

Role of Atopy in Interferon Production in Response to Rhinovirus and *Streptococcus pneumoniae* Infection in Mild Asthma

Jonathan David Robert Macintyre

Department of Respiratory Medicine
National Heart and Lung Institute
Faculty of Medicine
Imperial College London
Norfolk Place
London W2 1PG
UK

A report submitted for PhD examination

2014

Statement of Work

I declare that the work presented in this thesis was carried out by me and that any work that is not my own is appropriately referenced. I am grateful for some assistance in patient recruitment and bronchoscopy from Ajerico del Rosario. I am also grateful to Eteri Bakhsoliani for some assistance with RNA isolation, cDNA synthesis, Taqman RT-PCR and MSD and Vera Gielen for some assistance with RNA isolation and cDNA synthesis.

Jonathan Macintyre

The copyright of this thesis rests with the author and is made available under a Creative Commons Attribution Non-Commercial No Derivatives licence. Researchers are free to copy, distribute or transmit the thesis on the condition that they attribute it, that they do not use it for commercial purposes and that they do not alter, transform or build upon it. For any reuse or redistribution, researchers must make clear to others the licence terms of this work.

Abstract

Asthma exacerbations result in significant morbidity and rhinoviruses (RVs) are largely responsible. Asthmatics have increased susceptibility to RV infections and defective RV-induced interferon (IFN) production in atopic asthmatics (AA) has been observed in human bronchial epithelial cells (HBECs) and bronchoalveolar lavage (BAL) cells. 70-90% of asthmatics are atopic. Whether the previously observed IFN deficiency is asthma- or atopy-specific is unknown. Asthmatics have increased risk of invasive disease with *Streptococcus pneumoniae* (*Spn*) and proinflammatory/antibacterial cytokines are protective against bacterial infections. IFNs are anti-viral cytokines, but models of *Spn* infection have demonstrated type I IFNs are protective. Investigation of toll-like receptors (TLRs) might delineate the signalling pathways involved in IFN/proinflammatory cytokine deficiencies further.

This study hypothesised that RV, *Spn* and TLR stimulation of HBECs, BAL cells and peripheral blood mononuclear cells (PBMCs) would result in deficient IFN production in AA and non-atopic asthmatics (NAA), compared to atopic non-asthmatics (ANA) and non-atopic non-asthmatics (NANA). IFN and proinflammatory cytokine mRNA and protein expression was analysed by qPCR and the MesoScale Discovery platform.

IFNs were induced in all cell types following stimulation with RVs and TLR3/7/8 agonists. No differences were observed between asthmatics or controls; thus the role of atopy in IFN deficiency is still uncertain. IFNs were not induced from *Spn*-stimulated cells. Anti-bacterial cytokines were induced in PBMCs stimulated with *Spn*, but no differences were observed between asthmatics or controls. HBECs responded to TLR1/2/6/4/5/9 stimulation with non-significant increases in anti-bacterial cytokines and type III IFNs. PBMCs responded to TLR1/2/6/4/5/9 stimulation with no induction of type I or III IFNs, but increases in anti-bacterial cytokine proteins. Deficiency in asthmatics was not observed.

The lack of IFN/proinflammatory cytokine deficiency was suggested to be due to the asthmatics having relatively mild disease. Further studies in more severe forms of asthma are required to investigate these responses further.

Acknowledgements

I would like to thank my supervisors Professor Sebastian Johnston and Dr Onn Min Kon for all their assistance and guidance throughout this study. I am grateful for all the support of members of the department of respiratory medicine and particularly Dr Anny Sykes and Dr Vera Gielen. I am so appreciative of the support and patience I have had from my family and friends, particularly during the long writing-up stage of my project.

Contents

Statement of Work	2
Abstract.....	3
Acknowledgements.....	4
Abbreviations	17
Chapter 1: Introduction.....	20
1.1 Asthma	20
1.1.1 Epidemiology.....	20
1.1.2 Asthma exacerbations	21
1.2 Viruses and asthma.....	22
1.2.1 Respiratory virus infection and asthma exacerbations.....	22
1.2.2 Rhinoviruses and asthma.....	23
1.2.3 Rhinoviruses	24
1.2.4 Rhinovirus infection of airway cells.....	25
1.2.5 Rhinovirus infection and peripheral blood mononuclear cells (PBMCs).....	26
1.3 Bacteria and asthma	27
1.3.1 Atypical bacteria and asthma	27
1.3.2 Typical bacteria and asthma.....	28
1.3.3 <i>Streptococcus pneumoniae</i> (<i>Spn</i>)	30
1.3.4 <i>Spn</i> infection of airway cells	32
1.3.5 <i>Spn</i> infection and PBMCs	32
1.4 Immunology of RV-induced asthma exacerbations.....	33
1.4.1 Interleukin-6 (IL-6).....	33
1.4.2 Interleukin-8 (CXCL8/IL-8).....	34
1.4.3 Interleukin-1 β (IL-1 β)	35
1.4.4 Tumour Necrosis Factor- α (TNF- α).....	35
1.4.5 Other proinflammatory cytokines	36
1.5 Proinflammatory cytokine expression following airway infection with <i>Spn</i>	36
1.5.1 IL-6 and CXCL8/IL-8.....	36
1.5.2 IL-1 β	37
1.5.3 TNF- α	37
1.6 Role of interferons in virus and bacterial infection.....	37
1.6.1 Interferons	37

1.6.2 Is there a role for interferons in defence against bacteria?	43
1.7 Role of atopy in asthma	44
1.8 Interferon signalling pathways	46
1.8.1 Pattern recognition receptors	46
1.8.2 Toll like receptors	46
1.8.3 TLR signalling pathways	50
1.8.4 Pattern recognition receptors in <i>S. pneumoniae</i> cell signalling	53
1.9 Rationale, Hypothesis and Aims	54
1.9.1 Rationale.....	54
1.9.2 Specific Hypotheses	55
1.9.3 Aims.....	55
Chapter 2: Materials and Methods	56
2.1 Materials.....	56
2.1.1 Clinical Equipment	56
2.1.2 Laboratory instruments.....	57
2.1.3 Buffers and reagents.....	57
2.1.4 Media and supplements	59
2.1.5 Toll like Receptor (TLR) Ligands	60
2.1.6 Primers and Probes for Realtime RT-PCR.....	61
2.1.7 Antibodies	62
2.1.8 Commercial kits	62
2.1.9 Software.....	63
2.2 Clinical Methods.....	64
2.2.1 Participant Recruitment	64
2.2.2 Screening Investigations	64
2.3 Clinical Sample Collection.....	68
2.3.1 Peripheral Blood Collection	68
2.3.2 Bronchoscopy	68
2.4 Clinical Sample Processing	69
2.4.1 PBMC Processing	69
2.4.2 BAL processing.....	70
2.4.3 Bronchial epithelial cell (HBEC) processing.....	70
2.5 Cell Culture Methods.....	71
2.5.1 Tissue Culture.....	71

2.5.2 RV Culture	73
2.5.3 <i>Streptococcus pneumoniae</i> (<i>Spn</i>) culture	75
2.5.4 TLR ligands	76
2.6 Molecular Biology Techniques.....	79
2.6.1 RNA extraction and cDNA synthesis.....	79
2.6.2 Primers and probes for Realtime RT-PCR.....	80
2.6.3 Realtime RT-PCR.....	80
2.7 Protein analysis techniques	81
2.7.1 Enzyme-Linked ImmunoSorbent Assay (ELISA) to test RV induced production of IL-6 and IL-8 in BEAS2B cells, Primary HBECs (Lonza) and PBMCs	81
2.7.2 Development of a Meso Scale Discovery (MSD) prototype to measure IL-29	82
2.7.3 Meso Scale Discovery (MSD) to measure proinflammatory cytokines IL-1 β , IL-6, IL-8 and TNF- α , and interferons IFN- α , IFN- β and IL-29 (IFN- λ 1) in HBECs, BAL macrophages and PBMCs	83
2.8 Statistical analysis	85
Chapter 3: RV infection of BAL cells, HBECs and PBMCs, and TLR3/7/8 stimulation of HBECs and PBMCs	86
3.1 Introduction	86
3.1.1 Hypotheses	87
3.1.2 Aims.....	87
3.2 Results	89
3.2.1 Preliminary testing of RV.....	89
3.2.2 Testing the IFN- λ 1/IL-29 MSD prototype.....	91
3.2.3 Clinical characteristics of all participants	92
3.2.4 BAL cytospin composition in NANA, ANA, NAA and AA	95
3.2.5 RV infection of BAL cells: induction of type I and III IFN and proinflammatory cytokine mRNA and protein	96
3.2.6 RV infection of HBECs: induction of type I and III IFN and proinflammatory cytokines mRNA and protein	103
3.2.7 RV infection of PBMCs: induction of type I and III IFN and proinflammatory cytokines protein.....	114
3.2.8 RV infection induced RV replication and viral release.....	120
3.2.9 TLR 3, 7, 8 ligands stimulation of HBECs: induction of type I and III IFN and proinflammatory cytokines mRNA and protein	123
3.2.10 TLR 3, 7, 8 ligands stimulation of PBMCs: induction of type I and III IFN and proinflammatory cytokines protein	132
3.3 Discussion.....	140

3.3.1 Rhinoviruses	141
3.3.2 “Viral” TLR ligands.....	145
3.4 Conclusions	146
3.5 Summary.....	147
Chapter 4: <i>Streptococcus pneumoniae</i> infection of BAL cells, HBECs and PBMCs.....	148
4.1 Introduction	148
4.1.1 Hypothesis.....	149
4.1.2 Aims.....	149
4.2 Results	150
4.2.1 Pre-study investigation of <i>Spn</i>	150
4.2.2 <i>Spn</i> infection of BAL cells: induction of type I and III IFN and proinflammatory cytokine mRNA and protein	152
4.2.3 <i>Spn</i> infection of HBECs: induction of type I and III IFN and proinflammatory cytokine mRNA and protein	156
4.2.4 <i>Spn</i> infection of PBMCs: induction of type I and III IFN and proinflammatory cytokine protein.....	161
4.3 Discussion.....	165
4.4 Conclusions	168
4.5 Summary.....	168
Chapter 5: TLR 1/2/6/4/5/9 stimulation of HBECs and PBMCs	169
5.1 Introduction	169
5.1.1 Hypothesis.....	170
5.1.2 Aims.....	170
5.2 Results	171
5.2.1 Preliminary experiments: time course and concentration responses to TLR ligands in BEAS-2B cells, primary HBECs and PBMCs	171
5.2.2 TLR1, 2 and 6 stimulation of HBECs: type I and III IFN and proinflammatory cytokine mRNA and protein induction	178
5.2.3 TLR4 stimulation of HBECs: type I and III IFN and proinflammatory cytokine mRNA and protein induction.....	190
5.2.4 TLR5 stimulation of HBECs: type I and III IFN and proinflammatory cytokine mRNA and protein induction.....	193
5.2.5 TLR9 stimulation of HBECs: type I and III IFN and proinflammatory cytokine mRNA and protein induction.....	196
5.2.6 Summary of type I and III IFN and proinflammatory cytokine induction in HBECs stimulated with TLR1/2/4/5/6/9 ligands	197

5.2.7 TLR1, 2 and 6 stimulation of PBMCs: type I and III IFN and proinflammatory cytokine protein induction	201
5.2.8 TLR 4 stimulation of PBMCs: type I and III IFN and proinflammatory cytokine protein induction	217
5.2.9 TLR 5 stimulation of PBMCs: type I and III IFN and proinflammatory cytokine protein induction	221
5.2.10 TLR 9 stimulation of PBMCs: type I and III IFN and proinflammatory cytokine protein induction	224
5.2.11 Summary of type I and III IFN and proinflammatory cytokine induction in PBMCs stimulated with TLR1/2/4/5/6/9 ligands	227
5.3 Discussion.....	230
5.3.1 TLR1, 2 and 6 stimulation	230
5.3.2 TLR4 stimulation.....	232
5.3.3 TLR5 stimulation.....	232
5.3.4 TLR9 stimulation.....	233
5.4 Conclusions	234
5.5 Summary.....	234
Chapter 6: Discussion	235
6.1 Key study findings	235
6.1.1 RV16 induced type I and III IFN in BAL cells, whereas RV1B only induced type III IFN, but no deficiency was identified in asthmatics	235
6.1.2 IFN induction in HBECs differed for major and minor group RVs, but no difference was observed between asthmatics and non-asthmatics.....	235
6.1.3 RV16 and not RV1B infection induced type I and III IFN in PBMCs	236
6.1.4 TLR3 stimulation of HBECs resulted in induction of type I and III IFN, whereas TLR7/8 stimulation resulted in induction of type III IFN only	237
6.1.5 <i>Spn</i> failed to induce type I or III IFN mRNA or protein in BAL cells, HBECs or PBMCs	238
6.1.6 HBEC responses to stimulation of TLRs which recognise bacterial products	238
6.1.7 PBMC responses to stimulation of TLRs which recognise bacterial products.....	239
6.2 Future Work.....	239
6.3 Conclusions	240
References	241
Appendices.....	264
Appendix A: Supplementary figures	264
Appendix B: Patient information sheet, consent, screening proforma and ethics approval letter.....	270

Figures

Chapter 1:

Figure 1.1 <i>Streptococcus pneumoniae</i> virulence factors.....	31
Figure 1.2 Activation of JAK-STAT pathway by type I IFN.....	40
Figure 1.3 Toll-like receptor signalling.....	51

Chapter 3:

Figure 3.1 Testing the stock rhinovirus.....	89
Figure 3.2 IL-6 and CXCL8/IL-8 induction at 24h in PBMCs from NANA stimulated with varying dilutions of RV16 and RV1B.....	90
Figure 3.3 IL-6 and CXCL8/IL-8 induction at 24h in primary HBECs (Lonza) stimulated with varying dilutions of RV16 and RV1B.....	90
Figure 3.4 Comparing IFN- λ 1/IL-29 MSD prototype to ELISA.....	91
Figure 3.5 Clinical characteristics of participants with statistically significant differences between groups as a whole.....	94
Figure 3.6 BAL cytopins.....	95
Figure 3.7 Interferon mRNA induction in BAL macrophages at 8h post stimulation with RV16 and RV1B.....	97
Figure 3.8 Interferon protein induction in BAL macrophages at 8h post stimulation with RV16 and RV1B.....	99
Figure 3.9 IL-6 and CXCL8/IL-8 mRNA induction in BAL macrophages at 8h post stimulation with RV16 and RV1B.....	100
Figure 3.10 IL-1 β , IL-6, CXCL8/IL-8 and TNF- α protein induction in BAL cells stimulated with RV16 and RV1B.....	101
Figure 3.11 Interferon mRNA induction in HBECs post stimulation with RV16 at 8h and 24h.....	104
Figure 3.12 Interferon mRNA induction in HBECs post stimulation with RV1B at 8h and 24h.....	105
Figure 3.13 Interferon protein induction in HBECs post stimulation with RV16 at 24h and 48h.....	106
Figure 3.14 Interferon protein induction in HBECs post stimulation with RV1B at 24h and 48h.....	107
Figure 3.15 IL-6 and CXCL8/IL-8 mRNA induction in HBECs post stimulation with RV16 and RV1B.....	108
Figure 3.16 Proinflammatory cytokine protein induction in HBECs stimulated with RV16 at 24h and 48h.....	110
Figure 3.17 Proinflammatory cytokine protein induction in HBECs stimulated with RV1B at 24h and 48h.....	111
Figure 3.18 Interferon protein induction in PBMCs stimulated with RV16 at 24h and 48h.....	115
Figure 3.19 Interferon protein induction in PBMCs stimulated with RV1B at 24h and 48h.....	116
Figure 3.20 Proinflammatory cytokine protein induction in PBMCs stimulated with RV16 at 24h and 48h.....	117

Figure 3.21 Proinflammatory cytokine protein induction in PBMCs stimulated with RV1B at 24h and 48h.....	118
Figure 3.22 RV vRNA induction in HBECs after inoculation with RV16 and RV1B.....	121
Figure 3.23 Time courses demonstrating RV vRNA induction in HBECs after inoculation with RV16 and RV1B.....	121
Figure 3.24 RV release in supernatants of HBECs after inoculation with RV16 and RV1B.....	122
Figure 3.25 Time courses demonstrating RV release in supernatants of HBECs after inoculation with RV16 and RV1B.....	122
Figure 3.26 Interferon mRNA induction in HBECs post stimulation with poly(IC) at 8h and 24h.....	124
Figure 3.27 Interferon mRNA induction in HBECs post stimulation with R848 at 8h and 24h.....	125
Figure 3.28 Interferon protein induction in HBECs post stimulation with poly(IC) and R848 at 24h...	126
Figure 3.29 IL-6 and CXCL8/IL-8 mRNA induction in HBECs post stimulation with poly(IC) and R848.....	128
Figure 3.30 Proinflammatory cytokine protein induction in HBECs stimulated with poly(IC) and R848 at 24h.....	130
Figure 3.31 Interferon protein induction in PBMCs stimulated with poly(IC) at 24h and 48h.....	134
Figure 3.32 Interferon protein induction in PBMCs stimulated with R848 at 24h and 48h.....	135
Figure 3.33 Proinflammatory cytokine protein induction in PBMCs stimulated with poly(IC) at 24h and 48h.....	136
Figure 3.34 Proinflammatory cytokine protein induction in PBMCs stimulated with R848 at 24h and 48h.....	137
Figure 3.35 Proinflammatory cytokine protein induction at 48h in PBMCs from all groups combined stimulated with poly(IC) and R848.....	138

Chapter 4:

Figure 4.1 Concentration and time course response of <i>S. pneumoniae</i> (<i>Spn</i>) in BEAS-2B cells, primary HBECs and PBMCs.....	151
Figure 4.2 Interferon mRNA induction in BAL macrophages at 8h post stimulation with <i>Spn</i>	152
Figure 4.3 Interferon protein induction in BAL macrophages at 8h post stimulation with <i>Spn</i>	153
Figure 4.4 Proinflammatory cytokine mRNA induction in BAL macrophages at 8h post stimulation with <i>Spn</i>	154
Figure 4.5 Proinflammatory cytokine protein induction in BAL macrophages at 8h post stimulation with <i>Spn</i>	155
Figure 4.6 Interferon mRNA induction in HBECs at 8h and 24h post stimulation with <i>Spn</i>	157
Figure 4.7 Interferon protein induction in HBECs at 24h and 48h post stimulation with <i>Spn</i>	158
Figure 4.8 Proinflammatory cytokine mRNA induction in HBECs at 8h and 24h post stimulation with <i>Spn</i>	159
Figure 4.9 Proinflammatory cytokine protein induction in HBECs at 24h and 48h post stimulation with <i>Spn</i>	160

Figure 4.10 Interferon protein induction in PBMCs at 24h and 48h post stimulation with <i>Spn</i>	162
Figure 4.11 Proinflammatory cytokine protein induction in PBMCs at 24h and 48h post stimulation with <i>Spn</i>	164

Chapter 5:

Figure 5.1 Concentration and time course response of Pam ₃ CSK ₄ in BEAS-2B cells, primary HBECs and PBMCs.....	172
Figure 5.2 Concentration and time course response of lipoteichoic acid in BEAS-2B cells, primary HBECs and PBMCs.....	173
Figure 5.3 Concentration and time course response of synthetic lipoprotein in BEAS-2B cells, primary HBECs and PBMCs.....	175
Figure 5.4 Concentration and time course response of peptidoglycan in BEAS-2B cells, primary HBECs and PBMCs.....	176
Figure 5.5 Concentration and time course response of flagellin in BEAS-2B cells, primary HBECs and PBMCs.....	177
Figure 5.6 Interferon mRNA induction in HBECs post stimulation with Pam ₃ CSK ₄ at 8h and 24h.....	179
Figure 5.7 Interferon protein induction in HBECs post stimulation with Pam ₃ CSK ₄ at 24h.....	180
Figure 5.8 IL-6 and CXCL8/IL-8 mRNA induction in HBECs post stimulation with Pam ₃ CSK ₄ at 8h and 24h.....	181
Figure 5.9 Proinflammatory cytokine protein induction in HBECs post stimulation with Pam ₃ CSK ₄ at 24h.....	182
Figure 5.10 Interferon mRNA induction in HBECs post stimulation with LTA at 8h and 24h.....	183
Figure 5.11 IL-6 and CXCL8/IL-8 mRNA induction in HBECs post stimulation with LTA at 8h and 24h.....	184
Figure 5.12 Proinflammatory cytokine protein induction in HBECs post stimulation with LTA at 24h.....	185
Figure 5.13 Interferon mRNA induction in HBECs post stimulation with PGN at 8h and 24h.....	186
Figure 5.14 IL-6 and CXCL8/IL-8 mRNA induction in HBECs post stimulation with PGN at 8h and 24h.....	187
Figure 5.15 Proinflammatory cytokine protein induction in HBECs post stimulation with PGN at 24h.....	187
Figure 5.16 Interferon mRNA induction in HBECs post stimulation with FSL at 8h and 24h.....	188
Figure 5.17 IL-6 and CXCL8/IL-8 mRNA induction in HBECs post stimulation with FSL at 8h and 24h.....	189
Figure 5.18 Proinflammatory cytokine protein induction in HBECs post stimulation with FSL at 24h.....	190
Figure 5.19 Interferon mRNA induction in HBECs post stimulation with LPS at 8h and 24h.....	191

Figure 5.20 IL-6 and CXCL8/IL-8 mRNA induction in HBECs post stimulation with LPS at 8h and 24h.....	192
Figure 5.21 Proinflammatory cytokine protein induction in HBECs post stimulation with LPS at 24h.....	193
Figure 5.22 Interferon mRNA induction in HBECs post stimulation with FLA at 8h and 24h.....	194
Figure 5.23 IL-6 and CXCL8/IL-8 mRNA induction in HBECs post stimulation with FLA at 8h and 24h.....	195
Figure 5.24 Proinflammatory cytokine protein induction in HBECs post stimulation with FLA at 24h.....	195
Figure 5.25 Proinflammatory cytokine protein induction in HBECs post stimulation with CpG-C at 24h.....	196
Figure 5.26 Interferon protein induction in PBMCs post stimulation with Pam ₃ CSK ₄ at 24h and 48h.....	202
Figure 5.27 Proinflammatory cytokine protein induction in PBMCs post stimulation with Pam ₃ CSK ₄ at 24h and 48h.....	203
Figure 5.28 Proinflammatory cytokine protein induction at 24h and 48h in PBMCs from all groups combined stimulated with Pam ₃ CSK ₄	204
Figure 5.29 Interferon protein induction in PBMCs post stimulation with LTA at 24h and 48h.....	206
Figure 5.30 Proinflammatory cytokine protein induction in PBMCs post stimulation with LTA at 24h and 48h.....	207
Figure 5.31 Proinflammatory cytokine protein induction at 24h and 48h in PBMCs from all groups combined stimulated with LTA.....	208
Figure 5.32 Interferon protein induction in PBMCs post stimulation with PGN at 24h and 48h.....	210
Figure 5.33 Proinflammatory cytokine protein induction in PBMCs post stimulation with PGN at 24h and 48h.....	211
Figure 5.34 Proinflammatory cytokine protein induction at 24h and 48h in PBMCs from all groups combined stimulated with PGN.....	212
Figure 5.35 Interferon protein induction in PBMCs post stimulation with FSL at 24h and 48h.....	214
Figure 5.36 Proinflammatory cytokine protein induction in PBMCs post stimulation with FSL at 24h and 48h.....	215
Figure 5.37 Proinflammatory cytokine protein induction at 24h and 48h in PBMCs from all groups combined stimulated with FSL.....	216
Figure 5.38 Interferon protein induction in PBMCs post stimulation with LPS at 24h and 48h.....	218
Figure 5.39 Proinflammatory cytokine protein induction in PBMCs post stimulation with LPS at 24h and 48h.....	219
Figure 5.40 Proinflammatory cytokine protein induction at 24h and 48h in PBMCs from all groups combined stimulated with LPS.....	220

Figure 5.41 Proinflammatory cytokine protein induction in PBMCs post stimulation with FLA at 24h and 48h.....	222
Figure 5.42 Proinflammatory cytokine protein induction at 24h and 48h in PBMCs from all groups combined stimulated with FLA.....	223
Figure 5.43 Proinflammatory cytokine protein induction in PBMCs post stimulation with CpG-C at 24h and 48h.....	225
Figure 5.44 Proinflammatory cytokine protein induction at 24h and 48h in PBMCs from all groups combined stimulated with CpG-C.....	226

Tables

Chapter 1:

Table 1.1 Factors influencing the development and expression of asthma.....	21
Table 1.2 The interferon family members.....	38
Table 1.3 Summary of studies assessing the role of type I and III IFNs in asthma.....	42
Table 1.4 Toll like receptors: ligands, IFN induction pathways used and cellular location.....	47

Chapter 2:

Table 2.1 Clinical equipment used in the project in order of application.....	56
Table 2.2 Laboratory instruments used in the project in alphabetical order.....	57
Table 2.3 Buffers and reagents used in the project in alphabetical order.....	57
Table 2.4 Media and media supplements used in the project in alphabetical order.....	59
Table 2.5 TLR ligands in order of preference used in this study, with the receptor they bind to and the concentrations used in HBECs, BAL cells and PBMCs.....	60
Table 2.6 TaqMan Realtime RT-PCR primers and probes and their optimal concentrations used.....	61
Table 2.7 Antibodies used in the project and their working concentrations.....	62
Table 2.8 Commercially available kits used in the project, listed in alphabetical order.....	62
Table 2.9 Software used in the project listed in alphabetical order.....	63
Table 2.10 Inclusion and Exclusion criteria.....	66
Table 2.11 Asthma Control Questionnaire.....	67
Table 2.12 Components of the reaction for cDNA synthesis.....	79

Chapter 3:

Table 3.1 Samples obtained from participants.....	92
Table 3.2 Clinical characteristics of all participants.....	93

Table 3.3 Summary of IFN and proinflammatory cytokine mRNA and protein induction post RV16 infection in BAL cells.....	102
Table 3.4 Summary of IFN and proinflammatory cytokine mRNA and protein induction post RV1B infection in BAL cells.....	102
Table 3.5 Summary of IFN and proinflammatory cytokine mRNA induction post RV16 infection in HBECs.....	112
Table 3.6 Summary of IFN and proinflammatory cytokine protein induction post RV16 infection in HBECs.....	112
Table 3.7 Summary of IFN and proinflammatory cytokine mRNA induction post RV1B infection in HBECs.....	113
Table 3.8 Summary of IFN and proinflammatory cytokine protein induction post RV1B infection in HBECs.....	113
Table 3.9 Summary of IFN and proinflammatory cytokine protein induction post RV16 infection in PBMCs.....	119
Table 3.10 Summary of IFN and proinflammatory cytokine protein induction post RV1B infection in PBMCs.....	119
Table 3.11 Summary of IFN and proinflammatory cytokine mRNA and protein induction post poly(IC) stimulation in HBECs.....	131
Table 3.12 Summary of IFN and proinflammatory cytokine mRNA and protein induction post R848 stimulation in HBECs.....	131
Table 3.13 Summary of IFN and proinflammatory cytokine protein induction post poly(IC) stimulation in PBMCs.....	139
Table 3.14 Summary of IFN and proinflammatory cytokine protein induction post R848 stimulation in PBMCs.....	139

Chapter 4:

Table 4.1 Summary of IFN and proinflammatory cytokine mRNA and protein induction post <i>Spn</i> infection in BAL cells.....	156
Table 4.2 Summary of IFN and proinflammatory cytokine mRNA induction post <i>Spn</i> infection in HBECs.....	161
Table 4.3 Summary of IFN and proinflammatory cytokine protein induction post <i>Spn</i> infection in HBECs.....	161
Table 4.4 Summary of IFN and proinflammatory cytokine protein induction post <i>Spn</i> infection in PBMCs.....	165

Chapter 5:

Table 5.1 Summary of IFN and proinflammatory cytokine mRNA and protein induction post PAM stimulation in HBECs.....	197
Table 5.2 Summary of IFN and proinflammatory cytokine mRNA and protein induction post LTA stimulation in HBECs.....	198
Table 5.3 Summary of IFN and proinflammatory cytokine mRNA and protein induction post PGN stimulation in HBECs.....	198

Table 5.4 Summary of IFN and proinflammatory cytokine mRNA and protein induction post FSL stimulation in HBECs.....	199
Table 5.5 Summary of IFN and proinflammatory cytokine mRNA and protein induction post LPS stimulation in HBECs.....	199
Table 5.6 Summary of IFN and proinflammatory cytokine mRNA and protein induction post FLA stimulation in HBECs.....	200
Table 5.7 Summary of IFN and proinflammatory cytokine mRNA and protein induction post CpG stimulation in HBECs.....	200
Table 5.8 Summary of IFN and proinflammatory cytokine protein induction post PAM stimulation in PBMCs.....	227
Table 5.9 Summary of IFN and proinflammatory cytokine protein induction post LTA stimulation in PBMCs.....	227
Table 5.10 Summary of IFN and proinflammatory cytokine protein induction post PGN stimulation in PBMCs.....	228
Table 5.11 Summary of IFN and proinflammatory cytokine protein induction post FSL stimulation in PBMCs.....	228
Table 5.12 Summary of IFN and proinflammatory cytokine protein induction post LPS stimulation in PBMCs.....	229
Table 5.13 Summary of IFN and proinflammatory cytokine protein induction post FLA stimulation in PBMCs.....	229
Table 5.14 Summary of IFN and proinflammatory cytokine protein induction post CpG stimulation in PBMCs.....	230

Abbreviations

2BME	2-β mercaptoethanol
AA	Atopic asthmatic
AHR	Airway hyperresponsiveness
ANA	Atopic non asthmatic
ANOVA	Analysis of variance
BAL	Bronchoalveolar lavage
BEBM	Bronchial epithelial cell basal medium
BEGM	Bronchial epithelial cell growth medium
BSA	Bovine serum albumin
BTS	British Thoracic Society
cDNA	Complementary DNA
CFU	Colony forming unit
DMEM	Dulbecco's Modified Eagle's medium
DNA	Deoxyribonucleic acid
D-PBS	Dulbecco's phosphate buffered saline
ELISA	Enzyme-linked immunosorbent assay
ERS	European Respiratory Society
FEV₁	Forced expiratory volume in 1 second
FLA	Flagellin
FBS	Foetal bovine serum
FSL	Synthetic lipoprotein
FVC	Forced vital capacity
GINA	Global initiative for asthma
HBEC	Human bronchial epithelial cell
HEPES	Hydroxyethyl piperazine ethanesulphonic acid

HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
ICS	Inhaled corticosteroid
IFN	Interferon
IgE	Immunoglobulin E
IL	Interleukin
LABA	Long acting beta agonist
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MSD	Meso scale discovery
NAA	Non atopic asthmatic
NANA	Non atopic non asthmatic
OD	Optical density
ODN	Oligodeoxynucleotide
PAM	Pam ₃ CSK ₄
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PC₂₀	Provocative concentration causing a 20% drop in FEV ₁
PEF	Peak expiratory flow
PGN	Peptidoglycan
RLT	RNeasy lysis buffer
RNA	Ribonucleic acid
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute
rRNA	Ribosomal RNA
RT	Reverse transcriptase

RT-PCR	Reverse transcriptase polymerase chain reaction
RV	Rhinovirus
<i>Spn</i>	<i>Streptococcus pneumoniae</i>
SPT	Skin prick test
TCID₅₀	Tissue culture infectious dose 50
TLR	Toll like receptor
TMB	Tetramethylbenzidine

Chapter 1: Introduction

1.1 Asthma

1.1.1 Epidemiology

Asthma is a chronic inflammatory airway disease affecting approximately 300 million people worldwide ¹. The prevalence has increased in the last few decades ², particularly in westernised and English-speaking countries, with some studies finding up to a 40% increase in the past 20 years ^{3, 4}. Asthma is characterised by airway hyperresponsiveness, leading to widespread reversible airways obstruction with symptoms of wheeze, chest tightness and cough, particularly at night. The GINA guidelines classify asthma severity as mild intermittent, mild persistent, moderate persistent or severe persistent ⁵. There is a huge burden of healthcare associated with asthma, particularly with asthma exacerbations, and asthma is a major cause of absence from work in many countries. The WHO has estimated that 15 million disability-adjusted life-years are lost annually due to asthma, representing 1% of the total global disease burden ⁶. There are an estimated 250,000 worldwide deaths attributed to asthma ⁶.

