing additional regulatory mechanisms or co-stimulatory signals. Fourth, while our epithelial supernatant studies provide valuable insights into paracrine signalling, they represent a simplified model of the complex tissue microenvironment during SARS-CoV-2 infection, which involves multiple cell types, extracellular matrix components, and dynamic inflammatory mediators that may influence PMN responses. Lastly, while this study characterizes responses to the SARS-CoV-2 Delta variant, it does not comprehensively measure PMNs' responses to a full panel of variants, including Omicron sublineages or the effects of post-vaccine breakthrough infections. As SARS-CoV-2 continues to evolve, determining how neutrophil responses differ across viral variants and in the context of host immune status, including vaccination history, will be crucial for understanding evolving disease patterns and optimizing therapeutic interventions. Despite these limitations, this thesis establishes fundamental mechanistic principles of SARS-CoV-2-PMN interactions that provide a strong foundation for future translational studies and therapeutic development.

6.11 Future Directions

Beyond the mechanistic insights and methodological innovations discussed in this thesis, several unexplored or under-addressed areas warrant future investigation to expand the current understanding of PMN biology in the context of SARS-CoV-2 infection. One promising direction involves investigating sex- and age-dependent differences in PMN responses to SARS-CoV-2 components. Epidemiological data indicate that males and older individuals are more susceptible to severe COVID-19, yet the cellular and molecular basis for this disparity remains unclear [651-653]. Future studies should investigate whether age- or sex-related differences exist in the cytokine production, survival, or NETosis profiles of PMNs, which may help tailor personalized therapeutic approaches and explain observed demographic risk patterns. Another critical but underexplored area is the influence of the host metabolic state on PMN activation. PMN function is significantly affected by obesity, diabetes, and metabolic syndrome, which are also major risk factors for severe COVID-19. Examining the effects of metabolic alterations, such as hyperglycemia, insulin resistance, or lipid dysregulation, on PMN responses to SARS-CoV-2 may reveal new mechanisms of immune dysregulation and comorbidity susceptibility. Furthermore, future work should address the impact of COVID-19 vaccination on PMN activation during breakthrough infections and vaccine-induced immune conditioning. Whether prior immunisation alters the magnitude or nature of PMN responses to SARS-CoV-2 variants, including NET formation capacity, SARS-CoV-2 SP responses, or regulatory cytokine production such as TGF- β ,

remains unknown. Comparisons between vaccinated and unvaccinated donors may reveal immune training or tolerance phenomena that influence infection outcomes. To strengthen the clinical relevance of *in vitro* findings, *in vivo* confirmation using animal models or patient-derived samples is essential. Although mouse models of neutrophil biology have limitations, humanized or ACE2 transgenic systems may provide insights into how SARS-CoV-2 affects neutrophil migration, activation, and their contribution to disease pathogenesis during infection. Clinical validation in human settings could include analysis of lung biopsy samples or bronchoalveolar lavage fluid from COVID-19 patients, where our findings predict that SARS-CoV-2 SP would induce TGF- β and IL-8 *in vivo*. Beyond COVID-19, the insights gained here may inform studies of other acute respiratory viral infections in which PMNs have pathogenic or protective roles, including influenza, RSV, or emerging pathogens. Understanding how viral components interact with PMN receptors such as TLR8, or how epithelial–neutrophil communication shapes inflammation, could have broad applications across infectious disease immunology and contribute to the development of pan-viral therapeutic strategies. Finally, the role of neutrophils in post-viral immune remodelling and tissue repair remains poorly defined and represents a critical knowledge gap. Given the persistence of symptoms in long COVID and other post-viral syndromes, future studies should examine whether the prolonged presence of SARS-CoV-2 SP or viral components induces sustained TGF- β , IL-8, and NET formation in lung tissue through analyses of lung biopsies or bronchoalveolar lavage fluid from patients.