There are many factors involved in the development of asthma. These can be divided into host and environmental factors. Table 1.1 illustrates these factors ⁵. The major association with asthma is atopy, with 70-90% of adult asthmatics being atopic ⁷. There is overlap with environmental factors and their influence in the development of asthma and their role in triggering asthma symptoms. Much of the information about asthma risk factors comes from studies in children. Risk factors for the development of asthma in adults are less understood but the persistence of adult atopic asthma is strongly associated with childhood eczema and rhinitis ⁸.

For many years asthmatics have been subclassed into atopic (extrinsic) and non-atopic (intrinsic) on the basis of whether a known allergen provokes airway obstruction and the presence of positive skin tests ⁹. For the purposes of research the atopic status of asthmatics has long been determined on the basis of skin prick testing ¹⁰. Gender differences have been observed in the incidence of non-atopic asthma. In men the incidence rate is low until the age of 40 years, whereas the

incidence rate in women is relatively high between the ages of 20 and 30 years, and remains higher than in men throughout reproductive years ¹¹. The explanation for this is unclear. The prevalence of asthma is increasing, but this increase is largely due to the increase in the prevalence of atopy, and subsequently the prevalence of non-atopic asthma is unchanged over the last 20 years ¹². The prevalence of non-atopic asthma is also higher in lower socio-economic groups ^{13, 14}.

Table 1.1. Factors influencing the development and expression of asthma

Factors Influencing the Development and Expression of Asthma
HOST FACTORS Genetic – genes predisposing to atopy, genes predisposing to AHR Obesity Sex – males in childhood asthma, females in adult asthma
ENVIRONMENTAL FACTORS Allergens – mites, furred animals, cockroach, fungi, moulds, yeasts, pollen Infections – predominantly viral Occupational exposures – over 300 substances have been associated with occupational asthma Tobacco smoke Air pollution Diet – breast feeding, processed foods

1.1.2 Asthma exacerbations

Asthma exacerbations can be defined as a transient worsening of asthma symptoms following exposure to various risk factors ⁵. For research purposes there is no commonly accepted definition for asthma exacerbations and a definition could be obtained by phenotyping exacerbations based on clinical signs, lung function parameters and biomarkers ¹⁵. Exacerbations differ from poor asthma control and asthmatics are vulnerable to exacerbations, even when asthma is controlled ¹⁶.

They are common, with 56% of asthmatics reporting one or more exacerbation in the preceding 12 months between 2001 and 2003 in the US ¹⁷.

Treatments available for exacerbations are only partially effective. These can be divided into those that prevent exacerbations and those that treat exacerbations. Inhaled corticosteroids (ICS) and combinations of ICS and long acting beta-2 agonists (LABA) are the mainstay of preventative treatment. A combination of LABA/ICS in asthmatics results in improved lung function, symptoms and decreased use of reliever therapy compared to high dose ICS ¹⁸. There are no statistically significant differences between the different LABA/ICS combinations in the need for oral corticosteroids or number of adverse events ¹⁹. Other agents are also used in combination, particularly the leukotriene antagonists. Montelukast as an add-on treatment to ICS is more effective than ICS alone ²⁰. However, salmeterol/ICS is more effective than montelukast/ICS. Leukotriene antagonists have been shown to reduce exacerbation frequency; zafirlukast in mild-moderate adult asthmatics ²¹ and montelukast in children during the predictable September peak in asthma symptoms ²².

Exacerbations are treated using ICS, LABA and oral corticosteroids (OCS). Steroids act by binding and activating the glucocorticoid receptor, which is then translocated from the cytosol to the nucleus where it acts through a number of mechanisms, including upregulation of anti-inflammatory gene expression, suppression of proinflammatory cytokines, chemokines, arachadonic acid metabolites and adhesion molecules, and interaction with transcription factors such as nuclear factor kappa B (NF- κ B) ²³⁻²⁵.

1.2 Viruses and asthma

1.2.1 Respiratory virus infection and asthma exacerbations

Multiple studies have reported the association between asthma exacerbations and respiratory virus infection. Many use different study designs, methods of detection and definitions. Nine studies, in patients with a known diagnosis of asthma, a defined exacerbation and using the reverse transcriptase-polymerase

chain reaction (RT-PCR) for virus detection detected one or more respiratory viruses in 56% exacerbations, with a range of 26-80%²⁶⁻³⁴. Rhinoviruses were the most commonly identified virus. Other viruses identified were coronaviruses, influenza viruses, parainfluenza viruses, respiratory syncytial viruses (RSV) and adenoviruses.

1.2.2 Rhinoviruses and asthma

Rhinoviruses (RVs) are the major virus type associated with exacerbations of asthma. Studies have shown that, in adults and children, RVs are responsible for 60-65% of viral exacerbations. The peak period for RV infection is September in the northern hemisphere and peaks of asthma exacerbation requiring hospital treatment occur globally after school return. A time trend analysis of upper respiratory infections and hospital admissions for asthma identified a strong correlation between the seasonal patterns of infection and hospital admissions³⁵. This relationship was stronger for paediatric than for adult admissions. RVs were the major pathogens implicated in these infections. It was speculated that it was the children's school attendance that facilitates the spread of virus, leading to an increased number of asthma admissions. Another study showed human picornaviruses were detected in 52% of paediatric asthma admissions and 29% of controls and these cases were less likely to have been prescribed controller medication, suggesting that children with asthma who are prescribed anti-inflammatory medications are less likely to require emergency department treatment during this period of high risk of exacerbation²⁸.

Asthmatic individuals have increased susceptibility to RV infection. This was first addressed in the 1970s in prospective longitudinal studies comparing infection rates in cohabiting child and adult asthmatic and non-asthmatics^{36, 37}. Increased incidences of confirmed colds were identified in asthmatics. A more recent study of cohabiting couples, one of whom had asthma, showed that asthmatics were no more likely to develop RV infection, but had longer lasting and significantly more severe lower respiratory tract symptoms associated with RV infections compared to healthy controls³⁸. RV ribonucleic acid (RNA) has been detected in nasal aspirates from >40% of asthmatic children 6 weeks after an acute exacerbation³⁹. In patients who

had persistent RV RNA after 6 weeks, measurements of peak expiratory flow in the emergency room were significantly lower than in patients in whom RV RNA was present in the emergency room but absent after six weeks. This suggests that the severity of acute asthma may be linked to prolonged and possibly more severe RV infections. These studies identifying the importance of RV in the pathogenesis of asthma exacerbations highlight the importance of developing new therapeutic options for RV induced asthma exacerbations.

1.2.3 Rhinoviruses

RVs belong to the *Picornaviridae* family of viruses. They are small, non-enveloped, icosahedral, RNA viruses, with at least 100 serotypes known. The virus is composed of a single stranded positive sense RNA molecule encased by a capsid formed from 60 copies of four viral proteins (VP1-4)^{40, 41}. A protein shell, created by VP1-3, is on the exterior of the virus, with VP4 being in contact with the viral RNA. The structure of RVs include a well conserved region, a 'canyon'⁴¹, which is the site for interaction between the virus and its receptors⁴².

RVs can be classified into the major and minor groups based on the receptors they use to enter the host cell. The major group bind the surface glycoprotein human intercellular adhesion molecule-1 (ICAM-1)⁴³ and form the majority of RVs, with over 90 serotypes. The minor group bind to low density lipoprotein receptors (LDLR)⁴⁴. The alternative classification is according to VP4/VP2 nucleotide gene sequence analysis with type A, type B and the recently identified type C^{45, 46}. Type C has been identified to be an important cause of hospitalisations⁴⁷.

Following binding to the cell surface receptors intracellular internalisation of RV occurs. Viral replication occurs by the virus hijacking the host cell translational machinery. RVs have a protein, VPg, covalently linked to the 5'-ends of their genomes. Uridylation of VPg serves as the protein primer for RNA synthesis, linked to the presence of the internal *cis*-acting replication element (cre)^{48, 49}. Positive sense RNA undergoes translation by host ribosomes. The resulting product is cleaved by viral proteases and transcribed by viral RNA polymerase into negative sense RNA which is further transcribed into positive sense RNA. Viral proteins and

positive sense RNA form virions which are released from the cell ⁵⁰. RVs replicate in 6h, with 100,000 virions being released from infected cells *in vitro* ⁵¹.

1.2.4 Rhinovirus infection of airway cells

The bronchial epithelium is important in RV induced asthma exacerbations. Several genes that are associated with asthma susceptibility are expressed in the epithelium ⁵². RV is usually a mild infection in non-asthmatics, but is associated with cytotoxicity and delayed epithelial repair in asthmatics ⁵³. Human bronchial epithelial cells (HBECs) express ICAM-1 ⁵⁴⁻⁵⁶ and LDLR ⁵⁷. RVs infect HBECs *in vitro* and *in vivo* with the upper and the lower airways being infected and replication peaking at 24-48h post infection ⁵⁸. In situ hybridisation for RV16 on bronchial biopsy samples from an experimental model of infection identified RV16 localised predominantly to the columnar epithelial layer. Infection results in production of interleukin (IL)-6, CXCL8/IL-8, IL-16 and CCL5/RANTES, and RVs are cytotoxic to epithelial cells in culture ^{58, 59}. RV infection induces type I ⁶⁰ and type III ⁶¹ interferon (IFN) production with the IFN responses of HBECs from asthmatics being deficient.

The other key airway cell related to RV infection is the alveolar macrophage. These cells are resident in the lung and are the predominant immune effector cells in the airway and alveolar spaces. They are important for microbial clearance ⁶², decrease antigen presentation function of dendritic cells ⁶³, decrease humoral responses to inhaled antigens ⁶⁴ and promote a T helper (Th)-1 response ⁶⁵. Lung macrophages express high levels of both ICAM-1 ⁶⁶ and LDLR ⁶⁷. Clear interactions occur between RV and alveolar macrophages. Some reports confirm RV binding to macrophages with subsequent cytokine induction, but no productive RV replication or release of new virions ⁶⁸. More recently, RV replication has been demonstrated in a human monocyte cell line but was limited in primary human monocytes, the difference being suggested due to the robust type I IFN response in primary cells and not in the cell line-derived macrophages ⁶⁹. Though replication may or may not occur, exposure of alveolar macrophages to RV results in cell activation and induction of IFNs ⁶¹. Following activation macrophages secrete antiviral and

proinflammatory cytokines including CXCL8/IL-8, IL-10, IL-12, tumour necrosis factor (TNF)- α and CCL2/monocyte chemotactic protein 1 (MCP-1)⁷⁰⁻⁷³.

Differences in alveolar macrophage cytokine production between non-atopic non-asthmatics (NANA) and atopic asthmatics (AA) have been observed, with reduced IL-12⁷⁴, IL-18⁷⁵ and IL-15⁷⁶ production in AA. Macrophages from AA have been shown to have deficient RV-induced type III IFN production⁶¹. The important immune role alveolar macrophages have in host defence against viral and bacterial infection and the differences in their function in AA and NANA makes them an important cell type to study further, in particular whether the atopic status of the cell type is important.

1.2.5 Rhinovirus infection and peripheral blood mononuclear cells (PBMCs)

Intranasal inoculation of RV results in changes in peripheral blood leucocytes, with a significant decrease in total lymphocyte count and a strong correlation between decreases in lymphocytes and severity of symptoms⁷⁷. PBMC activation occurs without active RV replication and results in induction of IFN- γ , IL-12, IL-10 and IL-13^{78, 79}. Following infection with RV, production of type I and III IFNs have been observed in PBMCs, with IFN- α as the predominant type followed by IFN- β and IFN- λ ⁸⁰. There is data to suggest impaired IFN- α production in PBMCs stimulated with RV from adolescent asthmatics but not adults, and impaired IL-6, TNF- α and IL-10 production in adolescent and adult asthmatics⁸¹. A deficient type I IFN response has also been observed in PBMCs from AA following infection with RSV and Newcastle disease virus (NDV)⁸². Similarly, differences in RV-induced cytokine production by PBMCs between asthmatics and non-asthmatics have been observed, with NANA producing significantly more IFN- γ and AA producing significantly more IL-10⁷⁸.

The observed differences in IFN and cytokine production from PBMCs in asthmatics following respiratory virus infection warrant further investigation, and in particular whether atopy is important.

1.3 Bacteria and asthma

1.3.1 Atypical bacteria and asthma

Numerous studies have investigated the association of atypical bacteria such as *Chlamydophila pneumoniae* (*Cpn*) in both stable and exacerbations of asthma. Many studies rely on serology and are unable to distinguish between acute and chronic infection. A study of AA and NANA spouse pairs, with regular nasal aspirate sampling and detection of *Cpn* by RT-PCR showed that *Cpn* was positive in 6.4% of samples from atopic asthmatics compared to 2.3% of samples from controls, confirming that *Cpn* infection is detected more frequently among asthmatics than non-asthmatic participants ⁸³. Another study examined the association between immunoglobulin (Ig)G and IgA titres to *Cpn* and asthma severity ⁸⁴. Higher titres of antibodies were associated with markers of asthma severity. High dose inhaled steroid use was associated with a 74.1% increase in IgG titre and a 70.6% increase in IgA compared to low dose inhaled steroid use. The titre of antibody increased by 0.8% for every 1% decrease in % predicted forced expiratory volume in 1 second (FEV₁). An elevation in IgA was associated with a higher daytime symptom score. A study investigating the prevalence of *Cpn* infection in asthmatic patients, identified that the frequency of PCR positivity for *Cpn* in throat wash samples was higher in stable asthmatics (28.6%) than in healthy controls (11.8%) ⁸⁵. These studies confirm increased detection of *Cpn* in patients with asthma. The importance of this association requires further study.

There is also evidence suggesting a role for atypical bacteria in the aetiology of asthma exacerbations. A study of asthmatic children was performed with nasal aspirates taken when respiratory symptoms were reported and the presence of *Cpn* was tested by PCR and *Cpn*-specific IgA was detected by ELISA ⁸⁶. This showed that although *Cpn* detections were similar between symptomatic and asymptomatic episodes, those children who reported multiple episodes remained PCR positive, suggesting chronic *Cpn* infection. IgA antibodies were more than seven times greater in subjects who reported four or more exacerbations compared to those who reported just one. This demonstrates that immune responses to *Cpn* are positively associated with frequency of asthma exacerbations. Of interest, samples were

analysed from a previous study which had detected virus infections in 80-85% of exacerbations ²⁹, indicating that virus infections may interact with *Cpn* to increase risk or frequency of exacerbations. The mechanisms of this interaction are poorly understood.

Similarly, in an adult asthmatic population presenting to the emergency department who underwent sputum induction and spirometry at presentation and had acute and convalescent serology for *Cpn*-specific IgA and IgG, 20 (38%) subjects demonstrated an increase in *Cpn* antibody levels ⁸⁷. 15 of these had an increase in IgA levels on a background of raised IgG, suggesting reactivation of chronic infection, and 5 had a rise in IgG having been serologically negative at the first visit, indicating an acute infection. These patients with serological responses to *Cpn* had four times higher sputum neutrophil levels and sputum eosinophilic cationic protein concentrations than those patients who had exacerbation symptoms without serological responses, suggesting *Cpn* reactivation is associated with increased airway inflammation.

1.3.2 Typical bacteria and asthma

There is less evidence for a role for typical bacteria, such as *Streptococcus pneumoniae* (*Spn*), in asthma pathogenesis. Early studies suggest that infection with *Spn* is no more common in asthmatics than controls and with no difference between atopic and non-atopic asthmatics ¹⁰. *Spn* is commonly carried asymptomatically in the respiratory tract and is a major cause of community acquired pneumonia in children and adults. There is evidence from the Copenhagen birth cohort that neonates colonised with *Spn*, *Haemophilus influenzae*, *Moraxella catarrhalis* or any combination have increased risk of recurrent wheeze and early life asthma ⁸⁸. A recent study has identified house dust mite-sensitised children with lower IgG1 antibody titres to *Spn* surface antigens (PspA and PspC) which are associated with asthma. Of interest, antibacterial IgE was associated with a reduced risk for asthma ⁸⁹. The question of whether antibiotics in early life confer protection from the subsequent development of asthma has not been assessed by clinical trials, but population based cohort studies suggest that antibiotic administration early

in life does not reduce the risk of asthma ⁹⁰. Early studies suggest an association between asthma and bacterial sinusitis in children, with treatment of the bacterial infection resulting in improved asthma scores ⁹¹. IL-17 has a role in the pathogenesis of asthma. A polymorphism of IL-17 is associated with several asthma traits and detection of *Spn* has been shown to be higher in children with this polymorphism, suggesting a link between bacterial colonisation and the onset of asthma ⁹². Oropharyngeal pneumococcal carriage is more common in asthmatics than non-asthmatics ^{93, 94}. This may in part be explained by the fact that the use of inhaled corticosteroids in asthmatic children increases the risk of pneumococcal colonisation ⁹⁵. Of several risk factors, including asthma, smoking, allergic rhinitis, atopy and body mass index, the only significant risk factor for *Spn* oropharyngeal carriage was asthma (OR, 2.04) ⁹³.

While these studies demonstrate an association with asthma, the role of *Spn* in asthma exacerbations is less clear. Some older studies demonstrate bacteria being causative organisms in asthma exacerbations ⁹⁶ and vaccination against *Spn* reduces the number of hospital admissions for asthma exacerbations in children ⁹⁷ and the elderly ⁹⁸. Children admitted to the emergency department for asthma exacerbations have reduced IgG responses to house dust mite allergens ⁹⁹. In children following an acute exacerbation there is a marked increase in anti-bacterial IgE, with a reduced IgE response to house dust mite ¹⁰⁰. These studies support the concept of Th2 responses to bacterial antigens playing a role in asthma pathogenesis.

There are studies that suggest a protective role of *Spn* in the development of asthmatic symptoms, through induction of regulatory T cells ^{101, 102}. *Spn* has been observed to suppress allergic airways disease by inducing regulatory T cells, leading to an inhibition of T cell proliferation and Th2 cytokine release ¹⁰¹.

Asthma is a risk factor for invasive pneumococcal disease. Case-control studies to examine the association between asthma and invasive pneumococcal disease showed that asthma was an independent risk factor for invasive pneumococcal disease, with the risk among asthmatics at least double that of controls ^{103, 104}. These studies raise important questions about the relationship between asthma and *Spn*. The mechanisms for this increased susceptibility are unknown but asthmatics

have lower serotype-specific pneumococcal antibodies than non-asthmatics ¹⁰⁵ and interferon production has been found to be deficient in asthmatic bronchoalveolar macrophages following stimulation with lipopolysaccharide (LPS) ⁶¹. Of interest, atopic conditions other than asthma, particularly atopic dermatitis, have an increased risk of severe pneumococcal disease ¹⁰⁶.

There is some evidence that virus-induced asthma exacerbations may be worsened by concomitant bacterial infection. Studies have examined the effects of RV infection on *Spn* adherence to human nasal ¹⁰⁷ and tracheal epithelial cells ¹⁰⁸. With cells infected with RV-14, the number of *Spn* adhering increased and the use of NF-κB inhibitors decreased adherence. This suggests that RV infection stimulates bacterial adhesion to airway epithelial cells and this is, at least in part, mediated through activation of transcription factors. This increase in *Spn* adherence following RNA viral infection may be explained by the fact that the toll-like receptor (TLR)3 agonist polyinosinic:polycytidylic acid (poly(IC)) has been demonstrated to enhance platelet activating factor receptor (PAFR) expression on airway epithelial cells ¹⁰⁹. PAFR is used by *Spn* and PAFR-deficient mice are relatively resistant to *Spn* infection and have less pulmonary inflammation ¹¹⁰. Other respiratory viruses (RSV and human parainfluenza virus) have been shown to promote *Spn* adherence to the bronchial epithelium ¹¹¹. RV circulation within the community is associated with the development of invasive pneumococcal disease in children under 5 years of age ¹¹². A possible mechanism for this increased risk of bacterial infection is that RV infection results in impaired cytokine responses to bacterial LPS and lipoteichoic acid (LTA) by alveolar macrophages ¹¹³. This has important implications in the pathogenesis of asthma exacerbations.

1.3.3 *Streptococcus pneumoniae* (*Spn*)

To date there are more than 90 serotypes of *Spn* known. Each serotype has an individual polysaccharide capsule which functions as a key virulence factor by inhibiting phagocytosis, complement factor binding and entrapment by neutrophil extracellular traps ¹¹⁴⁻¹¹⁶. The exotoxin, pneumolysin, is another key pneumococcal virulence factor. It is cytotoxic to mammalian cells by binding to cell membrane

cholesterol and forming large pores in the membrane ¹¹⁷. This pore formation results in leakage of intracellular solutes and an influx of water, leading to osmotic lysis of the cell ¹¹⁸. It also induces separation of tight junctions of epithelial cells, thereby facilitating bloodstream invasion and inhibits polymorph chemotaxis ¹¹⁸. Expression of haemolytic pneumolysin by *Spn* inhibits dendritic cell maturation, thereby limiting induction of innate immune responses ¹¹⁹. Pneumolysin also inhibits ciliary beating on respiratory epithelial cells ¹²⁰ and activates the classical complement pathway ^{117, 121}. Other cell wall components, such as LTA lead to complement activation ¹¹⁸. Other pathogenic features of *Spn* include LPXTG-anchored surface proteins (hyaluronidase and neuraminidase which cleave sialic acid residues thus damaging host tissues, and serine protease) and various bacterial lipoproteins ¹¹⁷. *Spn* virulence factors are schematically illustrated in figure 1.1.

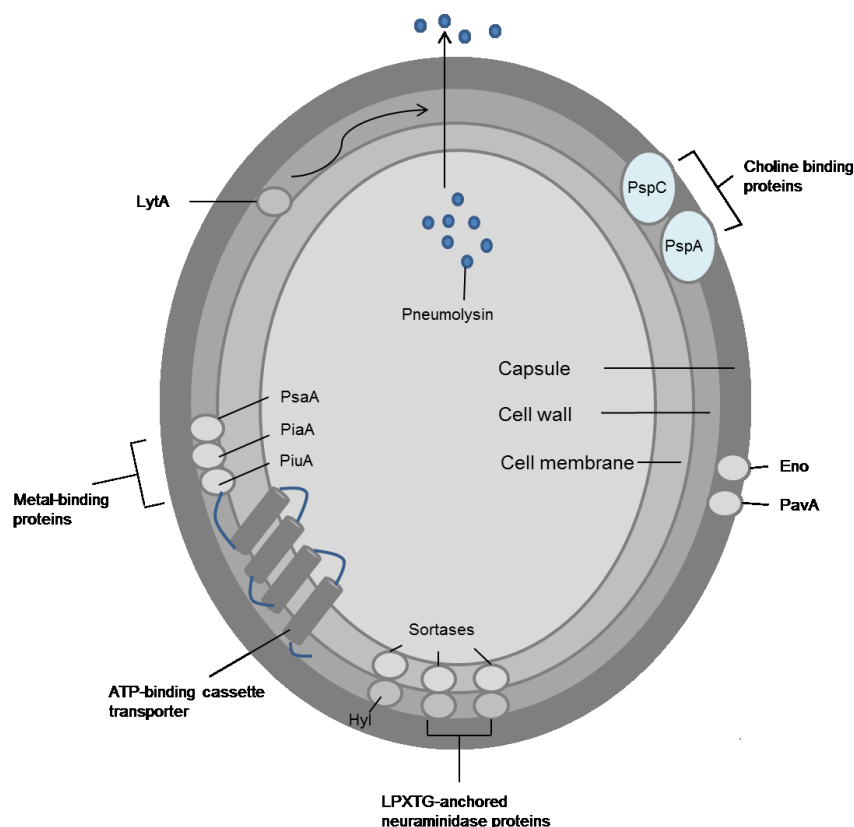


Figure 1.1. *Streptococcus pneumoniae* virulence factors. (adapted from Kadioglu et al. ¹²⁰) Virulence factors include the capsule, cell wall, pneumolysin, LPXTG-anchored neuraminidase proteins, hyaluronate lyase (Hyl), autolysin (LytA), enolase (Eno), pneumococcal adhesion and virulence A (PavA), pneumococcal surface proteins A and C (PspA and PspC), and the metal-binding proteins pneumococcal surface antigen A (PsaA), pneumococcal iron acquisition A (PiaA) and pneumococcal iron uptake A (PiuA) ¹²⁰. Several of these are recognised by pattern recognition receptors.

1.3.4 *Spn* infection of airway cells

Adhesion of bacteria to the bronchial epithelial cell is fundamental for colonisation and infection of the airway¹²². *Spn* has been shown to bind specifically to bronchial epithelial cells¹²³ and this binding is dependent on sialylated carbohydrate ligands¹²⁴. It is thought that changes in virulence factors allow for colonisation¹²⁵. *In vitro* *Spn* has been demonstrated to induce release of CXCL8/IL-8 from bronchial epithelial cells and to disrupt airway barrier function¹²⁶. The role of *Spn* and the bronchial epithelium in asthma is not well understood. A feature of chronic respiratory diseases such as asthma is mucus overproduction. *Spn* has been identified as an inducer of *MUC5AC* mucin expression from human epithelial cell lines¹²⁷.

There has been evidence for the involvement of alveolar macrophages in *Spn* infection since the 1970s, particularly identifying the phagocytic activity of macrophages¹²⁸⁻¹³⁰. *Spn* binds to alveolar macrophages and with opsonisation this results in internalisation and killing of the bacterium¹³¹. Alveolar macrophages are important for early proinflammatory cytokine release following *Spn* infection, suggesting a role for them in the innate immune response¹³². The NF- κ B protein RelA is important for host defence in *Spn* pneumonia and it has been demonstrated that RelA in alveolar macrophages is critical for the earliest innate immune response¹³³. *Spn* virulence factors such as LTA have been demonstrated to induce cytokine secretion, particularly TNF- α , from alveolar macrophages¹³⁴. A mouse model of *Spn* infection demonstrates the importance of pneumolysin in the secretion of IL-1 β from airway macrophages¹³⁵.

1.3.5 *Spn* infection and PBMCs

Pneumolysin from *Spn* results in stimulation of TNF- α and IL-1 β from human peripheral blood monocytes¹³⁶. Significant production of cytokines CXCL8/IL-8, IL-6, IL-1 β , CCL3/macrophage inflammatory protein (MIP)-1 α and TNF- α occurs following stimulation of PBMCs with a number of bacterial pathogens, including *Spn*, with the peak of cytokine production at 24h post stimulation^{137, 138}. Whether *Spn*

induces type I and III IFN production in PBMCs is unknown. Very little is known about *Spn* and PBMCs between asthmatic and non-asthmatics.

1.4 Immunology of RV-induced asthma exacerbations

The mechanisms of RV-induced asthma exacerbations have so far been elucidated largely from experimental infection models in asthmatics and non-asthmatics, *in vitro* studies of cell lines and studies of natural colds. RV infection of the upper respiratory tract and the resultant pathology is thought to be due to cytokine dysfunction as infection does not lead to extensive epithelial cytotoxicity ¹³⁹.

RV infection results in neutrophilic infiltration and in the upregulation of many proinflammatory cytokines, namely IL-6, CXCL8/IL-8, IL-16, CCL11/eotaxin, CXCL10/IP-10, CCL5/RANTES, CXCL5/ENA-78 and G-CSF ¹⁴⁰⁻¹⁴². Neutrophilic inflammation may have a role in persistent airway obstruction in asthma as higher sputum neutrophil counts are associated with lower post bronchodilator FEV₁ ¹⁴³. Other cell types involved in RV-induced airway inflammation include eosinophils and lymphocytes. In stable asthmatics sputum cell counts are normal, but during an exacerbation sputa become eosinophilic, neutrophilic or with a mixed cellular response ¹⁴⁴. RV infection is associated with increased submucosal lymphocytes and increases in bronchoalveolar lavage (BAL) lymphocytes have been observed in a human exacerbation model ⁷¹.