6.12 Concluding Remarks

This thesis presents unique and systematic insights into how SARS-CoV-2 affects and regulates the function of human PMNs. The study employs a comprehensive approach combining direct viral infection experiments, stimulation with viral components, and epithelial-PMN interaction models to reach these conclusions. These findings establish that PMNs are not passive bystanders in COVID-19 but active and central determinants of the innate immune landscape. Key findings include that SARS-CoV-2 SP selectively induces IL-8 and TGF- β while prolonging PMN survival without triggering classical activation, that live viral infection induces robust antiviral responses through STAT1/ISG upregulation, and that viral ssRNA triggers TLR8-mediated cytokine production through NF- κ B and MAPK signalling pathways. By using highly purified, endotoxin-free recombinant SARS-CoV-2 SP and developing a novel flow cytometry-based NETosis assay, this work successfully resolved methodological inconsistencies and provided significant technical innovations for

the field. Collectively, these findings demonstrate that PMNs are far more dynamic players in SARS-CoV-2 pathogenesis than previously recognized, capable of direct viral sensing, selective cytokine production, and sophisticated immunoregulatory signalling that balances inflammatory and anti-inflammatory responses. This work establishes a robust foundation for future studies on the mechanistic underpinnings of neutrophil-virus interactions and the targeted control of neutrophil activity in COVID-19 and other similar inflammatory diseases. The findings of this study also provide crucial information that can be used in the design of novel therapeutic strategies aimed at suppressing immunopathology without compromising the host defence against viral infection, ultimately contributing to more precise and effective treatments for COVID-19 and related respiratory viral diseases.

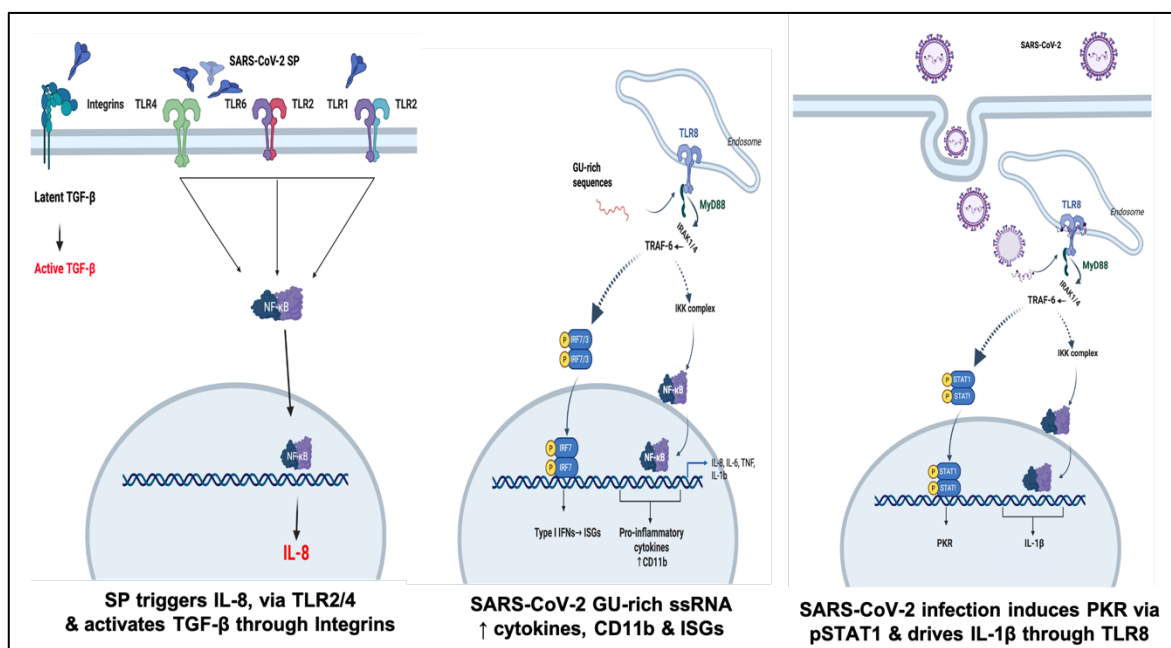


Figure 6.1. SARS-CoV-2-mediated innate immune signalling pathways in human PMNs.

Three mechanisms by which SARS-CoV-2 activates PMNs are illustrated. **(Left)** SARS-CoV-2 SP engages surface receptors (TLR2/4) and activates NF-κB to selectively induce IL-8. In parallel, SP activates Integrins, which convert latent TGF-β to its active form, contributing to immunomodulatory signalling. **(Middle)** Viral GU-rich ssRNA is sensed by endosomal TLR8, leading to parallel activation of IRF7/3 and NF-κB. This drives Type I IFN and ISG production alongside pro-inflammatory cytokines (IL-8, IL-6, TNF, IL-1β) and CD11b upregulation. **(Right)** Live SARS-CoV-2 infection triggers endosomal TLR8 signalling, activating pSTAT1, which induces PKR as part of the antiviral response. TLR8 signalling additionally drives IL-1β production through NF-κB activation.

Chapter 7

References

7. References

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