1.4.1 Interleukin-6 (IL-6)

IL-6 is a cytokine that acts both in an anti- and a pro-inflammatory manner in humans. It acts mainly through a cell surface receptor consisting of IL-6R α and the signal transducer gp130 ¹⁴⁵. Soluble IL-6 receptor has been recognised ¹⁴⁶. IL-6 is important in the acute phase and immune responses, by stimulating production of acute phase proteins, immunoglobulins, B-cell differentiation and T-cell activation ^{145, 147}.

A number of studies have demonstrated that IL-6 is produced following RV infection and that this production is mediated by an NF- κ B-dependent transcriptional pathway¹⁴⁸. Experimental RV infection in atopic asthmatics results in an increase in IL-6 in the sputum, with levels remaining elevated as long as 9 days after infection^{149, 150}. IL-6 is produced by bronchial epithelial cells¹⁵¹, monocytes and alveolar macrophages¹⁵². In comparison to non-asthmatics, asthmatic bronchial epithelial cells *in vitro* have been observed to produce higher levels of IL-6 spontaneously¹⁵¹, but IL-6 responses to RV infection in HBECs have been shown to be similar between asthmatics and non-asthmatics¹⁵³. IL-6 is also induced following RV infection in PBMCs and a recent study suggests the concentration is significantly reduced in asthmatics compared to non-asthmatic controls⁸¹. The important role IL-6 plays in inflammation is therefore of interest in asthma exacerbations.

1.4.2 Interleukin-8 (CXCL8/IL-8)

CXCL8/IL-8 is a major mediator of immune responses whose key function is in neutrophil chemotaxis. RV is a potent stimulator of CXCL8/IL-8 *in vivo* and *in vitro* and this production is, at least in part, mediated by an NF- κ B-dependent transcriptional pathway¹⁵⁴. CXCL8/IL-8 is produced following RV infection in HBECs¹⁵⁵, macrophages⁷³ and in PBMCs¹⁵⁶. Experimental RV infection in atopic asthmatics results in an increase in CXCL8/IL-8 in the sputum, with levels remaining elevated as long as 9 days after infection¹⁴⁹. However the inflammatory responses in the upper and lower airways following experimental RV infection of asthmatics are no different from non-asthmatics¹⁵⁰. Nasal lavage from asthmatics and non-atopic non-asthmatic subjects experimentally inoculated with RV had peak CXCL8/IL-8 levels correlating with cold scores and nasal neutrophil counts, highlighting the neutrophil chemotaxis function of CXCL8/IL-8¹⁵⁷. The role CXCL8/IL-8 has in the pathogenesis of RV infection and asthma exacerbations makes it an important cytokine to examine further.

1.4.3 Interleukin-1 β (IL-1 β)

IL-1 β has a major role in the communication between monocytes and epithelial cells. IL-1 β production from mononuclear cells has been identified as required for the inflammatory response from airway cells ¹⁵⁸. Studies have identified increased levels of IL-1 β in nasal lavage from volunteers experimentally infected with RV16 ¹⁵⁹ and differences have been observed between asthmatics and non-asthmatics ¹⁶⁰. RV infection of upper airway epithelial cells results in increased production of IL-1 β with a study demonstrating the use of neutralising antibodies to IL-1 β resulting in significantly decreased viral titres and ICAM-1 mRNA expression. This suggests that the upregulation of ICAM-1 by RV is mediated through the effects of IL-1 β ¹⁶¹. IL-1 β and myeloid differentiation factor (MyD) 88 play a crucial role in regulating RV replication and the inflammatory response to viral infection of airway cells and exogenous IL-1 β amplifies CXCL8/IL-8 release from RV-infected cells ¹⁶². IL-1 β is of interest in RV-induced asthma exacerbations.

1.4.4 Tumour Necrosis Factor- α (TNF- α)

TNF- α is produced largely from activated macrophages and plays an important part in the acute phase and immune response. It is produced by airway epithelial cells ¹⁶¹, macrophages ⁶⁹ and PBMCs ¹⁶³. RV infection of upper airway epithelial cells results in increased production of TNF- α ¹⁶¹ and this increased expression is at least in part mediated through NF- κ B ¹⁶⁴. Stimulation of tracheal epithelial cells with exogenous TNF- α results in significant upregulation of ICAM-1 and subsequent increases in neutrophilic adhesion ¹⁶⁵. TNF- α secretion results in increases in other proinflammatory cytokines and chemokines such as CXCL8/IL-8 from bronchial epithelial cells ¹⁶⁶. RV and TNF- α stimulation of primary HBECs result in cooperative increases in chemokine expression such as CXCL8/IL-8. This provides a possible cellular mechanism for RV-induced asthma exacerbations ¹⁶⁷. RV infection of human macrophages results in a time- and dose-dependent induction of TNF- α ⁶⁹.

TNF- α has been implicated in asthma for several years ¹⁶⁸. It is thought to play a key role in the initiation of airway inflammation in asthma and the resultant

bronchial hyperreactivity ¹⁶⁹ and much work has been carried out in the investigation of anti-TNF- α as a treatment modality in severe asthma ¹⁷⁰. Alveolar macrophages and PBMCs from asthmatics have been observed to produce significantly more TNF- α than in control subjects ¹⁷¹. The key role TNF- α has in the inflammatory response makes it an important cytokine to investigate further

1.4.5 Other proinflammatory cytokines

Cytokines which may be of importance in RV-induced asthma exacerbations include IL-16, CCL5/RANTES, CXCL10/IP-10, CXCL5/ENA and eotaxins. IL-16 is induced by RV and is a powerful lymphocyte chemoattractant and activator of eosinophils and macrophages ⁵⁸. CCL5/RANTES attracts eosinophils and lymphocytes and results in upregulation of other proinflammatory cytokines leading to airway hyperresponsiveness, inflammation and mucus secretion ¹⁴². CXCL10/IP-10 is involved in T cell recruitment and is stimulated by RV and secreted from airway epithelial cells *in vitro* and *in vivo* ¹⁷². An increased level of CXCL10/IP-10 in the serum of asthmatics has been observed ¹⁵³. CXCL5/ENA induces neutrophil migration ¹⁷³. Eotaxins are eosinophil chemoattractants and are secreted from airway epithelial cells following RV infection ¹⁴¹.

For the purposes of this study IL-6, CXCL8/IL-8, IL-1 β and TNF- α were chosen as the proinflammatory cytokines to be investigated.

1.5 Proinflammatory cytokine expression following airway infection with *Spn*

1.5.1 IL-6 and CXCL8/IL-8

Pneumococcal peptidoglycan-polysaccharides and lipopolysaccharides induce CXCL8/IL-8 production in bronchial epithelial cells via an NF- κ B and activation protein (AP)-1 dependent mechanism ¹⁷⁴. Pneumolysin interacts with TLR4. TLR4 deficient mice have increased susceptibility to pneumococcal infection ¹⁷⁵. Pneumolysin induces production of CXCL8/IL-8 in airway cells using signalling pathways involving calcium flux and activation of NF- κ B via TLR4 ¹⁷⁶. Indeed, pneumolysin is thought to be the major factor responsible for the induction of

inflammation and induces proinflammatory cytokine expression in a time-dependent manner ¹⁷⁷. The pneumococcal virulence factor choline binding protein A (CbpA) participates in the induction of IL-6 and CXCL8/IL-8 from bronchial epithelial cells, thereby having an immunopathological role ¹⁷⁸.

1.5.2 IL-1 β

The role of IL-1 β in the airway immune response to *Spn* is not well recognised. IL-1 β is thought to be important in the communication between airway epithelial cells and macrophages, regulating CXCL8/IL-8 release and enhancing neutrophil recruitment ¹⁷⁹. *Streptococcus* species other than *Spn* have been identified to stimulate release of IL-1 β from PBMCs ¹⁸⁰. IL-1 is secreted from human monocytes stimulated with pneumococcal cell wall components ¹⁸¹.

1.5.3 TNF- α

TNF- α has long been implicated in the pathogenesis of pneumococcal pneumonia, with its production being thought to be protective, preventing bacteraemia ¹⁸². Significant production of TNF- α from murine lung cells has been observed following *Spn* infection ¹⁸³. As well as having a protective role, TNF- α can be detrimental by inducing lung tissue injury. The role of TNF- α from *Spn*-stimulated human airway cells is not well recognised. With TNF- α playing an important role in asthma pathogenesis, further study is warranted.

1.6 Role of interferons in virus and bacterial infection

1.6.1 Interferons

IFNs are antiviral cytokines, first discovered in 1957 by their ability to 'interfere' with viral replication ¹⁸⁴. They are of central importance in the innate immune response to viral infections, including rhinoviruses. The antiviral activity of

IFNs is the result of upregulation of interferon stimulated genes (ISGs) leading to the production of IFN stimulated proteins. These proteins have antiviral, antimicrobial, antiproliferative, antitumour and immunomodulatory effects ¹⁸⁵.

There are three classes of IFN – type I, type II and type III. Type I IFNs are divided into various subtypes, the most important being IFN- α and IFN- β . IFN- α genes have more than 13 subtypes whereas IFN- β only has one subtype ¹⁸⁵. Virtually all cell types express type I and III IFNs. By contrast, Type II IFN or IFN- γ , which is structurally unrelated to type I IFNs, is induced by cytokines such as IL-12 and is expressed only in immune cells such as T cells, NK cells and monocytes/macrophages. Type III IFNs have been identified more recently and are known as IFN- λ 1, IFN- λ 2 and IFN- λ 3 (or IL-29, IL-28A and IL-28B respectively) ¹⁸⁶. Table 1.2 illustrates the different IFNs.

Table 1.2. The interferon family members. The different types, subtypes, receptor, gene locus and cell expression of the IFNs ¹⁸⁷.

Type	Subtype	Receptor	Gene locus	Expression pattern
I	IFN- α	IFNAR1/IFNAR2	9p21	Ubiquitous
	IFN- β	IFNAR1/IFNAR2	9p21	Ubiquitous
	IFN- ϵ	IFNAR1/IFNAR2	9p21	Uterus, ovary
	IFN- ω	IFNAR1/IFNAR2	9p21	Leucocytes
	IFN- κ	IFNAR1/IFNAR2	9p21	Selective expression in epidermal keratinocytes
	IFN- δ	IFNAR1/IFNAR2	None	Trophoblasts
	IFN- τ	IFNAR1/IFNAR2	None	Trophoblasts
	IFN- ζ	IFNAR1/IFNAR2	None	Spleen, thymus, lymph node
II	IFN- γ	IFNGR1/IFNGR2	12q24.1	Activated T cells, macrophages, NK cells
III	IFN- λ 1 (IL-29)	IL-28R α /IL-10R2	19q1	Ubiquitous
	IFN- λ 2 (IL-28A)	IL-28R α /IL-10R2	19q1	Ubiquitous
	IFN- λ 3 (IL-28B)	IL-28R α /IL-10R2	19q1	Ubiquitous

1.6.1.1 Type I interferons

Type I IFNs are a family of intronless genes, present in all vertebrates, which originated 250 million years ago ¹⁸⁸. The group includes IFN- α , IFN- β , IFN- ω , IFN- ϵ , IFN- κ , found in humans, and IFN- δ , IFN- τ , IFN- ζ , found in cattle and pigs ¹⁸⁹. In humans the gene is located on the short arm of chromosome 9 ¹⁹⁰. The IFNs of most importance in humans are IFN- α and IFN- β ¹⁸⁹. Type I IFNs interact with an evolutionary conserved single cell surface receptor, the IFN- α receptor (IFNAR), made up of an extracellular binding domain and an intracellular kinase domain. The receptor is composed of two subunits, IFNAR1 and IFNAR2, which are associated with two cytoplasmic tyrosine kinases, tyrosine kinase 2 (TYK2) and Janus kinase 1 (JAK1) respectively ¹⁹¹⁻¹⁹³. Both subunits are required for receptor function ¹⁹⁴. Type I IFN binding induces phosphorylation of these kinases, which activates signal transducers and activators of transcription (STAT) 1 and 2. In resting cells STATs reside in the cytoplasm as inactive homodimers ¹⁹⁵. Upon activation STATs form heterodimers and complex with IFN regulatory factor (IRF) 9 to form IFN-stimulated gene factor (ISGF) -3 for translocation to the cell nucleus where they upregulate the expression of ISGs by binding to the IFN stimulated response element (ISRE) enhancer family ^{196, 197} (Figure 1.2).

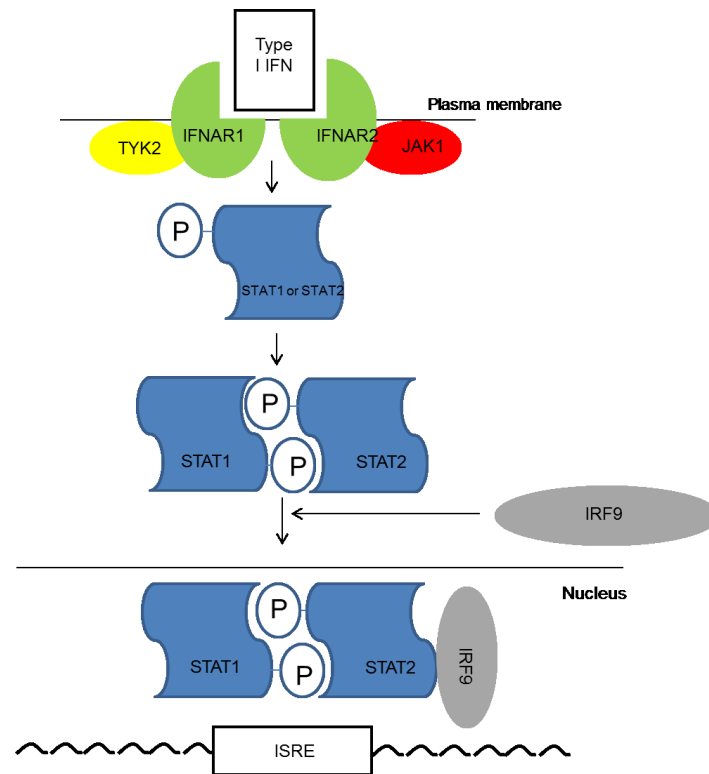


Figure 1.2. Activation of JAK-STAT pathway by type I IFN. (original figure) Type I IFN binds to the IFN receptor composed of IFNAR1 and IFNAR2. This associates with TYK2 and JAK1 resulting in phosphorylation of STAT1 and STAT2 which form a heterodimer before complexing with the translocation factor IRF9 to form ISGF-3. This complex translocates to the nucleus and binds to ISRE to initiate gene transcription.

1.6.1.2 Type III interferons

The IFN- λ s are known as the type III IFNs. They are structurally distinct from type I IFNs and bind to a different receptor. Their mechanisms of induction, signal transduction and biological actions are similar to type I IFNs^{186, 198}. The three functional IFN- λ genes are located on chromosome 19 and encode highly homologous proteins with IFN- λ 2 having 96% homology with IFN- λ 3 and IFN- λ 1 having 81% homology with IFN- λ 2¹⁹⁸. There is only a 20% homology between all IFN- λ s and type I IFNs¹⁸⁶. Whereas type I IFN genes have no introns, the coding regions of the IFN- λ genes are interrupted by 4 introns¹⁹⁹. IFN- λ s interact with a different cell surface receptor comprising IFN- λ R1 and IL-10R2. Despite signalling through distinct receptors, type I and III IFNs trigger similar signalling pathways, ending in the formation of ISGF-3 which binds to the ISRE¹⁹⁹. There is emerging evidence that IFN- λ s may have a role beyond innate antiviral protection with type III

IFNs having a unique function in antiviral protection of the gastrointestinal epithelium that is independent of, and not overlapping with, the type I IFN system ²⁰⁰, leading to the suggestion that type III IFNs may be involved in GI tract homeostasis.

1.6.1.3 Defective interferon production in asthmatics

Defective RV-induced IFN induction has been reported in HBECs and BAL cells from AA ^{60, 61, 201}. HBECs stimulated with RV produce significant concentrations of type I IFNs, particularly IFN- β , and IFN- λ ⁸⁰ and, compared to those from NANA, HBECs from AA have been shown to have significantly reduced IFN- β mRNA levels at 8h and protein at 48h following infection with RV16 ⁶⁰. In addition to this AA have increased virus release and virus RNA from infected cells and impaired apoptosis. HBECs from AA treated with exogenous IFN- β have responses to RV similar to that of NANA ⁶⁰. Likewise, HBECs from asthmatic donors have been shown to have a normal antiviral response to exogenous IFN- β , with exogenous IFN- β significantly suppressing RV replication and significantly reducing RV-induced CXCL10/IP-10, CCL5/RANTES and IL-6 expression ²⁰².

Induction of IFN- λ has also been reported as defective in HBECs and BAL cells from AA infected *ex vivo* with RV16 ⁶¹. This study was a human asthma exacerbation model and the IFN- λ response was related to BAL virus load, inflammation in BAL and sputum and positively correlated with reductions in lung function during *in vivo* infection. Table 1.3 is a summary of studies that do and do not demonstrate type I and III IFN deficiency in asthma.

Table 1.3. Summary of studies assessing the role of type I and III IFNs in asthma

Studies demonstrating Type I and III IFN deficiency in asthma		Studies not demonstrating Type I and III IFN deficiency in asthma	
Study	Key findings	Study	Key findings
Wark et al. ⁶⁰	Deficient IFN- β , impaired apoptosis and increased virus replication in atopic asthmatic HBECs	Lopez-Souza et al. ²⁰³	No significant differences between asthmatic and healthy subjects in RV-induced IFN- β expression in nasal epithelial cells and HBECs.
Contoli et al. ⁶¹	Deficient RV-induced production of IFN- λ in asthmatic HBECs and alveolar macrophages; highly correlated with severity of RV-induced asthma exacerbation	Bochkov et al. ²⁰⁴	Using microarrays and qPCR, no difference in RV-induced type I or III IFN expression between NANA and AA was observed
Gill et al. ²⁰⁵	Plasmacytoid dendritic cells secreted significantly less IFN- α upon exposure to influenza A in asthmatics		
Bufe et al. ²⁰⁶	Children with AA produced significantly lower levels of virus-induced IFN- α from blood cultures than healthy children and children with NAA		
Edwards et al. ²⁰⁷	HBECs from severe therapy resistant atopic asthmatic children have profoundly impaired RV-induced IFN- β and IFN- λ mRNA and protein production		
Sykes et al. ²⁰⁸	RV induction of type I IFNs in BAL cells is delayed and deficient in asthmatics and is associated with greater AHR and skin prick test positivity		
Baraldo et al. ²⁰⁹	In children, deficient RV-induced IFN responses present in asthmatics irrespective of their atopic status and in atopic patients without asthma		

1.6.2 Is there a role for interferons in defence against bacteria?

As well as asthma, *Cpn* has been associated with atherosclerosis. *Cpn* has been shown to induce IFN- β in bronchial and vascular smooth muscle cells, suggesting a possible role for *Cpn*-induced IFN- β production by smooth muscle cells in modulating inflammation²¹⁰. Mouse bone marrow-derived macrophages (BMMs) infected with *Cpn* have been shown to induce IFN- α/β , leading to expression of IFN- γ , which controls bacterial growth²¹¹. This is controlled by IRAK-4 and STAT-1 as is demonstrated in mouse knockout studies^{211, 212}.

Other intracellular bacteria such as *Listeria monocytogenes* can activate the type I IFN pathway through TANK-binding kinase 1 (TBK-1)²¹³. The importance of TBK-1 in antiviral immunity is well established, but much less is understood in bacterial infection. Studies of TBK-1 knockout mice showed increased *Salmonella*, *E. coli* and *S. pyogenes* replication in the cytosol and that TBK-1 appears important for the integrity of pathogen-containing vacuoles within the cell²¹⁴. Infection of mouse BMMs with *Listeria* results in the early induction of IFN- β and this expression is dependent on IRF-3 and TLR-independent as evidenced by the fact that TLR-deficient mice had similar levels of expression of IFN- β ²¹⁵.

Do extracellular bacteria induce interferons? Studies of the extracellular bacteria group B streptococcus have identified induction of type I IFNs via TBK-1 and IRF-3, via intracellular recognition of the bacterial DNA²¹⁶. Macrophages from mice homozygous for a Map3k8 gene mutation have been found to have a high susceptibility to group B streptococcus infection, with impaired TNF- α and type I IFN production²¹⁷. Interestingly the same mutation did not result in susceptibility to viral infections. *S. pyogenes* (group A streptococcus) has been shown to induce type I IFNs through the IRF-3 signalling pathway²¹⁸, through a MyD88-dependent receptor other than those typically used by bacteria²¹⁹. *S. pyogenes* has also been shown to upregulate TLR3 and TLR7 gene expression and that this upregulation is dependent on IFN- α/β ²²⁰.

There is evidence that IFNs are involved in *Spn* infection. A study using DNA microarray in a mouse model of asymptomatic carriage identified that *Spn* colonisation induces early expression of type I IFNs with increases in transcripts

associated with T cell development and activation ²²¹. Nasopharyngeal colonisation with *Spn* in mice results in a moderate increase in type I IFN mRNA ²²². *Spn* has been shown to activate the type I IFN cascade in airway epithelial and dendritic cells, a response dependent on the pore-forming toxin pneumolysin ²²³. IFN- α/β receptor null mice have significantly increased nasal colonisation of *Spn* than wild type mice, suggesting type I IFNs are important in the mucosal response to airway bacterial pathogens. More recent data suggest the epithelial cell is not the main producer of type I IFNs, but are activated in a paracrine manner to express ISGs by IFN- α/β produced by macrophages ²²⁴. *Spn* stimulates type I IFN in alveolar macrophages through a cytosolic DNA-sensing pathway involving the adaptor molecule STING and IRF-3. In contrast to this, but also suggesting a role for IFNs in bacterial infections another study suggests that type I IFNs mediate the development of post influenza bacterial pneumonia in mice ²²⁵. IFN- α/β receptor null mice had improved survival and clearance of *Spn* from the lungs and blood compared to wild type animals, demonstrating that an IFN inhibitory pathway on other CXC chemokine production (CXCL1/KC and CCL2/MCP-1) is important following a secondary challenge with *Spn*. Also of interest is the finding that mast cells, following TLR activation by Gram-positive and -negative bacteria, elicit proinflammatory, but not type I IFN responses ²²⁶.

These studies emphasise the importance of examining *C. pneumoniae* as an intracellular bacterium which has been established as important in asthma pathogenesis, and *S. pneumoniae* as a relevant respiratory tract extracellular bacterium.

1.7 Role of atopy in asthma

Most studies investigating risk factors for exacerbations of asthma define atopy as a positive allergen-specific IgE or skin test to common inhaled or food allergens. A significant proportion of individuals with positive tests have no evidence of allergic disease. Some investigators propose that a positive test should not be the sole marker of atopy ²²⁷. IgE-mediated sensitisation is not a simple yes/no phenomenon, demonstrated by the finding that the probability of childhood wheeze

and reduced lung function increases with increasing concentrations of serum specific IgE antibodies²²⁸.

Many studies have addressed the importance of allergen exposure in the first 2 years of life and the development of asthma^{229, 230}. Allergen exposure has also been implicated in exacerbations of asthma with high exposure to aeroallergens being associated with increased asthma related hospitalisations^{231, 232}.

Evidence for the involvement of atopy in interferon deficiency is less clear. A case report demonstrated undetectable IFN- α in the serum of a 3 year old child with atopic dermatitis²³³ and IFN- α treatment has been shown to improve severe atopic dermatitis with significant reductions in serum concentrations of IgE and in production of IgE by patients' PBMCs *in vitro*²³⁴. A study of children suggested an atopic phenotype (both asthmatic and non-asthmatic) was related to a reduction in virus induced IFN- α release from whole blood cultures²⁰⁶. Modest associations have been observed between asthma, bronchial hyperresponsiveness and atopy and variants of multiple genes in the type I IFN cluster on chromosome 9p22²³⁵. CD4⁺ Th₂ cells mediate atopic responses to allergens normally innocuous in non-atopic individuals by the selective secretion of various cytokines including IL-4, IL-5 and IL-13²³⁶. IL-4 is the key signal driving Th₂ commitment by recruitment and phosphorylation of STAT-6²³⁷ and regulation of induction of GATA3 transcription²³⁸. GATA3 expression is essential for the maintenance of the Th₂ phenotype²³⁹. Type I IFNs have been identified to inhibit GATA3 during Th₂ development, suggesting a potential therapeutic role for IFN α/β in asthma and atopy^{240, 241}. A study of the effects of IL-4 on mouse bone marrow-derived dendritic cells found that exogenous IL-4 renders dendritic cells less responsive to paracrine type I IFNs and less potent in sustaining the autocrine positive loop that normally amplifies the effects of type I IFNs²⁴². With atopy having a Th₂-skewed cytokine response, these effects of IL-4 could explain a deficient interferon response in asthma. A study to compare TLR9 mediated innate immune responses in dendritic cells between allergic and non-allergic individuals identified a six-fold less IFN- α production in cells from allergic subjects (both asthmatic and non-asthmatic) when stimulated with the TLR9 ligand CpG²⁴³.

The role of atopy in bacterial infection of the airway is less well understood. The presence of underlying Th₂-immune responses to bacteria, especially bacterial-specific IgE, in asthmatics has been known for many years^{244, 245}. These responses have been shown to be increased following severe asthma exacerbations in children¹⁰⁰. The significance of this is unclear. Of interest a more recent study suggested IgE titres against *Spn* surface antigens were independently associated with decreased asthma risk²⁴⁶. The role of atopy in bacterial infection in asthma requires further clarification.

1.8 Interferon signalling pathways

1.8.1 Pattern recognition receptors

Cells of the innate immune system express pattern recognition receptors (PRRs) which recognise pattern associated molecular patterns (PAMPs) found in viruses and bacteria. The aim of such a system is rapidly to detect and eliminate foreign pathogens²⁴⁷. The most important PRRs for virus-induced interferon production are the toll like receptors (TLRs), RNA helicases and RIG-I like receptors^{248, 249}. TLRs are found in the extracellular and endosomal compartments, whereas the RNA helicases patrol the cytoplasm for the presence of viral double-stranded DNA. More recently discovered and having a role in the recognition of bacterial cell products are the nucleotide-binding oligomerization domain (NOD)-like receptors²⁵⁰.

1.8.2 Toll like receptors

TLRs are major PRRs involved in induction of IFNs. The involvement of TLRs in innate immunity was first described in drosophila. There are 13 TLRs in mammals. 10 are known in humans, with TLR11-13 expressed in the mouse, but in humans are only represented as a pseudogene. TLRs are hetero- and homodimers of type I transmembrane glycoproteins. Each TLR mediates pattern recognition through extracellular leucine repeats, have a transmembrane domain which determines the location within the cell and have an intracellular Toll/IL-1 receptor

(TIR) domain which is required for intracellular signalling ²⁵¹⁻²⁵³. Recognition of PAMPs by TLRs occurs in different cellular locations: the plasma membrane, lysosomes, endosomes and endolysosomes. The location is important for ligand access and downstream signalling ²⁵². TLR signalling occurs through the MyD88 dependent and independent pathways. The ligands recognised, cellular location and IFN induction pathways used for each TLR is shown in table 1.4.

TLR stimulation in the airway has many effects on the immune pathway. It controls Th₁, Th₂ and Th₁₇ cell differentiation; results in cytokine production; results in eosinophil activation; and increases expression of major histocompatibility complex proteins and T-cell coreceptors in dendritic cells, resulting in T-cell activation ²⁵⁴.

Table 1.4. Toll like receptors: ligands, IFN induction pathways used and cellular location

Toll Like Receptor	Ligand	IFN induction pathway	Cellular location
TLR1	Forms heterodimer with TLR2 to recognise triacylated lipopeptides from gram negative bacteria	MyD88 dependent	Cell surface
TLR2	Forms heterodimer with TLR1 and TLR6 to recognise peptidoglycan, LTA and haemagglutinin	MyD88 dependent	Cell surface
TLR6	Forms heterodimer with TLR2 to recognise diacylated lipopeptides from gram positive bacteria	MyD88 dependent	Cell surface
TLR3	dsRNA, poly(IC)	MyD88 independent	Endosomal
TLR4	LPS, RSV fusion proteins, pneumolysin, paclitaxel	MyD88 dependent & independent	Cell surface
TLR5	Flagellin	MyD88 dependent	Cell surface
TLR7	ssRNA, R848	MyD88 dependent	Endosomal
TLR8	ssRNA, RNA40	MyD88 dependent	Endosomal
TLR9	CpG-ODN in bacterial DNA, CMV, HSV1 and HSV2	MyD88 dependent	Endosomal
TLR10	Unknown, may form heterodimers with TLR1 and TLR2 to recognise bacterial lipopeptides ²⁵⁵	Unknown	Cell surface

1.8.2.1 TLR1, TLR2 & TLR6

TLR2 recognises lipoproteins and lipoteichoic acid from bacteria and zymosan from fungi. Upon recognition of its ligands TLR2 forms a heterodimer with either TLR1 or TLR6. These heterodimers recognise distinct ligands (triacyl lipoproteins for TLR1/TLR2 and diacyl lipoproteins for TLR2/TLR6) and it is understood to be the formation of these heterodimers which results in bringing intracellular TIR domains close together to initiate the signalling pathway ²⁵⁶. Until recently TLR2 activation by bacterial ligands was thought to be associated with upregulation of proinflammatory cytokines but not with type I IFNs. Recent data suggest TLR2 activation induces type I IFNs from endolysosomal compartments ²⁵⁷. IRF2 has been shown to be activated by LTA resulting in upregulation of IRF1 and secretion of IFN- α ²⁵⁸. Another study demonstrates viruses but not bacteria can stimulate TLR2 to induce type I IFNs ²⁵⁹.

1.8.2.2 TLR3

TLR3 recognises dsRNA viruses and the synthetic analogue of dsRNA, poly(IC). TLR3-deficient mice have an impaired type I IFN and proinflammatory cytokine response to poly(IC) and poly(IC) was identified as an activator of NF- κ B ²⁶⁰. Human TLR3-deficiency results in increased susceptibility to herpes simplex encephalitis ²⁶¹ and may be implicated in polio infection ²⁶². It is expressed on macrophages, dendritic cells, fibroblasts and epithelial cells ²⁶³. dsRNA binds to TLR3 which forms a homodimer and induces IFN production through an MyD88-independent pathway (see section 1.8.3).

1.8.2.3 TLR4

TLR4 recognises LPS, the component of the outer membrane of gram negative bacteria, the virulence factor pneumolysin from *S. pneumoniae*, the RSV fusion protein and the cytotoxic drug paclitaxel ²⁶⁴. Several accessory molecules are used by TLR4 to bind LPS, including CD14 and LPS-binding protein (LBP). TLR4

complexes with the cell surface receptor myeloid differentiation factor 2 (MD2) and binds to a LPS-LBP complex to form a homodimer which is then internalised into the cytosol ²⁶⁵. TLR4 signalling then follows either the TIRAP/MyD88 pathway resulting in the production of proinflammatory cytokines, or the TRIF/TRAM, MyD88 independent pathway to result in the production of type I IFNs ²⁶⁴.

1.8.2.4 TLR5

TLR5 recognises the flagellar element of motile bacteria and is expressed by epithelial cells, monocytes and immature dendritic cells, particularly in the lungs and gut. Stimulation of TLR5 results in NF-κB upregulation and the production of proinflammatory cytokines as well as maturation of human dendritic cells ²⁶⁶. This is mediated through the MyD88 pathway ²⁶⁷. Although limited data suggest a role for TLR5 in type I IFN production, the TLR5 ligand flagellin has been observed to stimulate IFN-β expression in mouse bone marrow derived macrophages ²⁶⁸.

1.8.2.5 TLR7 & TLR8

TLR7 and TLR8 are found on the endolysosomes and both recognise ssRNA viruses and imidazoquinoline derivatives such as imiquimod and resiquimod (R848). Upon recognition an antiviral response is triggered resulting in the NF-κB activation and type I IFN production via the MyD88 dependent signalling pathway ²⁶⁴. TLR7 is expressed on dendritic cells and B cells and TLR8 is expressed highly on monocytes as well as dendritic cells, B cells and neutrophils.

1.8.2.6 TLR9

TLR9 recognises unmethylated 2'-deoxyribo CpG motifs found in bacterial and not human DNA. It is sequestered in the endoplasmic reticulum and upon stimulation of the cell moves to the endolysosome ²⁶⁹. TLR9 has been identified to play an early role in clearance of *Spn* from the lower respiratory tract of mice ²⁷⁰. *Spn* infection triggers a TLR9 and MyD88-dependent activation of phagocytic activity

from airway macrophages. TLR9 activation also leads to type I IFN responses in human primary cells ^{271, 272} via the MyD88 dependent pathway. There is some evidence that DNA viruses such as herpes viruses can stimulate production of type I IFNs through TLR9-dependent pathways ²⁷³. Different CpG ODNs (A, B & C) have different type I IFN inducing potencies and have been shown to have different effects on different cell types ²⁷⁴.

1.8.3 TLR signalling pathways

Recognition of PAMPs by TLRs leads to different signalling cascades and the upregulation of distinct genes. The different signalling cascades for individual TLRs can partly be explained by the TIR domain-containing adaptor molecules recruited to TLRs ²⁶⁴. TLR signalling is divided into two distinct pathways depending on the use of two adaptor molecules, namely MyD88 and TRIF (figure 1.3). The following description explains pathways used in the production of type I IFNs. Signalling pathways used in the production of type III IFN are not well understood.

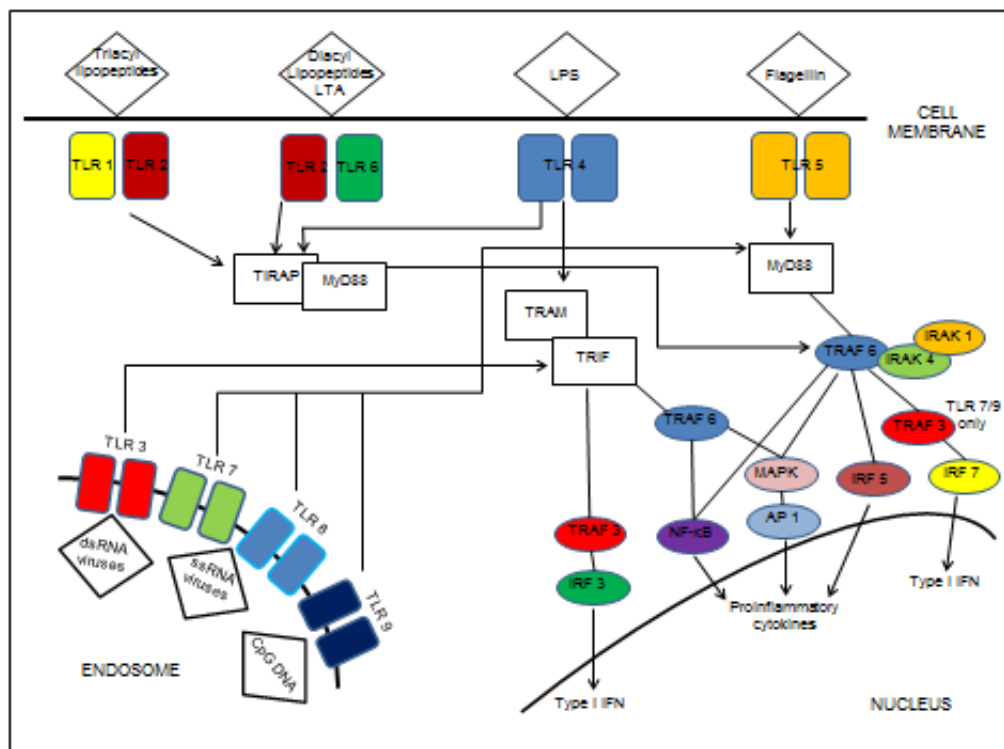


Figure 1.3. Toll-like receptor signalling. (adapted from O' Neill *et al.* ²⁷⁵) TLRs recognise microbial products. Resultant dimerisation of the receptor (or heterodimerisation for TLR2/6 and TLR1/6) is followed by downstream signalling. All TLRs engage the MyD88 adaptor molecule (except TLR3, which signals through TRIF). TLR4 signalling follows MyD88/TIRAP-dependent and/or TRIF/TRAM-dependent pathways. Signalling results in activation of NF- κ B, activator protein (AP)-1 and interferon regulatory factors (IRFs) which induce expression of genes encoding proinflammatory cytokines, type 1 IFN and IFN-inducible genes (MyD88 = Myeloid differentiation factor-88; TIRAP = TIR-associated protein; TRIF = TIR-domain-containing adapter-inducing interferon- β ; TRAM = TRIF-related adaptor molecule; TRAF = TNF receptor associated factor).

1.8.3.1 The MyD88-dependent signalling pathway

MyD88 is required for the downstream signalling of most TLRs, with the exception of TLR3 (figure 1.3). Children with autosomal recessive MyD88 deficiency suffer from life threatening and recurrent pyogenic bacterial infections including invasive pneumococcal disease ²⁷⁶. TIRAP is required as a bridge between TLR2 and TLR4, and MyD88 ²⁷⁷. MyD88 acts to facilitate the interaction of TNFR-associated factor 6 (TRAF6) with IL-1R-associated kinase (IRAK)-4. IRAK-4 activates other IRAK family members, IRAK-1 and IRAK-2. A series of ubiquitination and phosphorylation events results in the activation of I κ B kinase (IKK)- β and MAP

kinase (MAPK)-6. The resultant IKK complex, composed of IKK- α , IKK- β , and NF- κ B essential modulator (NEMO), phosphorylates I κ B. Phosphorylated I κ B undergoes polyubiquitination and degradation, thereby allowing the transcription factor NF- κ B to translocate into the nucleus and activate gene expression of proinflammatory cytokines. NF- κ B is a family of proteins, RelA (p65), RelB, C-Rel, p105 (NF- κ B1) and p100 which form homo or heterodimers to control upregulation of gene expression ²⁷⁸.

Activation of the MAP kinases such as c-Jun, N-terminal kinase (JNK), p38 and extracellular signal regulated kinase (ERK) is responsible for the formation of the transcription factor complex, AP-1, composed of heterodimers of Jun, Fos and ATF proteins, that target cytokine genes ²⁷⁹.

The other proteins implicated in the MyD88 pathway are the IRF family. The 9 IRFs in humans contain a conserved DNA binding domain which recognises the ISRE. Four IRFs (IRF1, IRF3, IRF5 and IRF7) have been identified as regulators of type I IFN production ²⁸⁰. IRF5 is involved in the MyD88-TRAF6 pathway and has a role in proinflammatory cytokine production as observed in that haematopoietic cells from IRF5-deficient mice have impaired cytokine production following stimulation of TLR4, TLR5, TLR7 and TLR9 ²⁸¹. IRF3 and IRF7 are important regulators of type I IFN induction. IRF3 is essential for LPS-mediated IFN- β induction ²⁸² and IRF3 deficient mice have impaired type I IFN responses to rotavirus ²⁸³. IRF3 is constitutively expressed whereas IRF7 is induced by virus infection ²⁸⁰. IRF7 is involved in the MyD88-TRAF6-TRAF3 pathway, whereas IRF3 activation is controlled by the MyD88-independent pathway ²⁸⁴.

1.8.3.2 The TRIF-dependent signalling pathway

Following stimulation with dsRNA, TLR3 recruits the adaptor protein TIR-domain-containing adapter-inducing interferon- β (TRIF). TLR4 signals through both MyD88 and TRIF. TLR4, but not TLR3, requires another adaptor, TRIF-related adaptor molecule (TRAM), to activate TRIF ²⁸⁵. TRIF associates with TRAF3 and TRAF6. TRAF3 activates TANK-binding kinase 1 (TBK1) and IKK- ϵ which leads to the phosphorylation of IRF3 ²⁸⁶. IRF3 dimers translocate to the nucleus, resulting in induction of type I IFNs and expression of IFN-inducible genes. TRAF6 and receptor

interacting protein 1 (RIP-1) activate NF- κ B through the activation of IKK α / β resulting in proinflammatory cytokine release.

1.8.4 Pattern recognition receptors in *S. pneumoniae* cell signalling

LTA and lipoproteins within the cell wall of *Spn* are recognised by TLR2^{287, 288}. Pneumococci have been shown to enhance TLR1 and TLR2 mRNA in BEAS-2B cells and stimulation of TLR1/2 leads to NF- κ B recruitment to the CXCL8/IL-8 promoter²⁸⁷. TLR2 modulates bacterial clearance in granulocytes, TLR2 deficient cells having impaired phagocytosis and oxidative killing of *Spn*²⁸⁹. LTA from *Spn* has been demonstrated to stimulate PBMCs via TLR2 with the help of CD14 and TLR1²⁹⁰. Blocking TLR2, 4 or 9 in PBMCs results in marked reductions in the inflammatory response to *Spn*²⁹¹. TLR9 detects pneumococcal DNA with TLR9 deficient mice having reduced survival from *Spn* pneumonia, with enhanced bacterial load in the lung²⁷⁰. TLR4 is best known for its detection of LPS of gram negative bacteria, but studies have identified TLR4 interaction with pneumolysin and subsequent inflammatory response mediated by MyD88^{175, 292}. TLR4 deficient mice have increased susceptibility and higher bacterial loads after *Spn* lung infection than wild type mice^{175, 293}. Pneumolysin can also activate TLR4-independent innate immune responses. While mouse studies of TLR2, TLR4 and TLR9 deficiencies reveal some susceptibility to pneumococcal infections, deficiency of the adaptor protein MyD88 leads to profound susceptibility to pneumococcus²⁹⁴. This is consistent with a human study reporting autosomal recessive deficiencies of MyD88 being associated with life threatening *Spn* infections in infancy and early childhood²⁷⁶.

NOD like receptors also have a role in the innate immune response to pneumococci. NOD1 is a host cytoplasmic peptidoglycan-recognition molecule²⁹⁵. NOD2 has been implicated in NF- κ B induction by *Spn*²⁹⁶ with upregulation of NOD1 and NOD2 mRNA following pneumococcal infection *in vivo* in C57BL/6 mice and *in vitro* in BEAS-2B cells. NOD2 in macrophages has been identified to sense digested peptidoglycan, requiring the pore-forming toxin pneumolysin²⁹⁷. This sensing results in release of the chemokine CCL2/MCP-1 with resulting recruitment of additional macrophages required for clearance of *Spn*.

The expression of NF- κ B-dependent proinflammatory genes such as IL-1 β requires a second signal from inflammasomes²⁹⁸. Inflammasomes are multiprotein complexes which mediate caspase-1 dependent cytokine maturation. The inflammasome best characterised is NOD-like receptor, pyrin domain containing 3 (NLRP3) which is activated by bacteria expressing pore forming toxins²⁹⁹. NLRP3 has also recently been shown to be activated by *Spn* and pneumolysin³⁰⁰.

It is unclear which PRR is involved in the IFN response following pneumococcal infection, but the response is dependent on cytosolic recognition of bacterial DNA and the transcription factor IRF3²²⁴. NOD2 contributes to type I IFN responses to pneumococcus *in vivo*²²². More investigation is needed to further determine the signalling pathways used in *Spn* infection.

1.9 Rationale, Hypothesis and Aims

1.9.1 Rationale

The rationale of this study is to investigate further the deficiency of IFN production in asthma. The majority of asthma morbidity, mortality and healthcare costs is due to exacerbations, commonly due to viral and particularly RV infection. Atopic asthmatics have been observed to have increased susceptibility to RV infection. To date defective IFN production in asthma has been observed in virus induced models *in vitro* and *in vivo* with HBECs from AA having impaired type I and III IFN response to RV infection and BAL cells having impaired type III IFN responses^{60, 61}. To date this deficiency in IFN production has been observed in AA, and the role of atopy in this process has not yet been elucidated. There is increasing evidence for a role for bacteria, both typical and atypical, in the pathogenesis of asthma and there is some evidence that bacteria may induce IFNs. The possibility that exacerbations are related to IFN deficiency in asthmatics requires further investigation. This project aims to investigate type I and III IFN and proinflammatory cytokine responses to RV and respiratory bacteria in HBECs, BAL cells and PBMCs in asthmatics, non-asthmatics, and atopics and non-atopics, specifically to investigate whether the atopic status has a role in IFN deficiency. Further

understanding of this IFN deficiency has the potential to identify therapeutic targets in the management of asthma exacerbations.

1.9.2 Specific Hypotheses

In this study I hypothesise that:

- (1) following rhinovirus infection of BAL cells and HBECs, deficiencies in type I and III interferon production will be observed in atopic and non-atopic asthmatics but not in atopic non-asthmatic and normal subjects
- (2) deficiencies will be observed following infection with bacteria such as *Streptococcus pneumoniae* and stimulation with bacterial TLR ligands
- (3) observations made in BECs and airway macrophages will be reflected in supernatants of stimulated PBMCs

1.9.3 Aims

The aim of this study is to further identify the defect(s) which results in the deficient production of interferons in asthma in BAL macrophages, PBMCs and HBECs

- (1) in response to rhinovirus infection and “viral” TLR ligand stimulation, focussing on IFN and proinflammatory cytokine responses
- (2) in response to bacterial infections and “bacterial” TLR ligand stimulation, examining IFN and proinflammatory cytokine responses, with an introduction of the concentration-timing experiments with *Spn* and “bacterial” TLR ligands in BEAS-2B cells, primary HBECs and PBMCs
- (3) to compare asthmatics and non-asthmatics
- (4) to compare atopics and non-atopics

Different cell types will be investigated to examine whether any deficiency is cell- or asthma-/atopy-specific, and specifically whether any observations made in airway cells are observed in PBMCs.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Clinical Equipment

Table 2.1 Clinical equipment used in the project in order of application

Name	Composition	Application	Source
Vacutainer blood tubes 9NC SST	Vacutainer tubes	Blood testing	BD, USA
Disposable sheathed cytology brush BC-202D-5010	5mm cytology brush	Bronchoscopy	Olympus
EndoJaw single use biopsy forceps FB-211D	Disposable endobronchial biopsy forceps	Bronchoscopy	Olympus
Keymed BF260 bronchoscope	Fibreoptic bronchoscope	Bronchoscopy	Olympus
Salbutamol nebulas	2.5mg Salbutamol sulphate in normal saline	Bronchoscopy	Allen & Hanburys, UK
Ipratropium bromide nebulas	0.25mg Ipratropium bromide in normal saline	Bronchoscopy	Allen & Hanburys, UK
Nebuliser (Porta Neb)	Portable air compressor	Drug administration	Profile Therapeutics
Histamine	Histamine Acid phosphate, 32mg/mL	PC ₂₀	NPH Pharmacy
ALK Lancet	Disposable micro-lancet	Skin Prick Tests	Boge Alle
Cat allergen (507)	Allergen solution	Skin Prick Tests	Modelo Harwell
Dog allergen (509)	Allergen solution	Skin Prick Tests	Modelo Harwell
6 grass mix allergen (688)	Allergen solution	Skin Prick Tests	Modelo Harwell
House dust mite allergen (315)	Allergen solution	Skin Prick Tests	Modelo Harwell
Alternaria alternata allergen (400)	Allergen solution	Skin Prick Tests	Modelo Harwell
Aspergillus fumigatus allergen (401)	Allergen solution	Skin Prick Tests	Modelo Harwell
Cladosporium herbarum allergen (414)	Allergen solution	Skin Prick Tests	Modelo Harwell
Birch allergen (615)	Allergen solution	Skin Prick Tests	Modelo Harwell
Tree mix allergen (718)	Allergen solution	Skin Prick Tests	Modelo Harwell
Mugwort allergen (605)	Allergen solution	Skin Prick Tests	Modelo Harwell
Spirometer	Portable spirometer	Spirometry	Micro medical, UK
Spiroguard bacterial/viral filters	Filter for spirometer	Spirometry	Spiroguard

2.1.2 Laboratory instruments

Table 2.2 Laboratory instruments used in the project in alphabetical order

Name	Application	Company
Acrodisc 0.2µm filters	Media and reagent filtering	Pall Life Sciences
Amicon Ultra-0.5, Ultracel-30 membrane, 30kDa filter unit	RV filtering	Millipore, Billerica, MA, USA
Cell strainer, 100µm	BAL processing	BD, USA Falcon
Centrifuge (Eppendorf 5415)	RNA isolation, RV filtering	Eppendorf, Hamburg, Germany
Centrifuge (Eppendorf 5810R)	Centrifugation of cultured cells	Eppendorf, Hamburg, Germany
Cytospin 3 system	BAL cytospin preparation	Shandon, USA
HERA Category II tissue culture hood	All sterile cell work	Kendro Lab. Products, Hanau, Germany
Hotplate and stirrer (Jenway 1000)	Buffer and solution preparation	Grant Instruments Ltd, Cambridge, UK
Incubator (NU 500E air jacketed automatic CO ₂)	Cell culture	NuAire Inc., Plymouth, MN, USA
Inverted microscope (Leica DME)	Cell quantification	Leica Microsystems, Wetzlar, Germany
Meso Scale Discovery SECTOR [®] Imager 2400	MSD	Meso Scale Discovery, Gaithersburg, MD, USA
Plate reader (spectramax plus 384)	ELISA	Molecular Devices Ltd, Wokingham, UK
Realtime PCR system (ABI7500)	Taqman RT-PCR	Applied Biosystems, Foster City, CA, USA
Shaker (R100) rotatest	RV infection of cells, HBEC experiments	Luckham Ltd, Sussex, UK
Taqman RT-PCR hood (Bioair instruments, Aura Mini)	Primer and probe handling	Wolf Laboratories Ltd, Pocklington, UK
UV crosslinker (Stratalinker240)	UV inactivation of RV	Stratagene, Amsterdam, Netherlands
Vortex	HBEC culture, reagent preparation	VWR International
Water bath	cDNA synthesis	Grant Instruments Ltd, Cambridge, UK

2.1.3 Buffers and reagents

Table 2.3 Buffers and reagents used in the project in alphabetical order

Name	Composition	Application	Source
3,3',5,5'-tetramethylbenzidine (TMB)	1x TMB. No other details given by manufacturer	ELISA substrate	Invitrogen, UK
Bovine Serum Albumin Powder (BSA)	1% BSA in 0.15M NaCl pH 7.0	ELISA, HBEC culture	Sigma Aldrich, USA
Diluent-1	RPMI-like medium. No other details given by manufacturer	MSD	MSD, USA

Diluent-100	Contains blocking and stabilizing agents. No other details given by manufacturer	MSD	MSD, USA
Dulbecco's PBS 1x (D-PBS)	Phosphate buffered saline 0.0014M KH_2PO_4 , 0.008M $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 0.0026M KCl, 0.137M NaCl	Cell culture	Invitrogen, UK
ELISA blocking solution	1% BSA in ELISA wash buffer	ELISA	Sigma Aldrich, USA
ELISA diluent buffer	0.2% BSA in ELISA wash buffer	ELISA	Sigma Aldrich, USA
ELISA stop solution	2M H_2SO_4	ELISA	Sigma Aldrich, USA
ELISA/MSD wash buffer	PBS, 0.05% Tween-20	ELISA, MSD	Sigma Aldrich, USA
Ethanol	96.6% Ethanol	RNA isolation	VWR International
Histopaque [®] -1077	Sterile solution of polysucrose, 57 g/L and sodium diatrizoate, 90 g/L. Density: 1.076–1.078 g/mL. Endotoxin: ≤ 0.3 EU/mL. pH: 8.8–9.0	PBMC processing	Sigma Aldrich, USA
MSD Read buffer T (4x)	4x Read buffer. No other details given by manufacturer	MSD	MSD, USA
MSD SULFO-TAG labelled Streptavidin	In PBS containing 2% BSA, 0.05% Azide and 0.02% Kathon CG	MSD (for IL-29 prototype)	MSD, USA
Nuclease-free water	Nuclease-free water	Primer and probe dilution, Taqman RT-PCR	Promega, USA
PBS (per tablet)	0.001M phosphate buffer, 0.0027M KCl, 0.137M NaCl in 200ml distilled H_2O	ELISA	Sigma Aldrich, USA
Quantitect probe PCR master mix	HotStar Taq DNA polymerase, quantitect probe PCR buffer (Tris-HCl, KCl, $(\text{NH}_4)_2\text{SO}_4$, 8mM MgCl_2 pH 8.7), dNTP mix	Taqman RT-PCR	Qiagen, UK
Random primers	Random hexadeoxynucleotides (0.5 $\mu\text{g}/\text{mL}$), in nuclease free H_2O	cDNA synthesis	Promega, USA
Recombinant human CXCL8/IL-8	Reconstituted at 100 $\mu\text{g}/\text{mL}$ in sterile PBS containing 0.1% BSA	ELISA	R&D systems, USA
Recombinant human IL-6	Reconstituted at 100 $\mu\text{g}/\text{mL}$ in sterile PBS containing 0.1% BSA	ELISA	R&D systems, USA
Streptavidin-HRP (Horseradish Peroxidase)	In PBS pH 7.2 containing 0.01% Thimerosal and 40% glycerol	ELISA	Biosource Int.
TE buffer (1x) Molecular grade	10mM Tris-HCl containing 1mM EDTA Na_2	Primer and probe resuspension	Promega, USA
Trypan blue	0.4% trypan blue in 0.85% saline solution	Cell viability	Invitrogen, UK
Tween-20 (polyoxyethylene-sorbitan monolaurate)	20 ethylene oxide units, 1 sorbitol unit, 1 lauric acid unit	ELISA, MSD	Sigma Aldrich, USA
β -mercaptoethanol (β -ME)	14.3M $\text{C}_2\text{H}_6\text{O}_5$	RNA isolation	Sigma Aldrich, USA

2.1.4 Media and supplements

Table 2.4 Media and media supplements used in the project in alphabetical order

Name	Composition	Application	Source
BEBM single quot additives	13g/L bovine pituitary extract, 5g/l insulin, 50g/L gentamicin, 0.1mg/L retinoic acid, 10g/L transferrin, 6.5mg/L triiodothyronine, 0.5g/L hydrocortisone, 0.5g/L epinephrine, 0.5mg/L human epidermal growth factor	HBEC culture	Lonza, USA
BEGM complete	BEBM with BEGM single quot additives, 5mL pen/strep	HBEC culture	Lonza, USA
BEGM infection+P/S	BEGM complete without 2mL bovine pituitary extract	HBEC culture	Lonza, USA
BEGM infection-P/S	BEGM complete without 2mL bovine pituitary extract or 5mL pen/strep	HBEC culture	Lonza, USA
Bovine serum albumin	1% BSA in filtered water	HBEC culture	Sigma Aldrich, USA
Bronchial epithelium basal medium (BEBM)	Refer to manufacturer's formulation	HBEC culture	Lonza, USA
Dulbecco's modified eagles medium (DMEM)	Refer to manufacturer's formulation	HeLa & virus culture, virus titration	Invitrogen, UK
Fibronectin (pure)	Lypophilizate from human plasma	HBEC culture	Roche, Switzerland
Foetal Bovine Serum (FBS)	Heat inactivated foetal bovine serum	HeLa, BEAS-2B, PBMC & BAL cell culture	Invitrogen, UK
HBEC collagen coating mx	9mL BEGM complete, 1mL 1% BSA, 100µL recombinant human like collagen, 100µL fibronectin (pure)	HBEC culture	Lonza, USA
HeLa cell complete medium	DMEM plus 10% FBS, 12.5mL HEPES buffer, 5mL Sodium bicarbonate	HeLa cell culture	Invitrogen, UK
HeLa cell titration medium	DMEM plus 4% FBS, 12.5mL HEPES buffer, 5mL Sodium bicarbonate, 5ml pen/strep	Virus titration	Invitrogen, UK
HEPES buffer	C ₈ H ₁₈ N ₂ O ₄ S 1M solution 4-(2-hydroxyethyl)piperazine-1-ethanesulphonic acid solution	HeLa, BEAS-2B, PBMC & BAL cell culture, virus culture & titration	Sigma Aldrich, USA
Hepes buffered saline solution	30mM HEPES	HBEC culture (cell splitting)	Lonza, USA
Penicillin/ Streptomycin	10,000 units/mL penicillin, 10,000µg/mL streptomycin	HBEC culture, virus titration, PBMC & BAL culture	Gibco, UK
Recombinant human like collagen	>95% human collagen from E. coli	HBEC culture	Biovision
RPMI complete	RPMI-1640 plus 10% FBS, 12.5mL HEPES buffer, 5ml sodium bicarbonate	BEAS-2B cell culture	Invitrogen, UK

RPMI infection	RPMI-1640 plus 2% FBS, 12.5mL HEPES buffer, 5ml sodium bicarbonate	BEAS-2B cell infection	Invitrogen, UK
RPMI+P/S	RPMI-1640 plus 10% FBS, 12.5mL HEPES buffer, 5mL pen/strep	PBMC & BAL culture	Invitrogen, UK
RPMI-1640	Refer to manufacturer's formulation	BEAS-2B, PBMC & BAL culture	Invitrogen, UK
RPMI-P/S	RPMI-1640 plus 10% FBS, 12.5mL HEPES buffer	PBMC & BAL culture	Invitrogen, UK
Sodium bicarbonate	7.5% sodium bicarbonate	HeLa, BEAS-2B cell culture	Invitrogen, UK
Trypsin EDTA (1x)	0.025% trypsin, 0.01% EDTA	HBEC culture (cell splitting)	Lonza, USA
Trypsin EDTA (10x)		HeLa, BEAS-2B cell culture	Invitrogen, UK
Trypsin neutralising solution (TNS)	No supplier information	HBEC culture (cell splitting)	Lonza, USA
Ultrosert TM G	Serum substitute – lyophilized Ultrosert G	HBEC culture	Pall Life Sciences
Virus culture medium	DMEM plus 12.5mL HEPES buffer, 5mL Sodium bicarbonate	Rhinovirus culture	Invitrogen, UK

2.1.5 Toll like Receptor (TLR) Ligands

Table 2.5 TLR ligands in order of preference used in this study, with the receptor they bind to and the concentrations used in HBECs, BAL cells and PBMCs

Name	Receptor	Source	Concentration		
			HBECs	BAL	PBMCs
Lipoteichoic Acid (LTA-SA)	TLR2	InvivoGen	25µg/mL	25µg/mL	25µg/mL
Lipopolysaccharide (LPS)	TLR4	Sigma Aldrich, USA	10µg/mL	10µg/mL	10µg/mL
CpG-C-ODN	TLR9	Eurogentec	3µM	3µM	3µM
Flagellin (FLA)	TLR5	InvivoGen	100ng/mL	100ng/mL	100ng/mL
Pam3CSK4 (PAM)	TLR1/2	InvivoGen	10µg/mL	10µg/mL	10µg/mL
Synthetic lipoprotein (FSL-1)	TLR2/6	InvivoGen	1µg/mL	1µg/mL	1µg/mL
Peptidoglycan (PGN-SA)	TLR2	InvivoGen	10µg/mL	10µg/mL	10µg/mL
R848 (Resiquimod)	TLR7/8	Eurogentec	1mM	1mM	1mM
Polyinosinic-Polycytidylic Acid (poly(IC))	TLR3	Sigma Aldrich, USA	10µg/mL	100µg/mL	100µg/mL

2.1.6 Primers and Probes for Realtime RT-PCR

Realtime RT-PCR primers were purchased from Invitrogen, UK (Paisley, UK) in a desalted, dry pellet form. All probes were purchased from Qiagen (Crawley, UK) in a HPLC purified, dry pellet form, and stored at -20°C until resuspension.

Table 2.6 TaqMan Realtime RT-PCR primers and probes and their optimal concentrations used.

Name	Sequence (FAM-5'-3'-TAMRA)
18S forward (300nM)	CGCCGCTAGAGGTGAAATTCT
18S reverse (300nM)	CATTCTTGGCAAATGCTTTTCG
18S probe (100nM)	ACCGGCGCAAGACGGACCAGA
IFN- β forward (300nM)	CACGGATACAGAACCTATGG
IFN- β reverse (900nM)	ACGAACAGTGTGCGCTACTA
IFN- β probe (100nM)	TCAGACAAGATTCATCTAGCACTGGCTGGA
IFN- α (A2) forward (50nM)	CTGGCACAAATGGGAAGAAT
IFN- α (A2) reverse (50nM)	CTTGAGCCTTCTGGAAGTGG
IFN- α (A2) probe (128nM)	TTTCTCCTGCCTGAAGGACAGACATGA
IL-28 (IFN- λ 2/3) forward (300nM)	CTGCCACATAGCCCAGTTCA
IL-28 (IFN- λ 2/3) reverse (900nM)	AGAAGCGACTCTTCTAAGGCATCTT
IL-28 (IFN- λ 2/3) probe (100nM)	TCTCCACAGGAGCTGCAGGCCTTTA
IL-29 (IFN- λ 1) forward (300nM)	GGACGCCTTGAAGAGTCACT
IL-29 (IFN- λ 1) reverse (900nM)	GGACGCCTTGAAGAGTCACT
IL-29 (IFN- λ 1) probe (100nM)	AGTTGCAGCTCTCCTGTCTTCCCCG
IL-6 forward (300nM)	CCAGGAGCCCAGCTATGAAC
IL-6 reverse (900nM)	CCCAGGGAGAAGGCA ACTG
IL-6 probe (100nM)	CCTTCTCCACAAGCG CCTTCGGT
IL-8 forward (900nM)	CTGGCCGTGGCTCTCTTG
IL-8 reverse (900nM)	CCTTGGCAAACTGCACCTT
IL-8 probe (100nM)	CAGCCTTCCTGATTTCTGCAGCTCTGTGT
Rhinovirus forward (50nM)	GTGAAGAGCCSCRTGTGCT
Rhinovirus reverse (300nM)	GCTSCAGGGTTAAGGTTAGCC
Rhinovirus probe (100nM)	TGAGTCCTCCGGCCCCCTGAATG

2.1.7 Antibodies

Table 2.7 Antibodies used in the project and their working concentrations (the antibodies are selective with known cross reactivity to other cytokines)

Antibody	Isotype	Working concentration	Application	Supplier
Capture, anti-human IL-6	Mouse IgG/6708	4µg/mL	ELISA	R&D systems, USA
Detection, anti-human IL-6	Biotinylated goat IgG	25ng/mL	ELISA	R&D systems, USA
Capture, anti-human IL-8	Mouse IgG1/6217	4µg/mL	ELISA	R&D systems, USA
Detection, anti-human IL-8	Biotinylated goat IgG	25ng/mL	ELISA	R&D systems, USA

2.1.8 Commercial kits

Table 2.8 Commercially available kits used in the project, listed in alphabetical order

Name	Components	Application	Source
Human IFN-α2a Tissue Culture Kit	Read Buffer T (4X), Diluent 1, Diluent 100, MULTI-SPOT® 96-well plates spotted with anti-IFNα2a capture antibody, SULFO-TAG™ labelled anti-IFNα2a detection antibody (50X), Human IFN-α2a Calibrator (1µg/mL)	MSD	MSD, USA
Human IFN-β Tissue Culture Kit	Read Buffer T (4X), Diluent 1, Diluent 100, MULTI-ARRAY® 96-well Avidin Plates, SULFO-TAG™ Anti-hIFN-β Antibody (50X), Anti-hIFN-β Biotinylated Capture Antibody (50X), Human IFN-β Calibrator	MSD	MSD, USA
Human IL-29/IL-28B DuoSet	Capture antibody (mouse anti-human IL-29/IL-28B); detection antibody (biotinylated goat anti-human IL-29/IL-28B); standard (recombinant human IL-29/IL-28B); streptavidin-HRP	MSD (IL-29)	R&D systems, USA
Human Proinflammatory 4-plex II Tissue Culture Kit	Read Buffer T (4X), Diluent 1, Diluent 100, MULTI-SPOT® 96-well 4-plex plates spotted with anti-IL-1β, anti-IL-6, anti-IL-8, anti-TNF-α capture antibody mix, SULFO-TAG™ labelled anti-IL-1β, anti-IL-6, anti-IL-8, anti-TNF-α detection antibody mix (50X), Human IL-1β, IL-6, IL-8, TNF-α Calibrator mix (1µg/mL)	MSD	MSD, USA
MULTI-SPOT® 96-well 4-spot prototype human IL-29	MULTI-SPOT® 96-well 4-plex plates spotted with anti-IL-29 capture antibody	MSD	MSD, USA

Omniscript RT (reverse transcriptase) kit	dATP, dCTP, dGTP, dTTP (5mM each) in water, 10x buffer RT, omniscript reverse transcriptase, RNase free water	cDNA synthesis	Qiagen, UK
REASTAIN quick-diff kit	Reastain Quick-Diff Fix: methanol 100%, methylene blue <0.1%; Reastain Quick-Diff Red: Eosin Y 0.12%, sodium azide <0.1% Reastain Quick-Diff Blue: Azur II 0.09%, glycerol 5%, sodium azide <0.1%;	Cytospin staining	Reagen, Finland
RNase free DNase set	1500 units RNase free DNase 1, RNase free buffer RDD	RNA isolation	Qiagen, UK
RNeasy Mini kit	Buffer RLT, Buffer RW1, Buffer RPE, RNase free water, RNeasy mini spin columns, collection tubes	RNA isolation	Qiagen, UK

2.1.9 Software

Table 2.9 Software used in the project listed in alphabetical order

Name	Application	Company
Softmax Pro v3.1.2	ELISA and Taqman RT-PCR data analysis	Molecular Devices Corporation, CA, USA
Discovery Workbench [®] 3.0 Software	MSD data analysis	MSD, USA
ABI Prism 7500 SDS software v1.4.0	Taqman RT-PCR data analysis	Applied Biosystems, USA
GraphPad Prism v4.0	Statistical analysis of data	GraphPad Software Inc., CA, USA
Primer Express v3.0	Primer and probe design	Applied Biosystems, USA

2.2 Clinical Methods

2.2.1 Participant Recruitment

Ethical approval for the study was obtained from the St Mary's Research Ethics Committee (reference 08/H0712/39). The aim was to recruit 48 subjects; 12 atopic asthmatics (AA), 12 non-atopic asthmatics (NAA), 12 atopic non-asthmatics (ANA) and 12 non-atopic non-asthmatics (NANA). A formal power calculation was not performed, however significant data had been observed in similar numbers of volunteers from other human studies ^{60, 61, 208}. Participants were recruited from records of previous studies performed in the department, from advertisements in local newspapers, and hospital and college websites, and attempts were made to recruit from established asthma clinics from 3 sites around London. Participants were telephone screened and then invited to St Mary's Hospital, London for screening investigations.

Clinical characterisation was determined by clinical history and examination, skin prick testing, serum total IgE, spirometry, histamine challenge, electrocardiogram and chest radiograph. If an electrocardiogram or chest radiograph had been performed in the previous year, this was not repeated and results were obtained from the participant's medical practitioner. Blood tests for full blood count, clotting (INR and APTT), urea and electrolytes and total IgE were performed. Table 2.10 illustrates the inclusion and exclusion criteria.

2.2.2 Screening Investigations

2.2.2.1 Spirometry

Spirometry was performed according to BTS/ARTP guidelines ³⁰¹ using Microlab medical spirometer. The same spirometer was used for all participants and it was regularly calibrated according to the manufacturer's instructions. The FEV₁, FVC, PEF and FEV₁/FVC ratio were recorded and the best of three attempts by the participant was used.

2.2.2.2 Histamine challenge

The histamine challenge was performed according to ERS guidelines³⁰² using a tidal breathing method. Asthmatic participants were asked to withhold LABA for 48 hours and SABA for 12 hours prior to the investigation. A diagnosis of asthma was supported by a $PC_{20} \leq 8$ mg/mL. Non-asthmatics were required to have $PC_{20} \geq 16$ mg/mL.

2.2.2.3 Skin prick testing

Atopy was defined by skin prick testing to a panel of common aeroallergens (Modelo Harwell) including: 6 grass mix, house dust mite (*Dermatophagoides pteronissinus*), *Cladosporium herbarum*, *Alternaria alternata*, *Aspergillus fumigatus*, Silver birch, 3 trees (*Betula verrucosa*, *Alnus glutinosa*, *Corylus avellana*), mugwort, cat dander and dog hair. Participants were asked to withhold antihistamine medication for 48 hours prior to the test. Drops of allergens, a positive control (histamine) and a negative control (normal saline) were placed on the participant's forearm and were introduced intraepidermally using an ALK sterile disposable lancet. A single wheal 3 mm greater than the negative control was considered diagnostic of atopy.

Table 2.10 Inclusion and Exclusion criteria

AA Inclusion Criteria	NANA Inclusion Criteria	Exclusion Criteria
• Age 18-55. • Clinical diagnosis of asthma. • Symptom questionnaires positive for asthma. • Skin Prick test Positive (1 positive reaction – wheal 3 mm greater than the control will be considered diagnostic of atopy) • PC₂₀ ≤8mg/ml or >20% variability over two weeks on peak flow testing. • No other chronic respiratory condition or systemic disease. • Up to step 3 BTS Guidelines / GINA intermittent or mild persistent • No respiratory tract infection or exacerbation for 6 weeks • Current non-smokers of >1 year (if ex-smokers <5 pack year history)	• Age 18–55. • Negative asthma symptom questionnaire. • IgE <50 IU/ml. • Skin Prick testing negative. • Minimal Peak flow variability in two weeks peak flow diary (<15%). • PC₂₀ >8mg/ml. • No history of atopy, asthma, eczema or allergic rhinitis or food allergy. • No chronic respiratory conditions. • No significant systemic disease. • No respiratory tract infection for 6 weeks. • Current non-smokers of >1 year (if ex-smokers <5 pack year history)	• Current smoker/smoker in the past year. • Current significant other chronic respiratory or systemic disease. • Severe or uncontrolled asthmatics (GINA – moderate persistent or severe persistent). • Significant other abnormalities on lung function testing i.e. restrictive defect etc.
NAA Inclusion Criteria	ANA Inclusion Criteria	
• Age 18-55. • Clinical diagnosis of asthma. • Symptom questionnaires positive for asthma. • No history of atopy, eczema, allergic rhinitis or food allergy. • Skin Prick test negative • IgE <50 IU/ml. • PC₂₀ ≤8mg/ml or >20% variability over two weeks on peak flow testing. • No other chronic respiratory condition or systemic disease. • Up to step 3 BTS Guidelines / GINA intermittent or mild persistent • No respiratory tract infection or exacerbation for 6 weeks • Current non-smokers of >1 year (if ex-smokers <5 pack year history)	• Age 18–55. • Negative asthma symptom questionnaire. • Skin Prick testing positive. • Minimal Peak flow variability in two weeks peak flow diary (<15%). • PC₂₀ >8mg/ml. • No chronic respiratory conditions. • No significant systemic disease. • No respiratory tract infection for 6 weeks • Current non-smokers of >1 year (if ex -smokers <5 pack year history)	

2.2.2.4 Asthma Control Questionnaire

In order to establish how well symptoms were controlled, asthmatics were asked to complete an asthma control questionnaire (ACQ) ³⁰³. ACQ scores were made on the basis of participants' answers to six questions (table 2.11), dividing the total score by six. Scores <1 were considered good control, >3 suboptimal and 6 poor control ³⁰⁴.

Table 2.11 Asthma Control Questionnaire

Question	Answer	Answer score
On average, during the past week, how often were you woken by your asthma during the night?	Never	0
	Hardly ever	1
	A few times	2
	Several times	3
	Many times	4
	A great many times	5
	Unable to sleep because of asthma	6
On average, during the past week, how bad were your asthma symptoms when you woke in the morning?	No symptoms	0
	Very mild symptoms	1
	Mild symptoms	2
	Moderate symptoms	3
	Quite severe symptoms	4
	Severe symptoms	5
	Very severe symptoms	6
In general, during the past week, how limited were you in your activities because of your asthma?	Not limited at all	0
	Very slightly limited	1
	Slightly limited	2
	Moderately limited	3
	Very limited	4
	Extremely limited	5
	Totally limited	6
In general, during the last week, how much shortness of breath did you experience because of your asthma?	None	0
	A very little	1
	A little	2
	A moderate amount	3
	Quite a lot	4
	A great deal	5
	A very great deal	6
In general, during the past week, how much of the time did you wheeze?	Not at all	0
	Hardly any of the time	1
	A little of the time	2
	A moderate amount of time	3
	A lot of the time	4
	Most of the time	5
	All of the time	6
On average, during the past week, how many puffs of short-acting bronchodilator (e.g. Ventolin, Bricanyl) have you used each day?	None	0
	1-2 puffs most days	1
	3-4 puffs most days	2
	5-8 puffs most days	3
	9-12 puffs most days	4
	13-16 puffs most days	5
	More than 16 puffs most days	6

2.3 Clinical Sample Collection

2.3.1 Peripheral Blood Collection

60ml of fresh blood was collected in heparinised tubes (BD, USA).

2.3.2 Bronchoscopy

Bronchoscopies were performed according to BTS guidelines³⁰⁵ in the endoscopy unit at St Mary's Hospital, London, using the Keymed BF260 bronchoscope (Olympus, UK). Participants were monitored by a separate doctor or nurse. Spirometry (FEV₁) was recorded before and after bronchoscopy. All participants received nebulised 5mg Salbutamol (Allen & Hanburys) and 500µg Ipratropium bromide (Allen & Hanburys) prior to the procedure. At bronchoscopy, HBECs were collected using bronchial brushes and bronchoalveolar lavage was performed to obtain BAL cells and BAL supernatant.

2.3.2.1 Bronchial Brushings

Seven bronchial brushings were taken with 5mm sheathed endobronchial brushes (Olympus BC-202D-5010) under direct vision by inserting the bronchoscope into a right lower lobe bronchial sub segment and brushing 5–10 strokes against bronchial wall. 6 sheathed brushes were removed from the bronchoscope and shaken into warmed BEGM in a single 50mL sterile tube. Brushes and sheaths were detached into the media and this was immediately transported to the laboratory for counting and culture. The processing team aimed to keep the HBECs at 37°C and into the incubator within 30 minutes of collection. The remaining sheathed brush was placed in a cryo-vial and snap frozen for storage in liquid nitrogen.

2.3.2.2 Bronchoalveolar lavage (BAL)

Sterile normal saline (at room temperature) was instilled into the right upper lobe bronchus in 6 x 20mL aliquots with a 10 second dwell time. BAL was collected in a single plastic bottle and transferred immediately to 50mL polypropylene tubes on ice for transport to the laboratory and further processing.

2.4 Clinical Sample Processing

2.4.1 PBMC Processing

The blood collected in heparinised tubes was diluted 1:1 with Dulbecco's phosphate buffered saline (D-PBS) and then layered over histopaque (Sigma Aldrich, USA). Tubes were centrifuged at 2500rpm, with the brake and accelerator turned off, for 30min. The layers of PBMCs were transferred to a single polypropylene tube and washed 2x with RPMI-1640 supplemented with 10% foetal bovine serum and 12.5mL HEPES buffer (RPMI-P/S) and centrifuged at 1500rpm for 10min. The wash supernatant was discarded and the cell pellet was resuspended in 10mL RPMI-P/S. The cell suspension was diluted 1:10 in 0.1% trypan blue (Invitrogen, UK) for counting and assessment of viability. A Neubauer haemocytometer with coverslip was filled with cell/trypan blue suspension (approx. 5-10 μ L) and viewed under a light microscope. Viable cells were counted and cells per mL calculated.

Cells at a concentration of 2x10⁶/mL were incubated at 37°C (5% CO₂) for 30min, then centrifuged at 1500rpm for 10 min. The supernatant was discarded and cell pellet resuspended in 1mL RPMI+/-P/S. Cells were stimulated with virus, bacteria or TLR ligands described in more detail below (see sections 2.5.2.4, 2.5.3.2 and 2.5.4). Cells were harvested at 8, 24 and 48h. This involved centrifugation for 10min at 1500rpm, removal and saving of the supernatant and lysis of the cell pellet in RLT/2 β ME (RLT buffer [Qiagen] and 1:100 2- β mercaptoethanol) for RNA analysis.

2.4.2 BAL processing

1mL of unfiltered BAL was removed and stored at -80°C, but not used in this study. The remaining BAL fluid was filtered through a 100µm cell strainer (BD Falcon, USA) and then centrifuged at 1500rpm for 10min to form a cell pellet. The supernatant was removed and stored at -80°C in 1.5mL aliquots. The cell pellet was resuspended and washed in 40mL RPMI-P/S and centrifuged again. The supernatant was discarded and cell pellet resuspended in 2mL RPMI-P/S. The cell suspension was diluted 1:10 in 0.1% trypan blue (Invitrogen, UK) for counting and assessment of viability. A Neubauer haemocytometer with coverslip was filled with cell/trypan blue suspension (approx. 5-10µL) and viewed under a light microscope. Viable cells were counted and cells per mL calculated. Cells at 2×10^6 /mL were incubated at 37°C (5% CO₂) for 20min, and processing continued as for PBMCs (see section 2.4.1).

2.4.2.1 BAL Cytospin

Two cytopspins of 20,000 cells were made using BAL cell suspension added to a cytopspin funnel attached to a Shandon cytoslide (Thermo Scientific) and centrifuged at 500rpm for 5 min using the Cytospin 3 system (Shandon, USA). Slides were air dried for at least 2h and stained using a REASTAIN Quick-Diff Kit (Reagent, Finland). After drying, cells were differentially counted, blinded to subject identification and differential cell count was expressed as a percentage.

2.4.3 Bronchial epithelial cell (HBEC) processing

BEGM containing the HBECs, brushes and sheaths were gently vortexed to detach cells. Brushes were passed through a pipette tip to remove adhered HBECs and the pipette tip and sheaths were flushed with BEGM. The BEGM/HBEC mix was then centrifuged at 1150rpm for 6min and the cell pellet was resuspended in 1mL BEGM through a 19G sterile needle to reduce clumping of the cells and made up to 10mL with BEGM. Cell counts were performed by diluting the cell suspension

in 0.1% trypan blue and counted using a Neubauer haemocytometer. Total cell count and ciliated cell count was performed. The cell suspension was placed in a 25cm² flask (Nunc), pre-coated for 4h with collagen mix (see table 2.4) and placed in a humidified incubator at 37°C (5% CO₂). After 48h, to allow maximal adherence of HBECs, cells were washed twice with warmed BEGM and the medium was replaced with 10mL fresh warmed BEGM + 100µL Ultrosor[®]-G (Pall Lifesciences, France). The medium was changed every 2-3 days, adding Ultrosor[®]-G to the BEGM until the HBECs were well established.

Once the HBECs were 80-90% confluent, cells were passaged. Cells were washed with 4mL HEPES buffered saline solution (Lonza, USA) which was then removed. 2mL trypsin-EDTA (Lonza, USA) was added and cells were incubated at 37°C until they detached with flask tapping. 4mL trypsin neutralising solution (Lonza, USA) was added, and cells were centrifuged at 1150rpm for 6min. The supernatant was discarded and the cells were resuspended in 4mL complete BEGM. 2mL cell suspension was added to 2 collagen pre-coated 75cm² flasks containing pre-warmed BEGM and returned to the incubator. The residual cells from the initial flask were washed with BEGM and more BEGM was added for further incubation and culture. Cells were further passaged to P2 and when 80% confluent, were split as described above, counted with a haemocytometer (Neubauer) and seeded onto collagen coated 24 well plates (Nunc) and Lab-Tek chamber slides with covers (Nunc) at a concentration of 0.8x10⁵ cells/mL for further experimentation.

2.5 Cell Culture Methods

2.5.1 Tissue Culture

All tissue culture was performed in a category II hood assigned for use with animal and human samples. All media used is listed in table 2.4.

2.5.1.1 BEAS-2B cell culture

BEAS-2B cells were purchased from ATCC, provided in frozen cryovials. Cells were stored in liquid nitrogen until resuscitated in pre-warmed RPMI complete medium and grown to confluence in a 75cm² tissue culture flask in a humidified incubator at 37°C (5% CO₂). This was subsequently passaged into 175 cm² tissue culture flasks. Cells were washed twice with D-PBS. 5mL trypsin-EDTA diluted 1:10 in D-PBS was added to coat the flask and then discarded. Cells were left for 30s at room temperature and were detached from the flask by tapping. Cells were resuspended in 10mL RPMI complete to deactivate the trypsin-EDTA. The cell suspension was divided between the required number of tissue culture flasks containing pre-warmed RPMI complete. For experiments, cells were plated into 24 well plates at a density of 1.7x10⁵cells/mL.

2.5.1.2 Primary HBEC culture

Primary HBECs were obtained from Lonza (USA). The cells had been obtained from a non-asthmatic, non-smoking, non-alcoholic subject and were provided in a frozen cryovial (source number 7F3143). Cells were stored in liquid nitrogen until resuscitated in pre-warmed BEGM complete medium and grown to confluence in a 25cm² tissue culture flask in a humidified incubator at 37°C (5% CO₂). Cells were passaged as in section 2.4.3. HBECs were plated into 24 well plates at a density of 1.0x10⁵cells/mL.

2.5.1.3 HeLa cell culture

HeLa (Ohio) cells were obtained from ATCC, stored in liquid nitrogen and resuscitated into 175cm² tissue culture flasks in pre-warmed HeLa cell complete medium. Cells were passaged into 175cm² tissue culture flasks by washing twice with D-PBS, adding 2mL trypsin-EDTA (diluted 1:10 in D-PBS), and incubating in a humidified incubator at 37°C (5% CO₂) until the cells detached. Cells were resuspended in 10mL HeLa cell complete medium to deactivate the trypsin-EDTA.

The cell suspension was divided between the required number of tissue culture flasks containing pre-warmed HeLa cell complete medium and incubated at 37°C (5% CO₂).

2.5.2 RV Culture

2mL RV16 and RV1B was expanded to produce 1150mL virus stock by passage through HeLa cell culture. This stock was stored in single use aliquots at -80°C.

Expansion was performed by culturing RV in 175cm² flasks of confluent HeLa cells. Cells were washed twice in 10mL HeLa cell infection medium. 2mL RV stock was added to 8mL HeLa infection medium. This was added to the HeLa cells and was shaken gently at room temperature for 1h, when a further 10mL of medium was added. The flask was incubated at 37°C (5% CO₂). About 24 hours later, when 90% cytopathic effect was observed, the cells were disrupted and contents of the flask were frozen at -80°C. Cells were lysed by 3x freeze/thawing. RV inoculum was produced by centrifuging 4000rpm for 15min at 4°C to pellet cell debris and filtering the supernatant through a 0.2µm filter (Acrodisc). This inoculum was used to infect further flasks of HeLa cells and the process repeated, generating the final volume of 1150mL filtered HeLa cell supernatant containing RV16 or RV1B.

RV was titrated in HeLa cells to estimate virus concentration, and the identity of the final virus stock was confirmed by neutralisation of cytopathological effect by RV16 or RV1B specific antisera. All virus stocks were tested for *Mycoplasma* contamination using commercial reagents (Roche, Switzerland). The potency of the virus was tested in BEAS-2B cells, with IL-6 and IL-8 being measured in the supernatants by ELISA.

2.5.2.1 Virus Titration in HeLa cells

Ohio HeLa cells were cultured, split, resuspended in HeLa cell titration medium, counted and diluted to give a concentration of 0.8×10^5 cells/mL. 150µL of

cells were placed in each well of a flat bottomed 96 well cell culture cluster plate (Costar). To establish virus concentration, virus was serially diluted 1:10 (neat to $1:10^{-7}$) in HeLa cell titration medium with each dilution being placed in a row of 8 wells, leaving 2 wells with cells alone to act as a control. For HBEC experiments to establish viral replication cell supernatants were serially diluted and protocol continued as above. Plates were incubated at 37°C (5% CO₂) for 4 days. Cytopathic effect (CPE) was determined by light microscopy and wells positive for CPE were counted on day 4. Tissue culture infective dose 50% (TCID₅₀)/mL value was determined by scoring the sum of positive wells and using the Spearman Karber formula ($M = x_k + d[0.5 - (1/n)(r)]$, wherein x_k = dose of highest dilution, r = sum of negative responses, d = spacing between dilutions and n = wells per dilution).

2.5.2.2 Filtration of virus stock to produce RV filter control

As a negative control for RV a filtered control was produced for in vitro experiments. 500µL RV (neat for RV16, 1:10 RV1B) was placed in an Amicon Ultra-0.5, Ultracel-30 membrane, 30kDa filter unit (Millipore) and centrifuged at 12000rpm for 8min.

2.5.2.3 Virus infection of HBECs and BEAS2B cells

HBECs (both obtained from study participants and primary HBECs from Lonza, USA) and BEAS-2B cells were cultured in 24 well plates until 80% confluent. Cells were serum starved overnight in BEGM infection+P/S medium (HBECs) or RPMI infection medium (BEAS-2Bs). The medium was removed and 100µL virus (neat RV16, 1:10 RV1B) or filtered virus was added to the cells. The plate was placed on a shaker for 1h at room temperature. The virus was removed and replaced with 0.5mL warmed BEGM infection+P/S medium (HBECs) or RPMI infection medium (BEAS-2Bs). For HBECs, wells being used for virus titrations were washed 3 times in BEGM infection+P/S medium prior to the addition of BEGM infection+P/S medium. Cells were incubated at 37°C (5% CO₂). Cells and supernatants were harvested at 8h, 24h and 48h.

2.5.2.4 Virus infection of PBMCs and BAL cells

PBMCs and BAL cells were processed as in sections 2.4.1 and 2.4.2. 1mL of cells, in sterile polypropylene tubes, at a concentration of 2×10^6 cells/mL was stimulated with 1mL RV (RV16, neat and RV1B, 1:10 dilution) or filtered RV, which was then incubated at 37°C (5% CO₂) for 1h. Tubes were then centrifuged at 1500rpm for 10min, the supernatant discarded and the cell pellet was resuspended in 2mL RPMI+P/S and incubated 37°C (5% CO₂) until harvesting at 8h, 24h and 48h, depending on cell number.

2.5.3 *Streptococcus pneumoniae* (*Spn*) culture

Spn stock (strain D39, an encapsulated human pathogen) was used from a gift of Professor Tracy Hussell, Imperial College, London. Briefly, *Spn* frozen stock was added to Todd Hewitt Broth + 0.05% yeast extract and grown until the OD_{600nm} was 0.4. At this point serial dilutions were made and plated onto blood agar to determine the concentration in colony forming units/mL (cfu/mL). The stock was aliquotted and stored in 10% glycerol at -80°C. Filtered *Spn* using a 0.2µm filter (Acrodisc) was used as a control. *Spn* was tested in BEAS-2B cells, primary HBECs and PBMCs prior to its use in the participants' samples.

2.5.3.1 *Spn* infection of HBECs and BEAS2B cells

Cells were grown to 80% confluence in 24 well plates. Cells were serum starved overnight in BEGM infection-P/S medium (HBECs) or RPMI infection medium (BEAS-2Bs). The medium was removed and 0.5mL *Spn* (diluted to 1×10^4 cfu/mL) or filtered *Spn* was added to the cells. The plate was placed on a shaker for 1h at room temperature. *Spn* was then removed and the cells were washed 3 times with the appropriate infection medium. Cells were incubated at 37°C (5% CO₂). Cells and supernatants were harvested at 8h, 24h and 48h.

2.5.3.2 *Spn* infection of PBMCs and BAL cells

PBMCs and BAL cells were processed as in sections 2.4.1 and 2.4.2. 1mL of cells, in sterile polypropylene tubes, at a concentration of 2×10^6 cells/mL was stimulated with 1mL *Spn* (1×10^4 cfu/mL, diluted in RPMI-P/S) or filtered *Spn*, which was then incubated at 37°C (5% CO₂) for 1h. Tubes were then centrifuged at 1500rpm for 10min, the supernatant discarded and the cell pellet was resuspended in 2mL RPMI-P/S and incubated 37°C (5% CO₂) until harvesting at 8h, 24h and 48h, depending on cell number.

2.5.4 TLR ligands

2.5.4.1 Lipoteichoic Acid (LTA)

LTA is found in the cell wall of most gram-positive bacteria and is linked to the cytoplasmic membrane and stimulates TLR2. LTA from *Staphylococcus aureus* was resuspended to a concentration of 1mg/mL in endotoxin-free water (InvivoGen) as per the manufacturer's instructions, aliquotted and stored at -20°C.

2.5.4.2 Lipopolysaccharide (LPS)

LPS is a toxic component of the outer leaflet of the outer membrane of gram-negative bacteria which stimulates TLR4. It was resuspended to a concentration of 1mg/mL in nuclease free water (Promega, USA), aliquotted and stored at -80°C. LPS was used at a concentration of 10µg/mL in all cell types. This dilution was chosen from protocols already established in the department (A. Sykes, unpublished data).

2.5.4.3 CpG-C ODN

Synthetic oligodeoxynucleotides (ODN) containing the CpG dinucleotides have been used to mimic bacterial CpG-DNA. These are ligands for TLR9. The sequence for CpG-C-ODN is (5'-3'): TCGTCGTTTTCGGCGCGCGCCG (the sequence underlined is a signature CpG sequence). CpG-C was diluted to a 3μM solution in the appropriate medium for the different cell types and was stored at -80°C until further use. This dilution was chosen from protocols already established in the department (A. Sykes, unpublished data).

2.5.4.4 Flagellin (FLA)

Flagellin is the major component of the bacterial flagellar filament, which confers motility on a wide range of bacterial species. It is recognised by TLR5. Flagellin from *Salmonella typhimurium* was resuspended to a concentration of 100μg/mL in endotoxin-free water (InvivoGen) as per the manufacturer's instructions, aliquotted and stored at -20°C.

2.5.4.5 Pam₃CSK₄ (PAM)

Pam₃CysSerLys₄ (Pam₃CSK₄) is a synthetic tripalmitoylated lipopeptide that mimics the acylated amino terminus of bacterial lipoproteins. Bacterial lipoproteins are found in both gram-positive and gram-negative bacteria. Pam₃CSK₄ is recognised by TLR2 which cooperates with TLR1. Pam₃CSK₄ was resuspended to a concentration of 1mg/mL in endotoxin-free water (InvivoGen) as per the manufacturer's instructions, aliquotted and stored at -20°C.

2.5.4.6 Synthetic Lipoprotein (FSL)

Pam₂CGDPKHPKSF (FSL-1) is a synthetic lipoprotein which contains a lipolyated N-terminal diacylated cysteine residue which is recognised by TLR2 and

TLR6. FSL-1 was resuspended to a concentration of 100µg/mL in endotoxin-free water (InvivoGen) as per the manufacturer's instructions, aliquotted and stored at -20°C.

2.5.4.7 Peptidoglycan (PGN)

Peptidoglycan is a surface component of gram-positive bacteria and is recognised by TLR2. There is evidence that peptidoglycan is recognised by other pattern recognition receptors such as NOD1 and NOD2. Peptidoglycan from *Staphylococcus aureus* was resuspended to a concentration of 200µg/mL in endotoxin-free water (InvivoGen) as per the manufacturer's instructions, aliquotted and stored at -20°C.

2.5.4.8 Resiquimod (R848)

R848 is an imidazoquinoline antiviral drug which stimulates TLR7/TLR8. It was diluted to a 1mM solution in the appropriate medium for the different cell types and was stored at -20°C until further use. This dilution was chosen from protocols already established in the department (A. Sykes, unpublished data).

2.5.4.9 Polyinosinic:polycytidylic Acid (poly(IC))

Poly(IC) is a synthetic, mismatched double stranded RNA with one strand of polyinosinic acid and one of polycytidylic acid. It is a TLR3 agonist. It was resuspended to a concentration of 1mg/mL in nuclease free water (Promega, USA) as per the manufacturer's protocol. It was aliquotted and stored at -80°C until further use. Poly(IC) was used at a concentration of 10µg/mL in HBECs and 100µg/mL in PBMCs. This dilution was chosen from protocols already established in the department (A. Sykes, unpublished data).

2.6 Molecular Biology Techniques

2.6.1 RNA extraction and cDNA synthesis

RNA was extracted from lysed cells using the RNEasy mini kits (Qiagen, UK) according to the manufacturer's protocol. Lysates were thawed, mixed with 350µL 70% ethanol and placed in the RNEasy spin column. This was spun at 10,000rpm for 1min. The flow through was discarded, 350µL buffer RW1 was added to the column and this was again spun at 10,000rpm for 1min. 80µL DNase 1 (diluted 1:8 with buffer RDD, Qiagen, UK) was applied directly to the column filter and incubated for 15min at room temperature. 350µL buffer RW1 was added to wash off the DNase 1 and the column was spun at 10,000rpm for 1min. The collection tubes were replaced and the spin filter was washed twice with 500µL buffer RPE, spinning at 10,000rpm once for 1min and once for 2min, followed by a dry spin for 1min to remove excess liquid. RNA was eluted from the column filter with 39µL nuclease free water (Qiagen, UK).

cDNA synthesis was performed using the Omniscript reverse transcriptase kit (Qiagen, UK) and random hexamer primers (Promega, USA) as per manufacturer's protocol. Table 2.12 lists the components of the kit and volumes required for each reaction. Samples were mixed and incubated at 37°C for 1.5h and stored at -80°C until further use.

Table 2.12 Components of the reaction for cDNA synthesis

Component	Volume per reaction (µL)
10 Buffer RT	6.0
dNTP mix (5mM each dNTP)	6.0
Random hexamer primers	3.0
RNase free water	3.0
Omniscript RT	3.0
Template RNA	39.0
Total volume	60.0

2.6.2 Primers and probes for Realtime RT-PCR

Primers and probes were all handled in a designated nuclease-free flow hood. All primers and probes were resuspended in sterile TE buffer to a stock concentration of 100µM and stored at -20°C. Working stocks of primers and probes were prepared by diluting to 5µM in nuclease-free water (Promega, USA, Madison, WI, USA) and stored at -20°C. Sequences of primers and probes and their optimal concentrations (nM) are listed in table 2.6. All primer and probe sequences were already established in the lab. Briefly, genomic and mRNA sequences of the gene of interest were found using NCBI PubMed and primers and probes overlapping an exon-exon boundary were selected using Primer Express v3.0. Optimal forward/reverse primer concentrations were already established.

2.6.3 Realtime RT-PCR

TaqMan™ RT-PCR (Applied Biosystems Fast 7500 machine) and specific primer and probe sequences were used to detect mRNA expression of IL-6, CXCL8/IL-8, RV, IFN-λ1, IFN-λ2/3, IFN-β and IFN-α(A2). A fast 96-well optical PCR plate (Applied Biosystems) was used. In each well 6.25µL 2x QuantiTect probe PCR Master Mix, forward and reverse primers (at the optimised nM ratio, Table 2.6), 175nM probe and 1µL cDNA was used. For 18S rRNA measurements, the cDNA was diluted 1/100 in nuclease-free water (Promega, USA). Non template controls (RNase free water in place of cDNA) were included. The PCR plate was sealed with a Microamp optical adhesive cover (Applied Biosystems) and was analysed with the Applied Biosystems Fast 7500 machine. The thermal cycle conditions consisted of 50°C for 2min, 95°C for 10min, then 45 cycles of 95°C for 15s and 60°C for 15s. Data was analysed using version 1.4.0 ABI Prism 7500 SDS software. Threshold cycle numbers from samples were compared to standard curves already established in the lab. Data was normalised to 18S rRNA and was presented as normalised copy number per µL of cDNA.

2.7 Protein analysis techniques

2.7.1 Enzyme-Linked ImmunoSorbent Assay (ELISA) to test RV induced production of IL-6 and IL-8 in BEAS2B cells, Primary HBECs (Lonza) and PBMCs

IL-6 and CXCL8/IL-8 production was measured by ELISA to test stocks of RV16 and RV1B in BEAS-2B cells, and to test a concentration response of serial dilutions of RV16 and RV1B in primary HBECs (Lonza) and PBMCs in order to establish the optimal concentration of RV for use in the remaining study.

Paired antibodies to IL-6 and CXCL8/IL-8 (R&D systems, USA) were used. 96 well microtitre plates (Nunc) were coated overnight with 100µL of anti-human IL-6 or CXCL8/IL-8 coating antibody (4.0µg/mL, diluted in D-PBS). Plates were washed 3 times with ELISA wash buffer, blocked with blocking buffer (1% BSA/0.1% Tween 20/PBS) for 2h, followed by 3 washes with ELISA wash buffer. IL-6 or CXCL8/IL-8 recombinant protein standard was serially diluted from 1000pg/mL to 3.9pg/mL and 100µL of standard or sample was added to the plate and incubated at room temperature for 1h. Plates were washed 3 times with wash buffer and 100µL of 25ng/mL IL-6 or CXCL8/IL-8 biotinylated detection antibody was applied and incubated at room temperature for 1h. After 3 further washes 100µL Streptavidin-HRP (diluted 1:5000 in diluent buffer) was added, incubated for a further 15min in the dark with shaking and washed 3 times. 100µL TMB substrate was added to the wells and incubated in the dark until colour began to develop in background wells. The reaction was stopped with 50µL 2M H₂SO₄ and the absorbance was measured at 450nm on a microplate reader. The absorbance readings were compared to those of the linear portion of the standard curve to give concentrations in pg/mL, using the Softmax Pro v3.1.2 software (Molecular Devices Corporation, USA).

2.7.2 Development of a Meso Scale Discovery (MSD) prototype to measure IL-29

MULTI-SPOT[®] 96 4 spot plates were pre-coated with human IL-29 capture antibody (DY1598, R&D systems, USA) by MSD. In order to test the IL-29 MSD plates samples of supernatants from polarised monocyte-derived macrophages stimulated with differing MOI of RV were acquired from a colleague in the department. Comparisons were made to data from these samples analysed with ELISA using the same human IL-29 antibody kit (DY1598, R&D systems, USA; 35.8% cross reactivity with IL-28A, no cross-reactivity with IFN- α or IFN- β). Reverse pipetting was used throughout the procedure to ensure no bubbles would interfere with the plate reading. Half the plate was blocked with 1% BSA (in D-PBS), 150 μ L/well, for 1h at room temperature. The plate was washed 3 times with 150 μ L/well 0.05% Tween-20/PBS. IL-29 standard was serially diluted 1:4 from 20,000pg/mL to 0pg/mL in Diluent 1 (MSD). 25 μ L of sample, both undiluted and a 1:10 dilution (in Diluent 1) were added to the blocked wells. Only diluted samples were added to the unblocked wells. 25 μ L of standards, in duplicate, were added to both blocked and unblocked wells. The plate was sealed with an adhesive plate seal and incubated for 2h with vigorous shaking (600rpm) at room temperature. The plate was washed 3 times with 0.05% Tween-20/PBS. 25 μ L IL-29 detection antibody (0.5 μ g/mL, R&D systems, USA)/SULFO-TAG[™] labelled streptavidin (1 μ g/mL, MSD) was added to each well. The plate was sealed and incubated for a further 1.5h with vigorous shaking at room temperature. The plate was washed 3 times with 0.05% Tween-20/PBS. 150 μ L of 2x Read Buffer T (MSD) was added to each well and the plate was analysed immediately on the SECTOR[®] Imager 2400. Data was analysed using the Discovery Workbench[®] 3.0 Software (MSD; the basis of which is the signal detected on the imager is compared to the standard curve of known cytokine concentration to give a sample concentration).

2.7.3 Meso Scale Discovery (MSD) to measure proinflammatory cytokines IL-1 β , IL-6, IL-8 and TNF- α , and interferons IFN- α , IFN- β and IL-29 (IFN- λ 1) in HBECs, BAL macrophages and PBMCs

MSD for proinflammatory cytokines, IFN- α 2a and IFN- β was performed according to the manufacturer's protocols. An MSD prototype assay for IL-29 was manufactured by MSD and tested as above (see section 2.7.2).

2.7.3.1 Proinflammatory cytokines (IL-1 β , IL-6, CXCL8/IL-8, TNF- α)

IL-1 β , IL-6, CXCL8/IL-8 and TNF- α were quantified with an MSD human proinflammatory-4 II Tissue Culture Kit (K11025B-4, MSD). The manufacturer's protocol was followed. Serial 1:4 dilutions of human ProInflammatory-4 II calibrator stock were made in Diluent 1 (MSD) starting at 10,000pg/mL to 0pg/mL. Samples were diluted 1:10 in Diluent 1 (MSD). 25 μ L/well of calibrator (in duplicate) or sample was added. The plate was sealed and incubated for 2h with vigorous shaking at room temperature. The plate was washed 3 times with 0.05% Tween-20/PBS. 25 μ L of human proinflammatory-4 II detection antibody (diluted to a final concentration of 1x in Diluent 100, MSD) was added to each well; the plate was again sealed and incubated for 1.5h with vigorous shaking at room temperature. The plate was washed 3 times with 0.05% Tween-20/PBS. 150 μ L of 2x Read Buffer T (MSD) was added to each well and the plate was analysed immediately on the SECTOR[®] Imager 2400. Data was analysed using the Discovery Workbench[®] 3.0 Software (MSD). The average sensitivities were 1.4pg/mL (IL-1 β), 1.0pg/mL (IL-6), 1.0pg/mL (CXCL8/IL-8), 3.3pg/mL (TNF- α).

2.7.3.2 IFN- α 2a

IFN- α 2a was quantified with an MSD human IFN- α 2a tissue culture kit (K151ACB-4, MSD). The manufacturer's protocol was followed. Serial 1:4 dilutions of IFN- α 2a calibrator stock were made in Diluent 1 (MSD) starting at 10,000pg/mL down to 0pg/mL. 25 μ L/well of calibrator (in duplicate) or sample was added. The

plate was sealed and incubated for 2h with vigorous shaking at room temperature. The plate was washed 3 times with 0.05% Tween-20/PBS. 25µL of anti-hIFN-α2a antibody (diluted to a final concentration of 1x in Diluent 100, MSD) was added to each well; the plate was again sealed and incubated for 1.5h with vigorous shaking at room temperature. The plate was washed 3 times with 0.05% Tween-20/PBS. 150µL of 2x Read Buffer T (MSD) was added to each well and the plate was analysed immediately on the SECTOR® Imager 2400. Data was analysed using the Discovery Workbench® 3.0 Software (MSD). The average sensitivity was 1.2pg/mL.

2.7.3.3 IFN-β

IFN-β was quantified with an MSD human IFN-β tissue culture kit (K111ADB-4, MSD). The manufacturer's protocol was followed. The plate was washed 3 times with PBS. 25µL anti-hIFN-β biotinylated capture antibody (diluted to a final concentration of 1x in Diluent 100, MSD) was added to each well; the plate was sealed and incubated for 1h with vigorous shaking at room temperature, followed by 3 washes with PBS. Serial 1:4 dilutions of IFN-β calibrator stock were made in Diluent 1 (MSD) starting at 100,000pg/mL down to 0pg/mL. 50µL/well of calibrator (in duplicate) or sample was added; 20µL of anti-hIFN-β antibody (diluted to a final concentration of 1x in Diluent 100, MSD) was added to each well; the plate sealed and incubated for 2h with vigorous shaking at room temperature. The plate was washed 3 times with 0.05% Tween-20/PBS. 150µL of 1x Read Buffer T (MSD) was added to each well and the plate was analysed immediately on the SECTOR® Imager 2400. Data was analysed using the Discovery Workbench® 3.0 Software (MSD). The average sensitivity was 8.6pg/mL.

2.7.3.4 IL-29 (IFN-λ1)

IL-29 was quantified using the MSD prototype human IL-29 MULTI-SPOT® 96 4 spot plates as described in section 2.7.2. Plates were not blocked prior to the addition of standard/sample and undiluted standard/sample was used. The average sensitivity was 20.2pg/mL.

2.8 Statistical analysis

Statistical analysis was carried out using non-parametric tests, at a 95% confidence interval with the Kruskal-Wallis test (one way ANOVA equivalent); if data were significant (p value < 0.05), differences were further analysed using Dunn's multiple comparison test. Statistics were analysed using GraphPad Prism software, v4.0 (USA).

Chapter 3: RV infection of BAL cells, HBECs and PBMCs, and TLR3/7/8 stimulation of HBECs and PBMCs

3.1 Introduction

RVs are responsible for a significant proportion of viral-induced asthma exacerbations. AA have increased susceptibility to RV infection, with longer lasting and more severe symptoms compared to non-asthmatics ³⁰⁶. The bronchial epithelium has an important role in the innate immune system's response to viral infection, expressing the cell surface receptors for RV, ICAM-1 and LDLR ^{56, 57}. RV results in the production of type I and III IFN and proinflammatory cytokines from the bronchial epithelium. Exposure of BAL cells and PBMCs to RV results in induction of type I IFNs ^{61, 80}. IFN production following RV infection has been reported to be defective in AA. HBECs from AA infected with RV have defective IFN- β production ⁶⁰ and HBECs and BAL cells infected with RV have defective IFN- λ production ⁶¹. The deficient IFN responses may impair an infected cell's ability to undergo apoptosis and thereby allow increased viral replication ⁶⁰. Addition of exogenous IFN- β to AA cells restores anti-viral responses to levels seen in cells from non-asthmatics ⁶⁰. A more recent study has identified deficient type I IFN production in BAL cells from AA and this deficiency may be related to asthma severity ²⁰⁸. PBMCs from adult and child AA have been reported as having deficient IFN- α 2 production following RSV and NDV infection ^{82, 206}.

Asthmatics are classed as atopic or non-atopic. The role of atopy in deficient IFN production is unknown, and whether it is the asthmatic or atopic status which results in this deficiency is unclear. Observations have been made identifying IFN- α deficiency in cases of atopic disease ^{234, 307}. A recent study reported deficient IFN- β and - λ responses to rhinovirus infection in BECs in asthmatic children irrespective of their atopic status and surprisingly, in atopic children without asthma ²⁰⁹. However, these atopic children without asthma had similar degrees of IL-4 staining and epithelial damage in their bronchial biopsies as did the children with asthma, thus the roles of atopy and asthma in IFN deficiency remain uncertain. To date whether adult NAA and/or ANA have deficient IFN responses has not been identified.

TLR3, TLR7 and TLR8 are receptors linked to viral cell signalling. TLR3 recognises dsRNA ²⁶⁰, is important in the recognition of RV ³⁰⁸ and is a potent inducer of type I IFNs ²⁶⁰. It is possible that the defective IFN response to RV in AA involves TLR3, and whether this extends to NAA or ANA is of interest. A polymorphism of TLR3 has been identified as a susceptibility gene for asthma and atopy ³⁰⁹. TLR7/8 recognise viral ssRNA ³¹⁰. These TLRs are expressed on the endolysosomes of HBECs, dendritic cells and monocytes. TLR7 function has been observed to be reduced in adolescents with asthma, with a significant negative correlation between total serum IgE and TLR7 stimulated CXCL10/IL-10 production ³¹¹. Polymorphisms of TLR7 and TLR8 have been linked to asthma and atopic diseases ³¹² and administration of TLR7/8 agonists has been reported to suppress inflammation and airway remodelling in rodent models of allergic airways disease, postulating the use of TLR7/8 agonists as a treatment modality in asthma ^{313, 314}.

3.1.1 Hypotheses

- RV infection of BAL cells, HBECs and PBMCs will result in deficient type I and III IFN production in AA and NAA, compared to NANA and ANA.
- Stimulation of TLR3, TLR7 and TLR8 will induce type I and III IFNs and proinflammatory cytokines in HBECs and PBMCs and will result in deficient type I and III IFN production in AA and NAA, compared to NANA and ANA.

3.1.2 Aims

- To confirm or refute previous reports of deficient RV induced type I and III IFN production in HBECs and PBMCs from a different group of AA and to determine whether similar deficiencies are seen in NAA and not in NANA and ANA.
- To confirm or refute previous reports of deficient RV induced type I and III IFN production in BAL cells from AA and to determine whether similar deficiencies are seen in NAA and not in NANA and ANA.

- To characterise the type I and III IFN and proinflammatory cytokine response to TLR3, TLR7 and TLR8 stimulation in HBECs and PBMCs (only HBECs and PBMCs were used in experiments with the TLR ligands due to the limited number of BAL cell samples being collected from the volunteers).
- To determine whether this response is deficient in AA and NAA compared to NANA and ANA.

3.2 Results

3.2.1 Preliminary testing of RV

In preparation for the main study, a stock of RV16 and RV1B was made and tested in BEAS-2B cells. IL-6 and CXCL8/IL-8 protein expression was examined using ELISA. Compared to medium- and filtered virus-treated BEAS-2B cells, RV16 induced expression of IL-6 and CXCL8/IL-8 (figure 3.1 A and B). Likewise, RV1B induced expression of IL-6 and CXCL8/IL-8 (figure 3.1 C and D). UV-inactivation of virus appeared incomplete as CXCL8/IL-8 was comparable to live virus, while filtered virus appeared an effective means of inactivation. Filtered virus was therefore used as control virus for subsequent experiments.

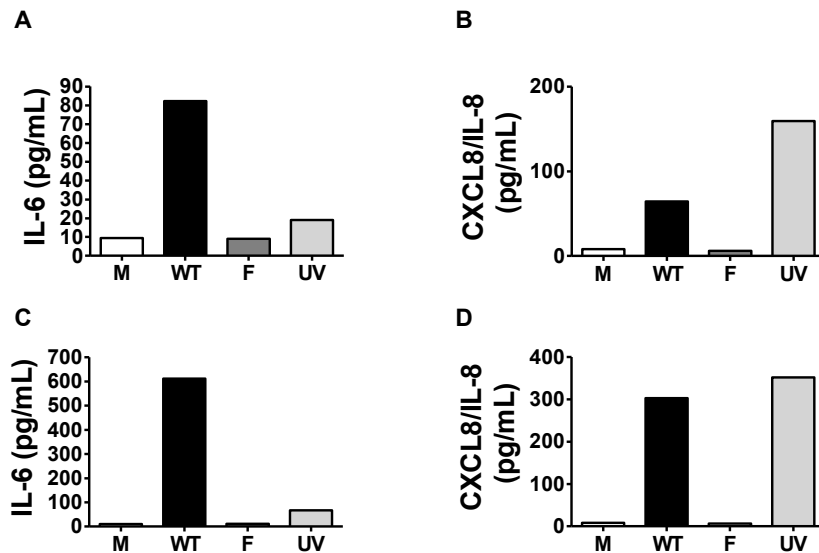


Figure 3.1. Testing the stock rhinovirus. IL-6 and CXCL8/IL-8 production at 24h in BEAS-2B cells stimulated with stock RV16 (A and B) and RV1B (C and D) (M = medium, WT = wild type, F = filtered virus, UV = UV inactivated virus) (N=1)

In order to assess the optimal concentration of RV for use in *in vitro* studies, a concentration response study was performed in PBMCs and primary HBECs (figures 3.2 and 3.3). Cells were stimulated with varying concentrations of RV16 and RV1B (wild type, 1:2, 1:5 and 1:10). At 24h the supernatants were taken and analysed by ELISA for IL-6 and CXCL8/IL-8 protein (figures 3.2 and 3.3). In PBMCs the optimal concentration for RV16 was a 1:2 concentration (Figure 3.2 A and B) and for RV1B was a 1:10 concentration (figure 3.2 C and D). In primary HBECs, wild type RV16

(figure 3.3 A and B) and a 1:10 concentration of RV1B (figure 3.3 C and D) were optimal. For all subsequent experiments wild type RV16 and 1:10 RV1B was used.

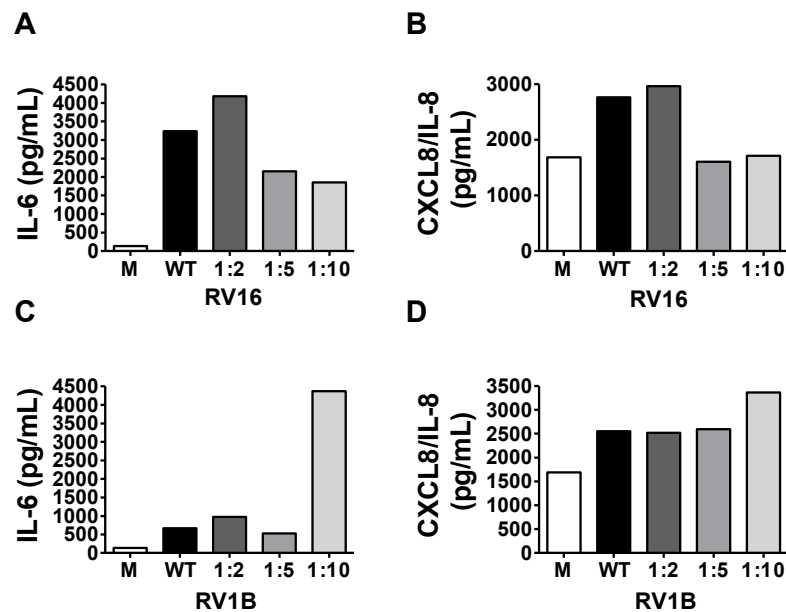


Figure 3.2. IL-6 and CXCL8/IL-8 induction at 24h in PBMCs from NANA stimulated with varying dilutions of RV16 (A and B) and RV1B (C and D) (M = media, WT = wild type) (N=1)

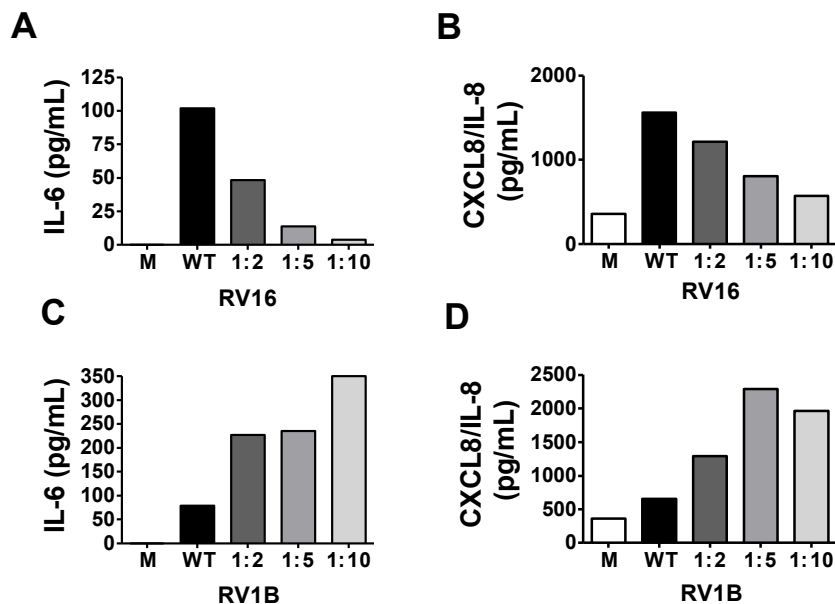


Figure 3.3. IL-6 and CXCL8/IL-8 induction at 24h in primary HBECs (Lonza) stimulated with varying dilutions of RV16 (A and B) and RV1B (C and D) (M = media, WT = wild type) (N=1)

3.2.2 Testing the IFN- λ 1/IL-29 MSD prototype

A prototype IFN- λ 1/IL-29 MSD was developed using R&D IFN λ 1/IL-29 duoset antibodies and used according to the manufacturer's instructions. The efficacy of the MSD was analysed and compared to ELISA. IFN- λ 1/IL-29 protein was analysed from supernatants from polarised monocyte-derived macrophages stimulated with varying MOI of RV16 at 8, 24h and 48h post stimulation (courtesy of A. Nikonova). The MSD prototype was compared to standard ELISA using blocked and unblocked MSD plates, with diluted and undiluted samples. Figure 3.4 demonstrates IFN- λ 1/IL-29 production at 8h with RV16 MOI 2 (figure 3.4 A) and 24 and 48h with RV16 MOI 0.5 and 2 (figure 3.4.B and C). MSD and ELISA did not produce equivalent concentrations of IFN- λ 1/IL-29, but the pattern of expression was similar. No marked differences were observed between the use of blocked or unblocked plates or using diluted or undiluted samples.

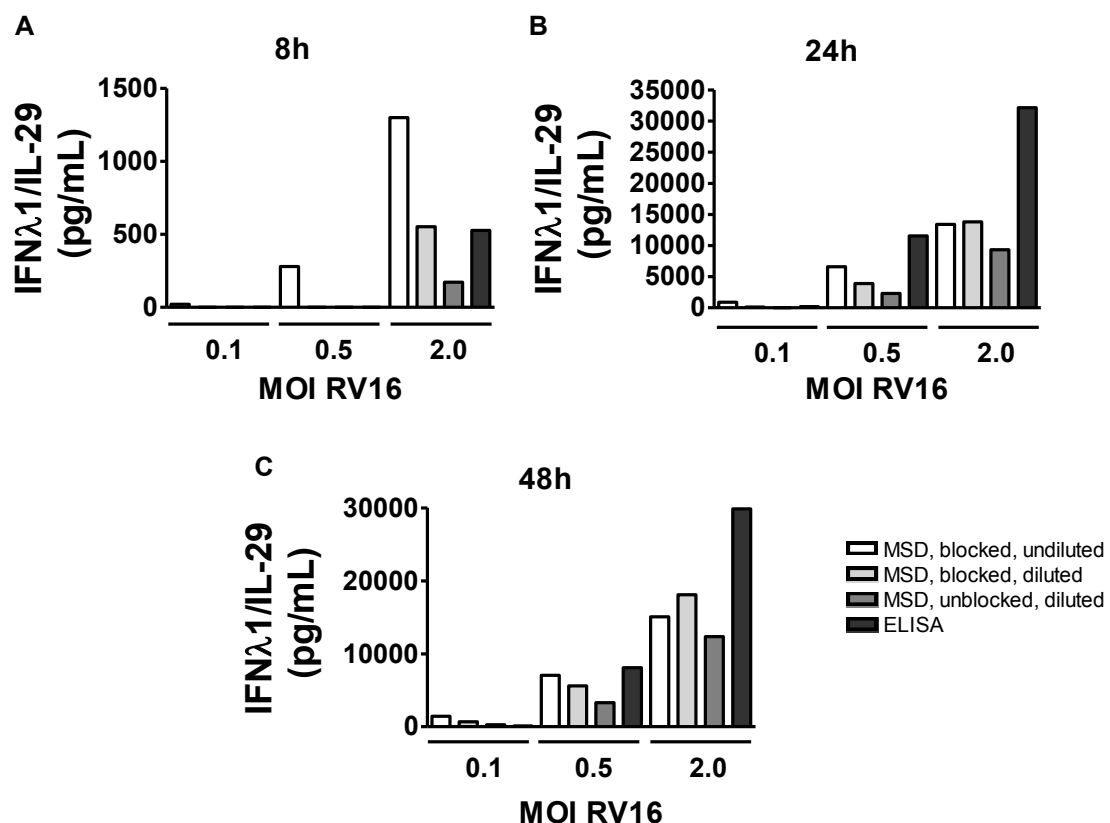


Figure 3.4. Comparing IFN- λ 1/IL-29 MSD prototype to ELISA. IFN- λ 1/IL-29 protein production was assessed in polarised monocyte-derived macrophages (MDMs) stimulated with differing MOI of RV16 at 8h (A), 24h (B) and 48h (C). Comparisons were made between various set ups of MSD and standard ELISA used within the department (N=1).

3.2.3 Clinical characteristics of all participants

A total of 41 subjects (12 non-atopic non-asthmatics, 13 atopic non-asthmatics, 4 non-atopic asthmatics and 12 atopic asthmatics) were screened and samples obtained. 23 other volunteers were screened but either withdrew from the study or were excluded. Table 3.1 illustrates the numbers of samples obtained from the participants. Recruitment of non-atopic asthmatics proved to be extremely difficult so the target N=12 was not reached. HBECs were prone to contamination, at various stages along the culture pathway, hence the reduced numbers of HBEC samples, particularly in NAA. In 3 participants (2 AA, 1 ANA) whose HBECs became contaminated a repeat bronchoscopy was performed to gain more samples (days in between bronchoscopies - 27, 93 and 150). On these second bronchoscopies only bronchial brushes for HBECs were performed, with no BAL or PBMC sampling. Of the 2 AA subjects who had a repeat bronchoscopy, 1 had an ACQ = 0, normal spirometry and PC₂₀ = 4 (very mild disease), the other had ACQ = 1.3, reduced spirometry and PC₂₀ = 0.24 (mild disease).

Table 3.1. Samples obtained from participants

	NANA	ANA	NAA	AA
Target sample number	12	12	12	12
BAL samples obtained and analysed	11	13	4	12
PBMC samples obtained and analysed	11	13	4	12
HBEC samples obtained and analysed	12	12	2	12

The clinical characteristics of the participants are demonstrated in table 3.2, with significant differences being highlighted in figure 3.5.

Table 3.2. Clinical characteristics of all participants. Values are expressed as median (IQR) except sex, treatments and atopic symptoms which are expressed as a percentage of the total in the individual groups. ACQ=asthma control questionnaire score, SABA=short acting β_2 agonists, ICS=inhaled corticosteroids, LABA=long acting β_2 agonists, SPT=number of wheals on skin prick testing. N = NANA-12, ANA-13, NAA-4, AA-12. For p values <0.05 see figure 3.5 for differences between groups.

Clinical characteristic	NANA	ANA	NAA	AA	p value
Sex	25% M 75% F	69.2% M 30.8% F	25% M 75% F	75% M 25% F	
Age	25.5 (19.5-29.5)	27.0 (24.5-32.0)	37.0 (28.0-44.5)	29.5 (28.0-34.0)	0.1675
ACQ	0	0	0.75 (0.33-1.08)	0.917 (0.417-1.67)	<0.001
SABA alone	0	0	1 (25%)	3 (25%)	
ICS	0	0	3 (75%)	9 (75%)	
ICS and LABA	0	0	1 (25%)	3 (25%)	
Exacerbations/year	0	0	0.5 (0.0-1.0)	0.0 (0.0-1.0)	0.0113
PEF (L/min)	490.5 (440.5-561.0)	586.0 (484.0-646.5)	518.5 (449.0-594.5)	537.5 (443.5-585.0)	0.3907
PEF % predicted	107.5 (97.5-112.5)	102.0 (91.5-112.0)	107.5 (106.0-130.0)	94.50 (89.5-99.0)	0.0869
FEV ₁ (L)	3.770 (3.495-4.410)	4.310 (3.715-4.840)	3.195 (3.115-3.660)	3.860 (3.170-4.290)	0.0739
FEV ₁ % predicted	106.5 (102.0-112.5)	103.0 (99.5-109.5)	106.5 (101.5-108.5)	93.50 (85.0-103.5)	0.0205
FVC (L)	4.250 (3.860-4.850)	4.750 (4.445-5.320)	3.815 (3.585-4.400)	5.065 (3.870-5.405)	0.1496
FVC % predicted	104.5 (96.5-110.0)	102.0 (92.5-110.5)	107.0 (104.5-112.0)	99.00 (93.0-105.5)	0.1764
FEV ₁ /FVC ratio	90.85 (87.0-92.5)	88.60 (83.90-91.65)	82.35 (81.55-88.95)	81.80 (74.20-84.05)	0.0044
PC ₂₀ (mg/mL)	≥16 (16-16)	≥16 (16-16)	4.0 (2.12-6.00)	3.0 (0.74-4.00)	<0.001
IgE (units/mL)	8.5(5.0-15.5)	90.0 (39.5-213.5)	6.0(3.5-14.5)	149.0 (44.5-235.5)	<0.001
SPT (number)	0 (0-0)	2.0 (1.5-3.5)	0 (0-0)	4.0 (2.5-4.5)	<0.001
Atopic symptoms	100% N	46.2% Y 53.8% N	100% N	91.7% Y 8.3% N	
% eosinophils in BAL	0 (0-0.6)	0.3 (0-0.55)	0 (0-0.225)	0 (0-1.875)	0.4554
% neutrophils in BAL	0.6 (0.4-2.4)	0.5 (0.15-1.95)	1.11 (0.38-2.645)	1.45 (1-2.475)	0.3541

There were no significant differences between all groups for age, sex, PEF, % predicted PEF, absolute FEV₁ and absolute and % predicted FVC. Expected significant differences were observed in ACQ score between AA and NANA and AA and ANA (p<0.001), but no difference between AA and NAA (figure 3.5 A). Expected differences were observed in exacerbation rate in the 4 groups as a whole (p=0.013),

but no differences were observed between individual groups (figure 3.5 B). A statistically significant difference in % predicted FEV₁ was observed between the NANA and AA groups (p<0.05). No differences were observed between the other groups (figure 3.5 C). A statistically significant difference in FEV₁/FVC ratio was observed between the NANA and AA groups (p<0.01). No differences were observed between the other groups (figure 3.5 D). The mean ratio of 79.94% in AA does not indicate an obstructive spirometry (FEV₁/FVC ratio <70%). Expected differences in PC₂₀ were observed between non-asthmatics and asthmatics, with no significant difference between AA and NAA (figure 3.5 E). Expected significant differences were observed in total IgE and number of wheals on skin prick tests between atopics and non-atopics (figure 3.5 F and G) with a significant difference in the number of wheals on skin prick tests observed between AA and ANA (p<0.01).

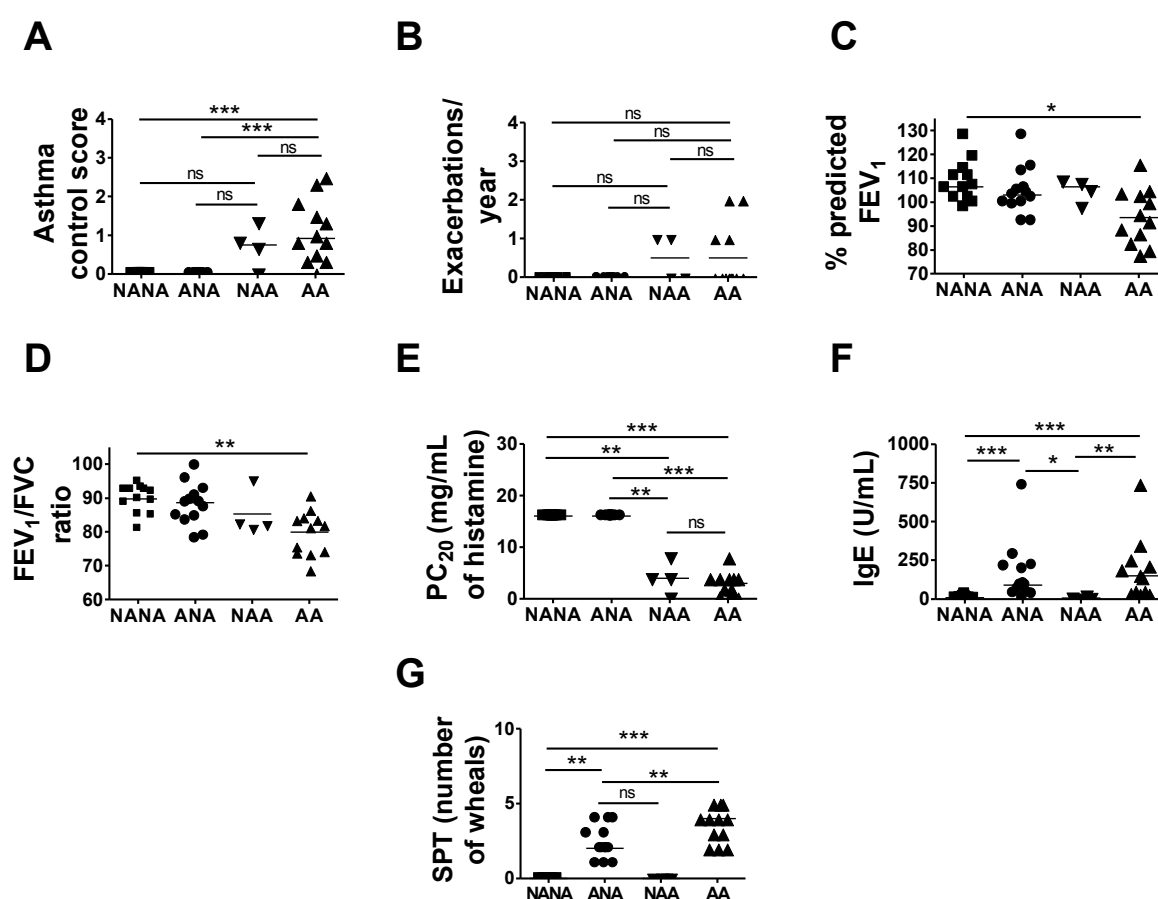


Figure 3.5. Clinical characteristics of participants with statistically significant differences between groups as a whole. Asthma control score (A), number of exacerbations per year (B), % predicted FEV₁ (C), FEV₁/FVC ratio (D), PC₂₀ (E), serum total IgE (F) and number of positive wheals on skin prick tests (G); * = p<0.05, ** = p<0.01, *** = p<0.001, ns = non-significant.

3.2.4 BAL cytopsin composition in NANA, ANA, NAA and AA

To assess which cells were obtained in BAL fluid cytopsin were performed on each participant. Figure 3.6 illustrates BAL cell composition. There was no significant difference in total cell number between the 4 groups, though a non-significant possible reduction in cell number was observed in NAA (figure 3.6 E). BAL cells were predominantly macrophages (~95%) There were no significant differences in any particular cell type between groups.

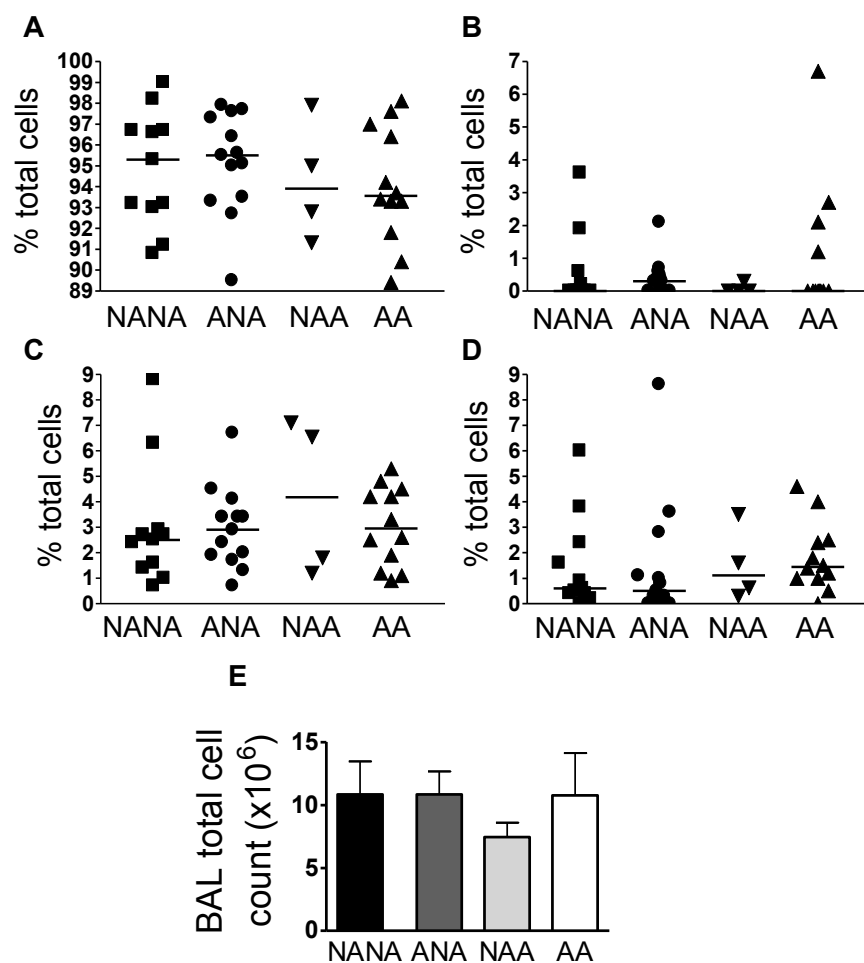


Figure 3.6. BAL cytopsin (A, B, C and D). Data presented as a % of total cell count. Macrophages (A), eosinophils (B), lymphocytes (C), polymorphonuclear cells (D). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA. Total BAL cell count (E). No differences were observed between all cell types in all groups. Within each cell type no significant differences were observed between groups. A non-significant reduction in total cell count in NAA was observed (E). No differences in total cell count were observed between other groups.

3.2.5 RV infection of BAL cells: induction of type I and III IFN and proinflammatory cytokine mRNA and protein

To assess whether the reported deficiency in RV induced IFN induction in BAL cells from atopic asthmatic subjects^{61, 208} was observed in this newly recruited group of atopic asthmatic subjects and whether there is a difference between atopic and non-atopic asthmatic and non-asthmatic groups, RV induced IFN- α , IFN- β and IFN- λ mRNA and protein production by BAL cells was measured at 8h. The aim was to measure at 24h also, but there were insufficient cells obtained at bronchoscopy for this.

The primer and probe sets for IFN- α detect IFN- α subtypes 1, 6 and 13, named IFN- α (A2). As the primer and probe pairs span multiple subtypes mRNA copy number could not be accurately determined, so data was presented as fold induction over medium. Significant induction of IFN- α mRNA was observed in NANA ($p<0.001$) and AA ($p<0.01$) with RV16. Non-significant increases were observed in ANA and NAA (figure 3.7 A). There were no significant differences between groups. With RV1B a 10-fold increase in IFN- α mRNA was observed in NANA but this failed to reach statistical significance. Induction was not observed in other groups and there were no statistical differences between the 4 groups (figure 3.7 B).

IFN- β mRNA was significantly induced by RV16 in NANA and AA ($p<0.001$). Increases were observed in ANA and NAA but these failed to reach statistical significance (figure 3.7 C). Small increases were observed in all groups with RV1B but these were not statistically significant (figure 3.7 D). No differences were observed between groups with either virus.

IFN- λ 2,3/IL-28 mRNA was significantly induced by both viruses in NANA ($p<0.01$) and AA ($p<0.001$). Induction was observed in ANA with RV16 ($p<0.05$) and a non-significant increase was observed with RV1B. Clear induction was observed with both viruses in NAA but these failed to reach statistical significance. No differences were observed between groups (figure 3.7 E and F).

IFN- λ 1/IL-29 mRNA was significantly induced by both viruses in NANA ($p<0.001$ with RV16, $p<0.01$ with RV1B) and AA ($p<0.001$). Clear induction was

observed in ANA and NAA but these failed to reach statistical significance. No differences were observed between groups (figure 3.7 G and H).

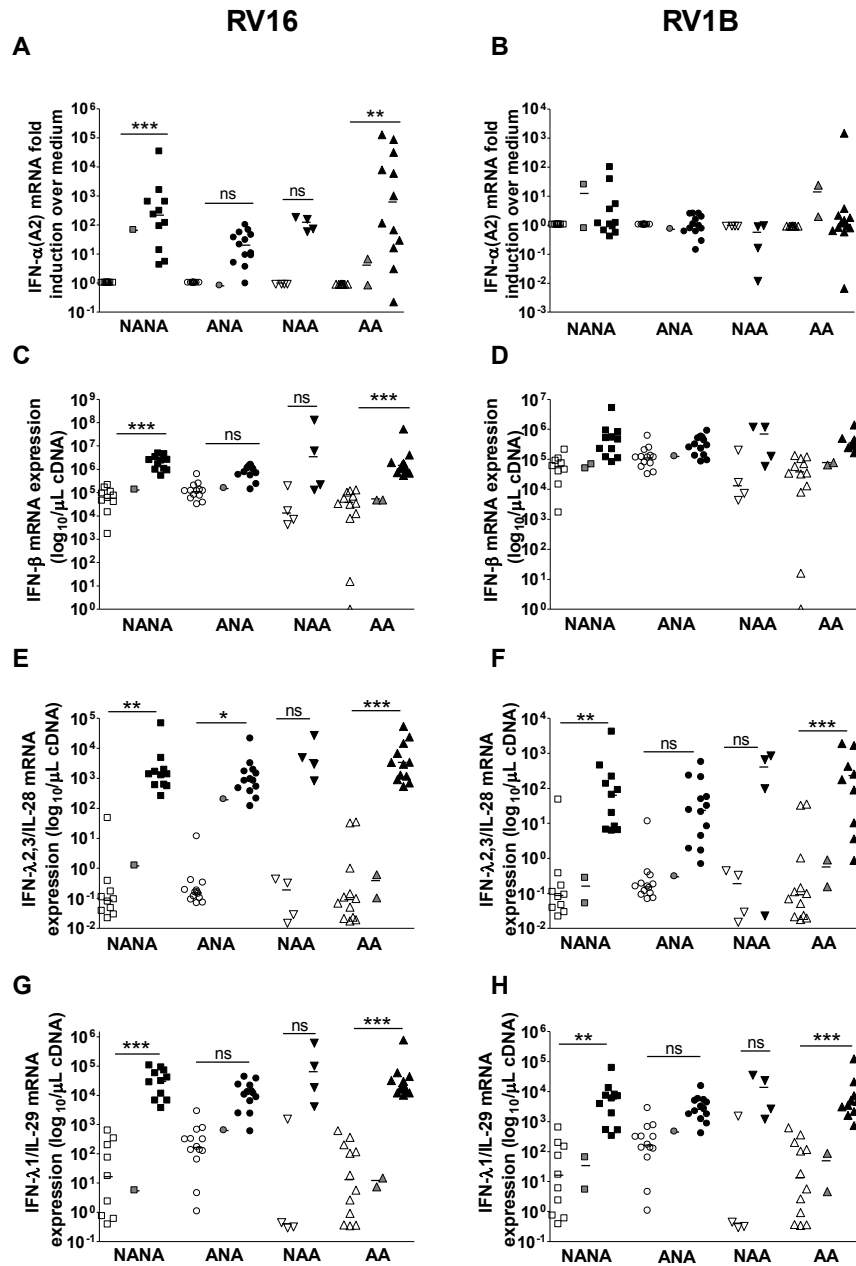


Figure 3.7. Interferon mRNA induction in BAL macrophages at 8h post stimulation with RV16 (A, C, E and G) and RV1B (B, D, F and H). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, grey shapes denote filtered virus, black shapes denote virus; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, ns = non-significant. Significant induction of type I IFN mRNA was observed in NANA and AA stimulated with RV16 (A and C), but not with RV1B (B and D). A non-significant increase was observed in ANA and NAA. Significant induction of IFN-λ2,3/IL-28 mRNA was observed in NANA, ANA and AA in BAL cells stimulated with RV-16 (E) and in NANA and AA in cells stimulated with RV1B (F). Non-significant increases were observed in the other groups. Significant induction of IFN-λ1/IL-29 mRNA in BAL cells stimulated with RV16 (G) and RV1B (H) was observed in NANA and AA, with non-significant increases in ANA and NAA. No differences in all IFN mRNA induction were observed between groups.

To assess interferon protein production in BAL cell supernatants MSD was employed. The commercially available IFN- α 2a and IFN- β MSD assays were used as these had been fully assessed and quality controlled by the company. The prototype IFN λ 1/IL-29 MSD assay (see section 3.2.2) was used to assess IFN λ 1/IL-29 protein production.

Type I IFNs were induced by RV16. Significant induction of IFN- α 2a was observed in NANA ($p < 0.01$), ANA ($p < 0.001$) and AA ($p < 0.001$). An increase was observed in NAA but this failed to reach statistical significance (figure 3.8 A). Significant induction of IFN- β by RV16 was also observed in NANA ($p < 0.01$) and AA ($p < 0.001$). An increase was observed in ANA and NAA but this failed to reach statistical significance (figure 3.8 C). There were no significant differences in the production of type I IFNs between any groups. Type I IFN protein was not significantly induced by RV1B (figure 3.8 B and D). A weak induction of IFN- λ 1/IL-29 was observed in asthmatics following infection with RV16 (figure 3.8 E), but this failed to reach statistical significance. No such induction was observed with RV1B (figure 3.8 F).

IL-6 and CXCL8/IL-8 mRNA expression in BAL cells, and IL-1 β , IL-6, CXCL8/IL-8 and TNF- α protein production in BAL cell supernatant was measured at 8h post stimulation to establish whether any deficiency in proinflammatory cytokines was identified in asthmatics, which could lead on to investigation of other innate immune responses.

IL-6 and CXCL8/IL-8 mRNA was not induced at 8h post stimulation with RV16 or RV1B (figure 3.9). High baseline mRNA levels of both cytokines were present and no significant difference was observed between any group. Likewise, production of IL-1 β , IL-6, CXCL8/IL-8 and TNF- α protein in BAL cell supernatants was not observed at 8h post stimulation with both RV16 and RV1B (figure 3.10) and there were no differences between the groups. Similar to mRNA, high baseline levels of proinflammatory protein were observed in all groups.

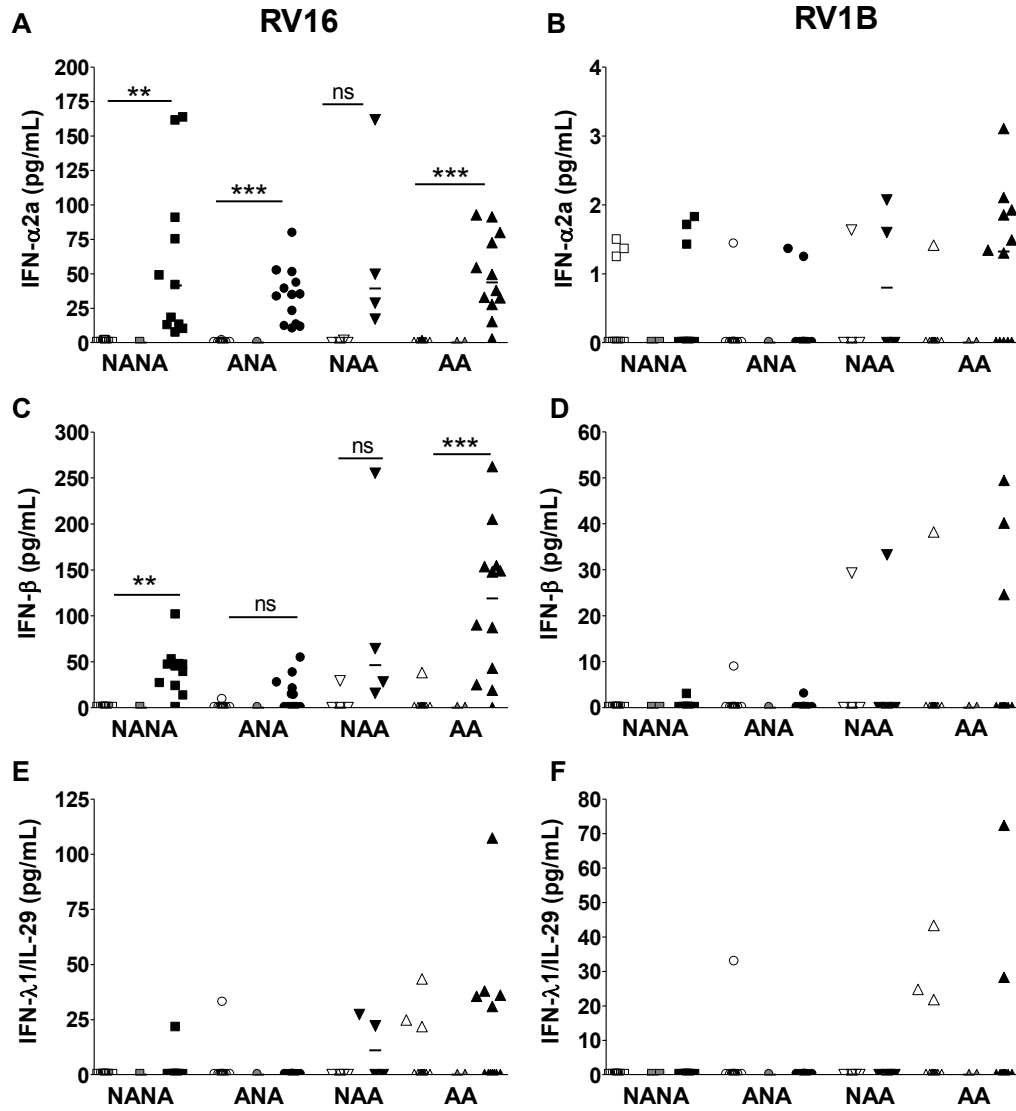


Figure 3.8. Interferon protein induction in BAL macrophages at 8h post stimulation with RV16 (A, C and E) and RV1B (B, D and F). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, grey shapes denote filtered virus, black shapes denote virus; ** = $p < 0.01$, *** = $p < 0.001$, ns = non-significant. Induction of IFN- α 2a and IFN- β was observed in all groups stimulated with RV16, with no differences between groups. A weak induction of IFN- λ 1/IL-29 was observed, but this was non-significant. There was no induction of IFNs in BAL cells stimulated with RV1B.

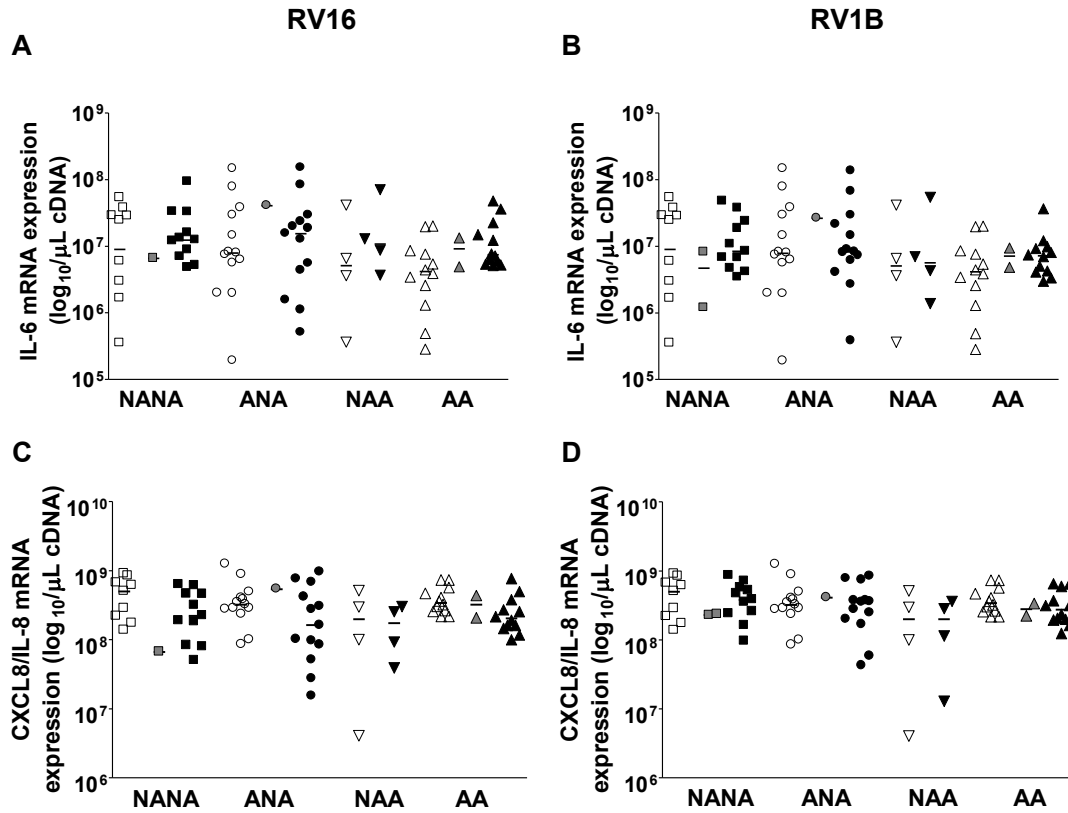


Figure 3.9. IL-6 and CXCL8/IL-8 mRNA induction in BAL macrophages at 8h post stimulation with RV16 (A and C) and RV1B (B and D). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, grey shapes denote filtered virus, black shapes denote virus. Induction of IL-6 and CXCL8/IL-8 mRNA was not observed in BAL cells from any group stimulated with RV16 or RV1B at 8h.

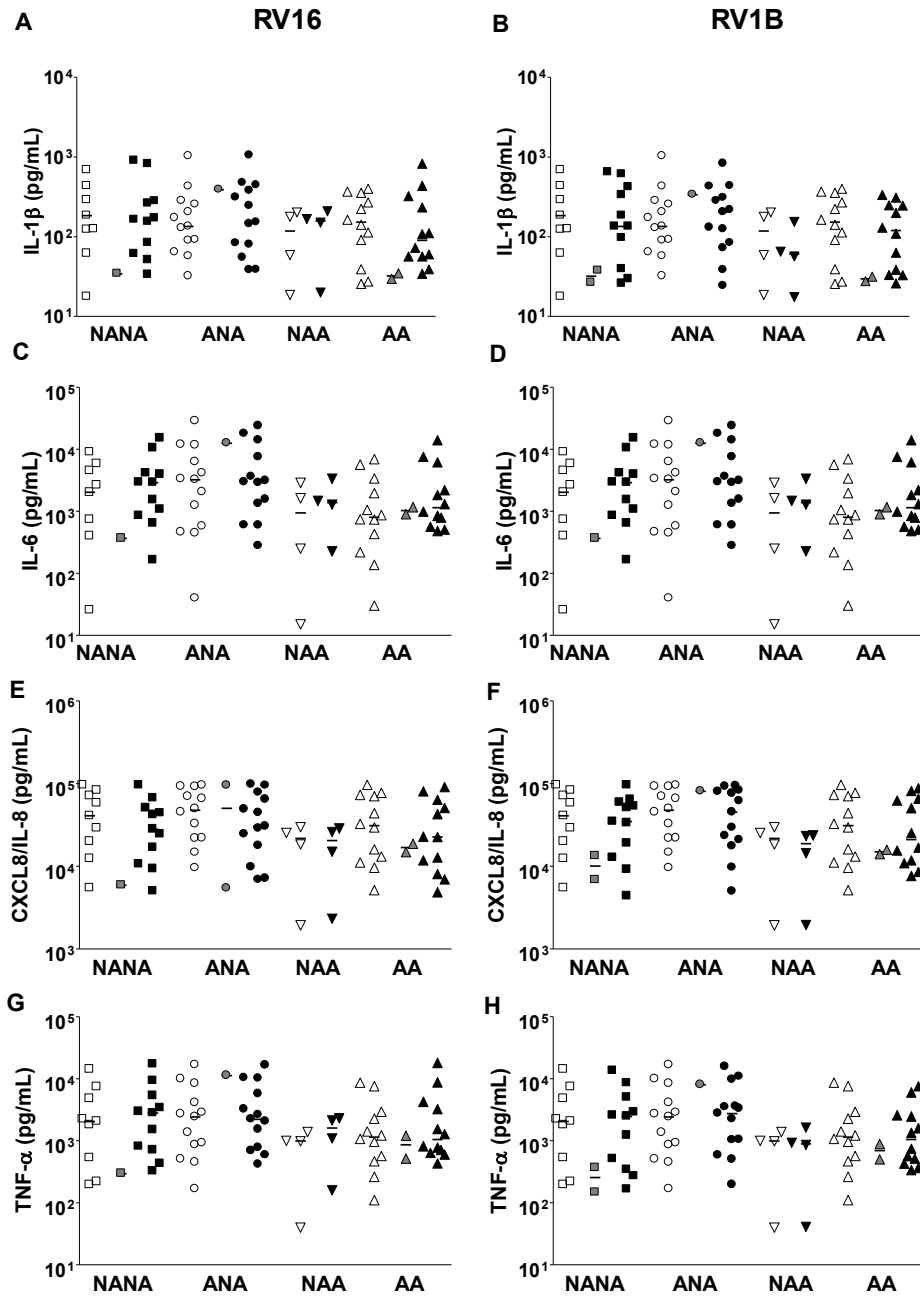


Figure 3.10. IL-1 β (A and B), IL-6 (C and D), CXCL8/IL-8 (E and F) and TNF- α (G and H) protein induction in BAL cells stimulated with RV16 (A, C, E and G) and RV1B (B, D, F and H). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, grey shapes denote filtered virus, black shapes denote virus. Production of proinflammatory cytokine protein was not observed in BAL cells from any group stimulated with RV16 or RV1B at 8h.

Tables 3.3 and 3.4 summarise IFN and proinflammatory cytokine protein and mRNA induction in BAL cells at 8h following infection with RV16 and RV1B.

Table 3.3 Summary of IFN and proinflammatory cytokine mRNA and protein induction post RV16 infection in BAL cells ↑ = significant induction, ns = non-significant increase observed, - = no induction

	8h			
	NANA	ANA	NAA	AA
IFN-α mRNA	↑	ns	ns	↑
IFN-α protein	↑	↑	ns	↑
IFN-β mRNA	↑	ns	ns	↑
IFN-β protein	↑	ns	ns	↑
IFNλ1/IL-29 mRNA	↑	ns	ns	↑
IFNλ1/IL-29 protein	-	-	-	-
IFNλ2,3/IL-28 mRNA	↑	↑	ns	↑
IL-6 mRNA	-	-	-	-
CXCL8/IL-8 mRNA	-	-	-	-
IL-1β protein	-	-	-	-
IL-6 protein	-	-	-	-
CXCL8/IL-8 protein	-	-	-	-
TNF-α protein	-	-	-	-

Table 3.4 Summary of IFN and proinflammatory cytokine mRNA and protein induction post RV1B infection in BAL cells ↑ = significant induction, ns = non-significant increase observed, - = no induction

	8h			
	NANA	ANA	NAA	AA
IFN-α mRNA	ns	-	-	-
IFN-α protein	-	-	-	-
IFN-β mRNA	ns	ns	ns	ns
IFN-β protein	-	-	-	-
IFNλ1/IL-29 mRNA	↑	ns	ns	↑
IFNλ1/IL-29 protein	-	-	-	-
IFNλ2,3/IL-28 mRNA	↑	ns	ns	↑
IL-6 mRNA	-	-	-	-
CXCL8/IL-8 mRNA	-	-	-	-
IL-1β protein	-	-	-	-
IL-6 protein	-	-	-	-
CXCL8/IL-8 protein	-	-	-	-
TNF-α protein	-	-	-	-

3.2.6 RV infection of HBECs: induction of type I and III IFN and proinflammatory cytokines mRNA and protein

To establish whether the previously reported defective RV induced IFN- β and IFN- λ in AAs was present in HBECs from other patient groups type I and III IFN mRNA was measured at 8h and 24h, and protein at 24h and 48h in response to RV16 and RV1B. HBECs have previously been shown not to produce IFN- α ⁸⁰.

RV16 induction of IFN- β mRNA was not observed at 8h (figure 3.11 A), but was observed later at 24h (figure 3.11B). Significant induction was observed in AA ($p < 0.01$) and non-significant increases were observed in NANA, ANA and NAA. No differences were observed between groups. RV1B induction of IFN- β mRNA was observed at 8h and 24h (figure 3.12 A and B). At 8h, significant induction in ANA ($p < 0.01$) and AA ($p < 0.05$), and non-significant increases in NANA were observed. Induction was not observed in NAA. At 24h, significant induction in NANA ($p < 0.05$) and AA ($p < 0.001$), and non-significant increases in ANA and NAA were observed. No differences were observed between groups.

RV16 induction of IFN- λ 2,3/IL-28 mRNA was observed at 8h and 24h (figure 3.11 C and D). At 8h, significant induction was observed in NANA ($p < 0.01$) and AA ($p < 0.05$) and non-significant increases were observed in ANA and NAA. At 24h, significant induction was observed in NANA ($p < 0.05$), ANA ($p < 0.01$) and AA ($p < 0.001$) and non-significant increases were observed in NAA. No differences were observed between groups. RV1B induction of IFN- λ 2,3/IL-28 mRNA was observed at 8h and 24h (figure 3.12 C and D). At 8h, significant induction was observed in NANA ($p < 0.05$) and AA ($p < 0.01$) and non-significant increases were observed in ANA and NAA. At 24h, significant induction was observed in NANA ($p < 0.01$), ANA ($p < 0.01$) and AA ($p < 0.001$) and non-significant increases were observed in NAA. No differences were observed between groups.

RV16 induction of IFN- λ 1/IL-29 mRNA was observed at 8h and 24h (figure 3.11 E and F). At 8h, significant induction was observed in ANA ($p < 0.05$) and AA ($p < 0.01$) and non-significant increases were observed in NANA and NAA. At 24h, significant induction was observed in NANA ($p < 0.01$), ANA ($p < 0.01$) and AA

($p < 0.001$) and non-significant increases were observed in NAA. No differences were observed between groups. RV16 induction of IFN- λ 1/IL-29 mRNA was observed at 8h and 24h (figure 3.12 E and F). At 8h, significant induction was observed in NANA ($p < 0.05$), ANA ($p < 0.05$) and AA ($p < 0.01$) and non-significant increases were observed in NAA. At 24h, significant induction was observed in NANA ($p < 0.001$), ANA ($p < 0.001$) and AA ($p < 0.001$) and non-significant increases were observed in NAA. No differences were observed between groups.

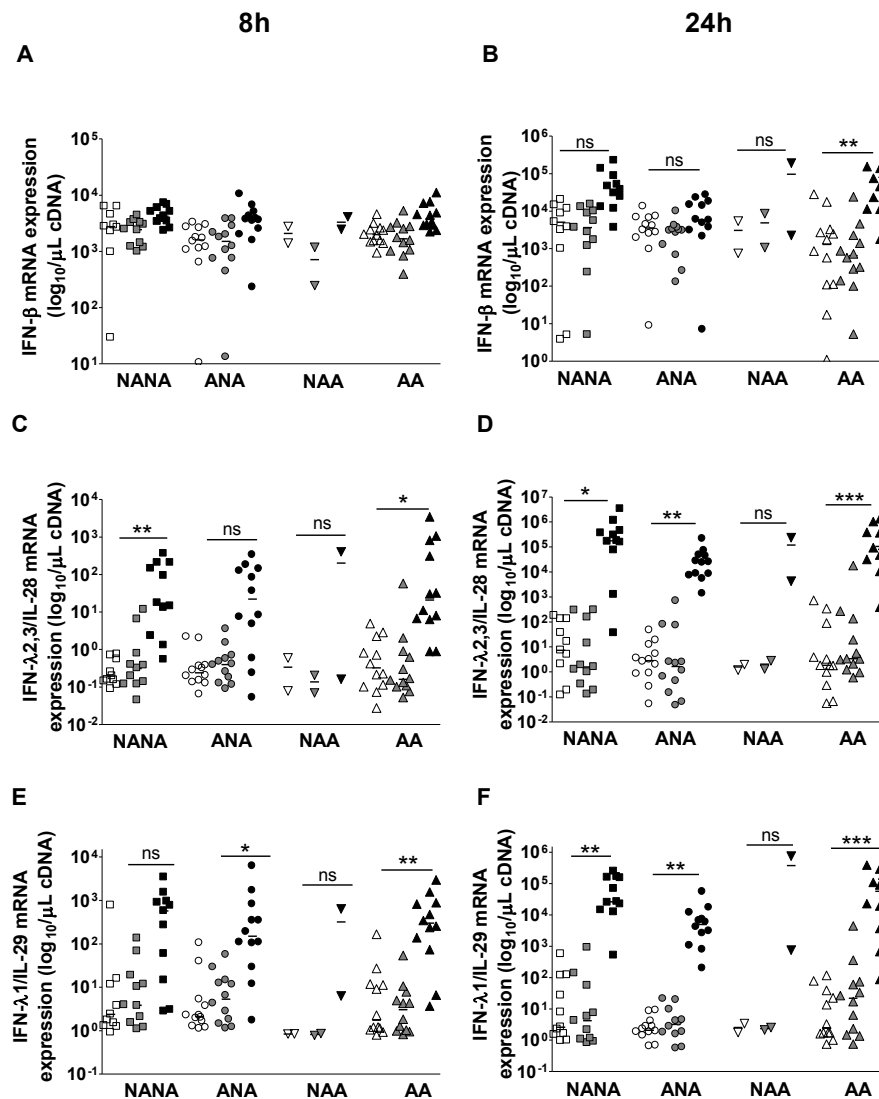


Figure 3.11. Interferon mRNA induction in HBECs post stimulation with RV16 at 8h (A, C and E) and 24h (B, D and F). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, grey shapes denote filtered virus, black shapes denote virus; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, ns = non-significant. IFN- β (A and B), IFN- λ 2,3/IL-28 (C and D), IFN- λ 1/IL-29 (E and F). Induction of IFN- β mRNA by RV16 was observed at 24h and type III IFN mRNA at both time points. No significant differences were observed between groups.

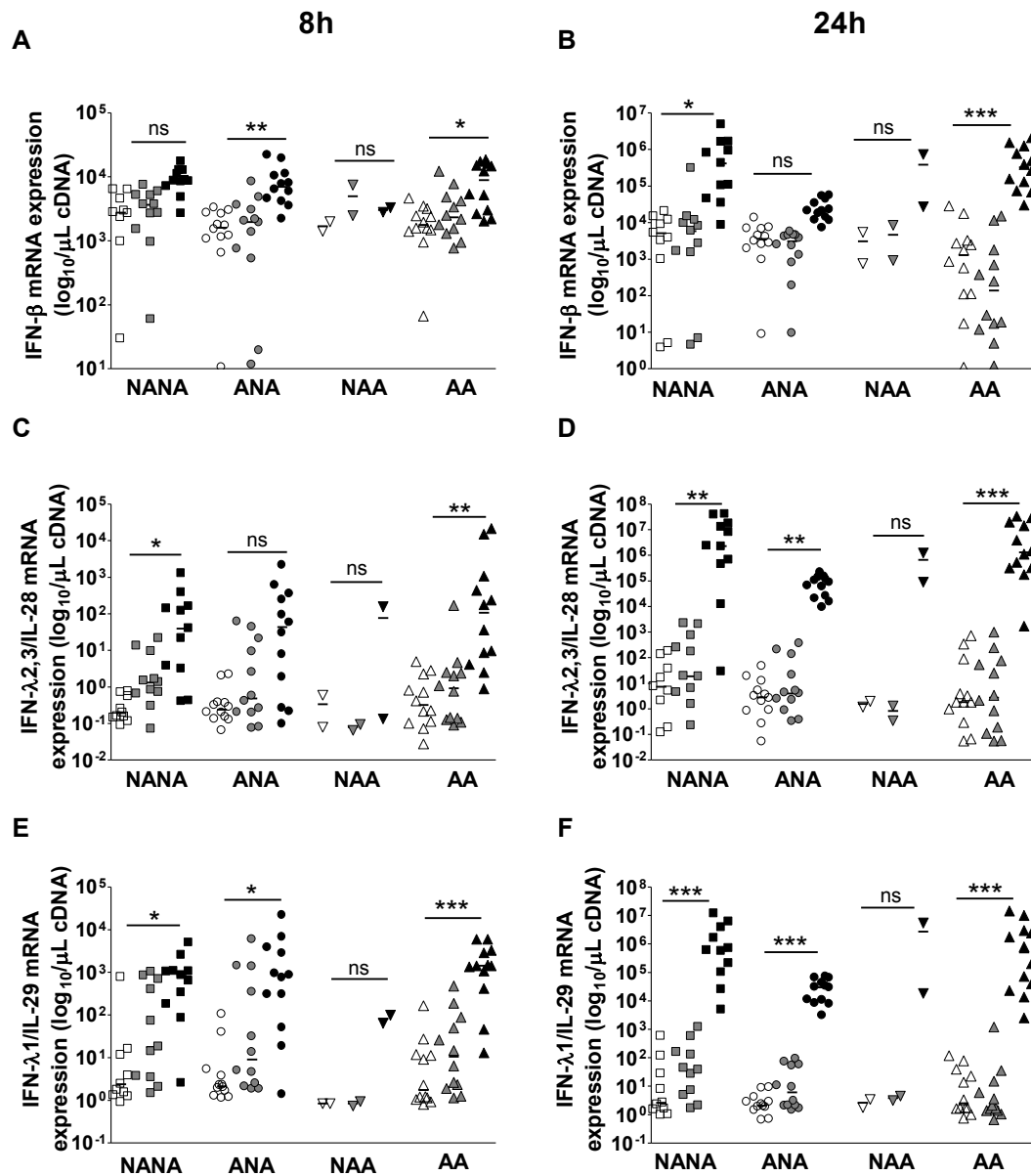


Figure 3.12. Interferon mRNA induction in HBECs post stimulation with RV1B at 8h (A, C and E) and 24h (B, D and F). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, grey shapes denote filtered virus, black shapes denote virus; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, ns = non-significant. IFN- β (A and B), IFN- λ 2,3/IL-28 (C and D), IFN- λ 1/IL-29 (E and F). Induction of type I and III IFN mRNA by RV1B was observed at both time points. No significant differences were observed between groups.

IFN protein levels in HBEC supernatant were measured using MSD. At 24h RV16 did not induce IFN- β protein (figure 3.13 A). RV16 induced production of IFN- β at 48h post stimulation, with significant induction in AA ($p < 0.05$) and non-significant increases in NANA, ANA and NAA (figure 3.13 B). IFN- λ 1/IL-29 protein was not

observed at 24h (figure 3.13 C), but significant production was observed at 48h in NANA ($p<0.001$), ANA ($p<0.01$) and AA ($p<0.001$) with non-significant increases observed in NAA (figure 3.13 D). At both time points no differences were observed between groups in the RV16 induced production of type I or III IFNs.

Stimulation of HBECs with RV1B resulted in no induction of IFN- β protein at 24h (figure 3.14 A) and a non-significant increase in IFN- β protein at 48h (figure 3.14 B). Increases in IFN- λ 1/IL-29 protein were observed at 24h and 48h (figure 3.14 C and D) with significance in NANA at 24h ($p<0.05$), and at 48h in ANA ($p<0.01$) and AA ($p<0.001$). At both time points no differences were observed between groups in the RV1B induced production of type I or III IFNs.

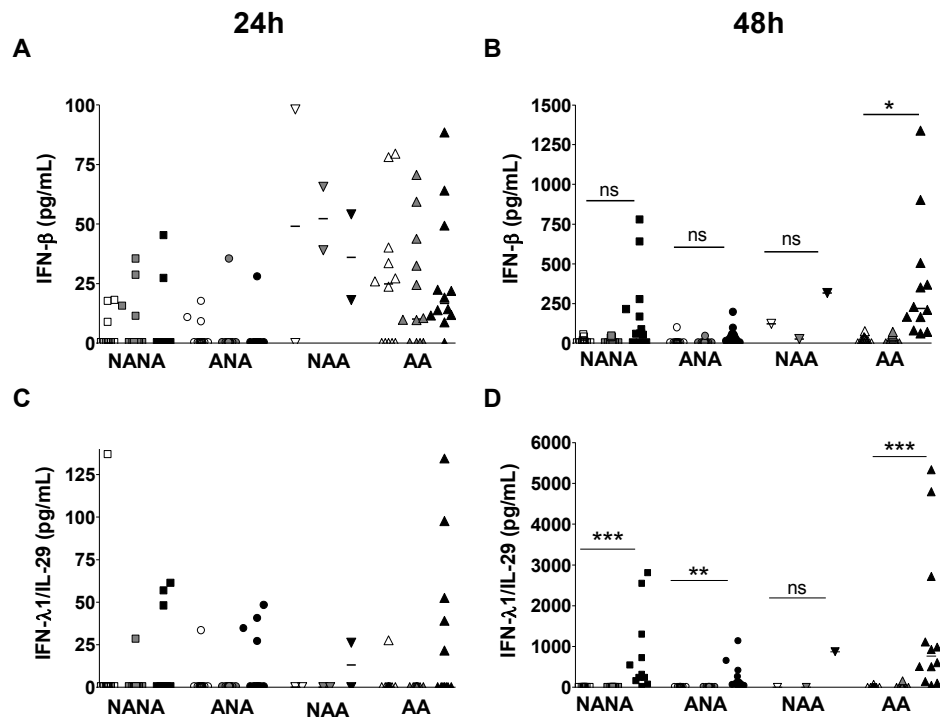


Figure 3.13. Interferon protein induction in HBECs post stimulation with RV16 at 24h (A and C) and 48h (B and D). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, grey shapes denote filtered virus, black shapes denote virus; * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, ns = non-significant. IFN- β (A and B), IFN- λ 1/IL-29 (C and D). No production of type I or type III IFN was observed at 24h. Production was observed at 48h, but no significant differences were observed between groups.

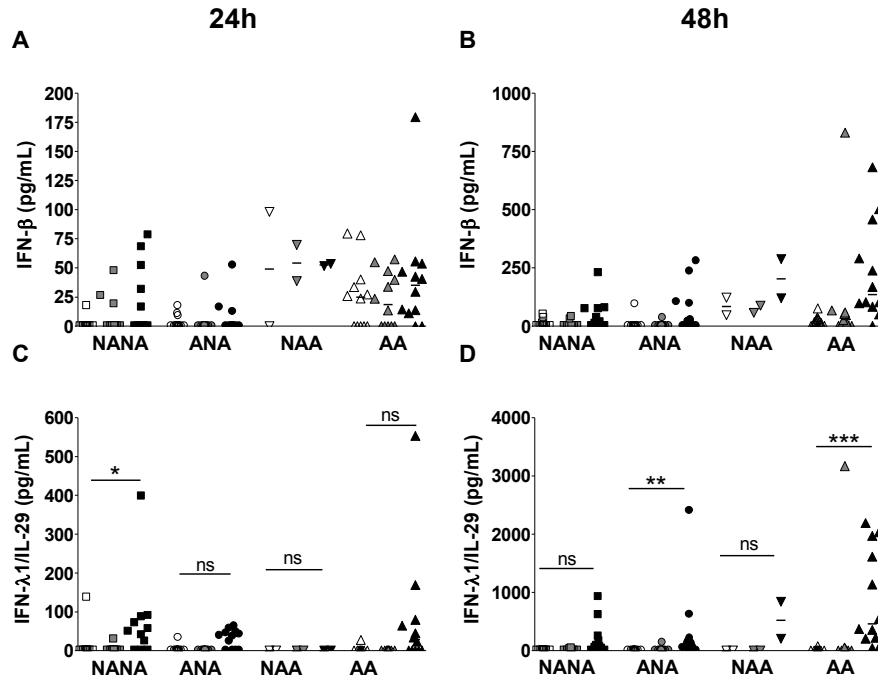


Figure 3.14. Interferon protein induction in HBECs post stimulation with RV1B at 24h (A and C) and 48h (B and D). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, grey shapes denote filtered virus, black shapes denote virus ; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, ns = non-significant. IFN- β (A and B), IFN- $\lambda 1$ /IL-29 (C and D). No production of IFN- β protein was observed at 24h or 48h. Production of IFN- $\lambda 1$ /IL-29 was observed at 24h in NANA and at 48h in ANA and AA. No significant differences were observed between groups.

To evaluate differences in HBEC proinflammatory cytokine production between NANA, ANA, NAA and AA IL-6 and CXCL8/IL-8 mRNA at 8h and 24h was measured post RV16 and RV1B infection (figure 3.15).

At 8h induction of IL-6 and CXCL8/IL-8 mRNA was not observed with either RV16 or RV1B (figure 3.15 A, C, E and G). At 24h, increases in IL-6 mRNA in HBECs stimulated with RV16 were observed, but these failed to reach statistical significance (figure 3.15 B). Significant increases in CXCL8/IL-8 mRNA, following RV16 stimulation were observed in AA ($p < 0.05$) with non-significant increases in NANA, ANA and NAA (figure 3.15 D). With RV1B at 24h, significant increases in IL-6 mRNA were observed in AA ($p < 0.05$) with non-significant increases in NANA, ANA and NAA (figure 3.15 F), and significant increases in CXCL8/IL-8 mRNA were observed in NANA ($p < 0.01$), ANA ($p < 0.01$) and AA ($p < 0.01$) with non-significant increases in NAA (figure 3.15 H). No significant differences were observed between groups.

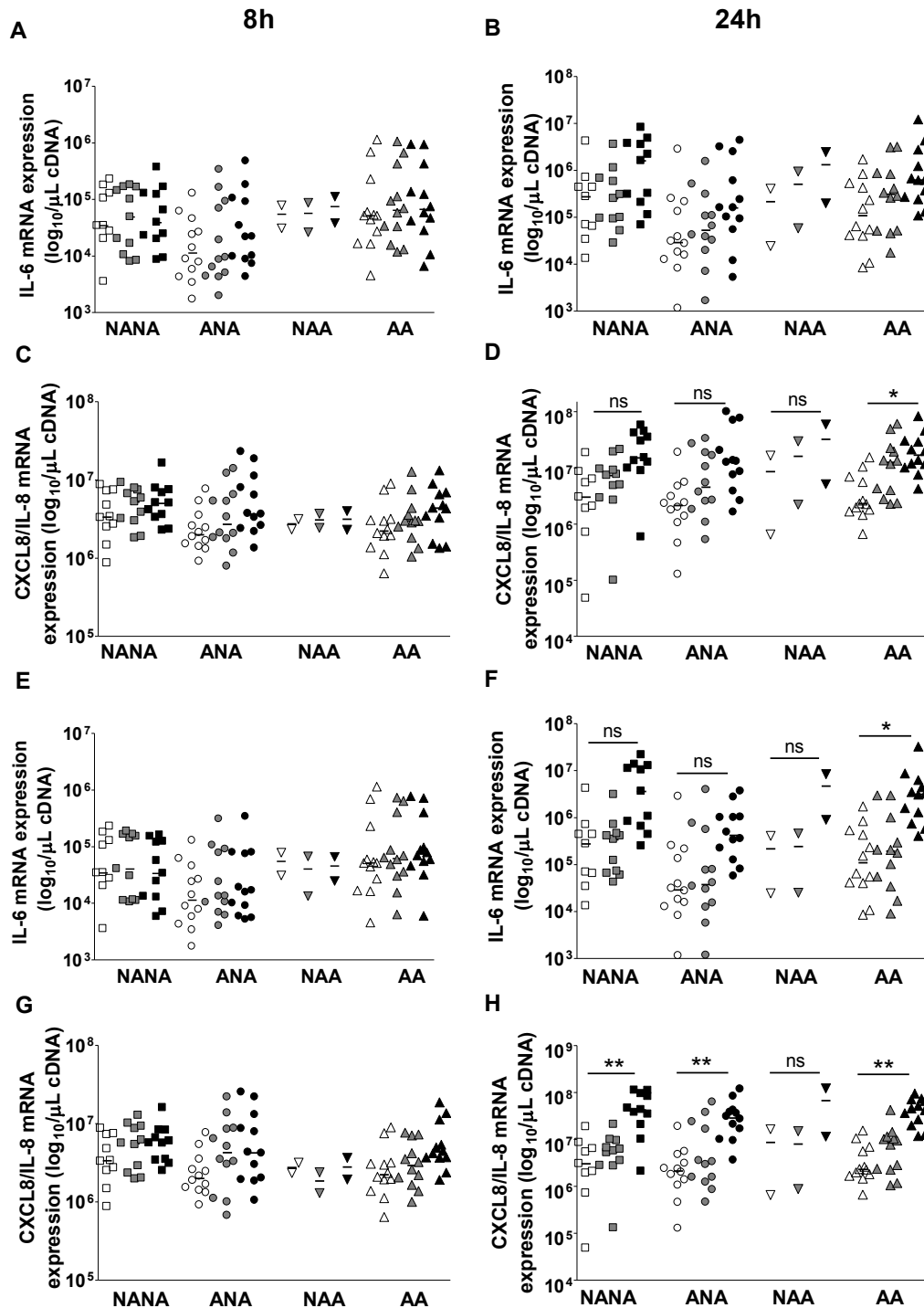


Figure 3.15. IL-6 mRNA induction in HBECs post stimulation with RV16 at 8h (A) and 24h (B) and RV1B at 8h (E) and 24h (F); CXCL8/IL-8 mRNA induction in HBECs post stimulation with RV16 at 8h (C) and 24h (D) and RV1B at 8h (G) and 24h (H). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, grey shapes denote filtered virus, black shapes denote virus; * = $p < 0.05$, ** = $p < 0.01$, ns = non-significant. At 8h induction of IL-6 and CXCL8/IL-8 mRNA was not observed. At 24h, with RV16 non-significant increases in IL-6 mRNA were observed (B) and significant increases in CXCL8/IL-8 mRNA were observed in AA (D). At 24h, with RV1B significant increases in IL-6 mRNA in AA and CXCL8/IL-8 mRNA in NANA, ANA and AA were observed (F and G). No significant differences were observed between groups.

To evaluate differences in HBEC proinflammatory cytokine production between NANA, ANA, NAA and AA IL-1 β , IL-6, CXCL8/IL-8 and TNF- α protein at 24h and 48h was measured post RV16 and RV1B infection (figure 3.16 and 3.17).

IL-1 β protein was produced at 24h post RV16 infection, but this failed to reach statistical significance (figure 3.16 A). At 48h, significant increases in IL-1 β protein were observed in NANA ($p<0.01$), ANA ($p<0.05$) and AA ($p<0.05$) with non-significant increases in NAA (figure 3.16 B). IL-6 protein was produced at 24h and 48h post RV16 infection, but this failed to reach statistical significance (figure 3.16 C and D). CXCL8/IL-8 protein was produced at 24h post RV16 infection in NANA ($p<0.01$), ANA ($p<0.05$) and AA ($p<0.05$) with a non-significant increase observed in NAA (figure 3.16 E). At 48h significant increases were observed in NANA ($p<0.01$), ANA ($p<0.001$) and AA ($p<0.05$) with a non-significant increase observed in NAA (figure 3.16 F). TNF- α protein was produced at 24h post RV16 infection in NANA, ANA, AA and not in NAA, but this failed to reach statistical significance (figure 3.16 G). At 48h significant increases were observed in NANA ($p<0.05$) and AA ($p<0.05$) with a non-significant increase observed in ANA and NAA (figure 3.16 H). With all cytokines and at both time points no differences were observed between groups.

IL-1 β protein was induced at 24h post RV1B infection, in NANA ($p<0.05$) and ANA ($p<0.05$) with non-significant increases observed in NAA and AA (figure 3.17 A). At 48h, significant increases in IL-1 β protein were observed in NANA ($p<0.05$), ANA ($p<0.01$) and AA ($p<0.01$) with non-significant increases in NAA (figure 3.17 B). IL-6 protein was induced at 24h and 48h post RV1B infection, but this failed to reach statistical significance (figure 3.17 C and D). CXCL8/IL-8 protein was induced at 24h post RV1B infection in NANA ($p<0.01$), ANA ($p<0.01$) and AA ($p<0.05$) with a non-significant increase observed in NAA (figure 3.17 E). At 48h significant increases were observed in ANA ($p<0.01$) and AA ($p<0.05$) with a non-significant increase observed in NANA and NAA (figure 3.17 F). TNF- α protein was induced at 24h post RV1B infection in NANA ($p<0.05$), with a non-significant increase observed in ANA and AA but not in NAA (figure 3.17 G). At 48h significant increases were observed in AA only ($p<0.05$) with a non-significant increase observed in NANA, ANA and NAA (figure 3.17 H). With all cytokines and at both time points no differences were observed between groups.

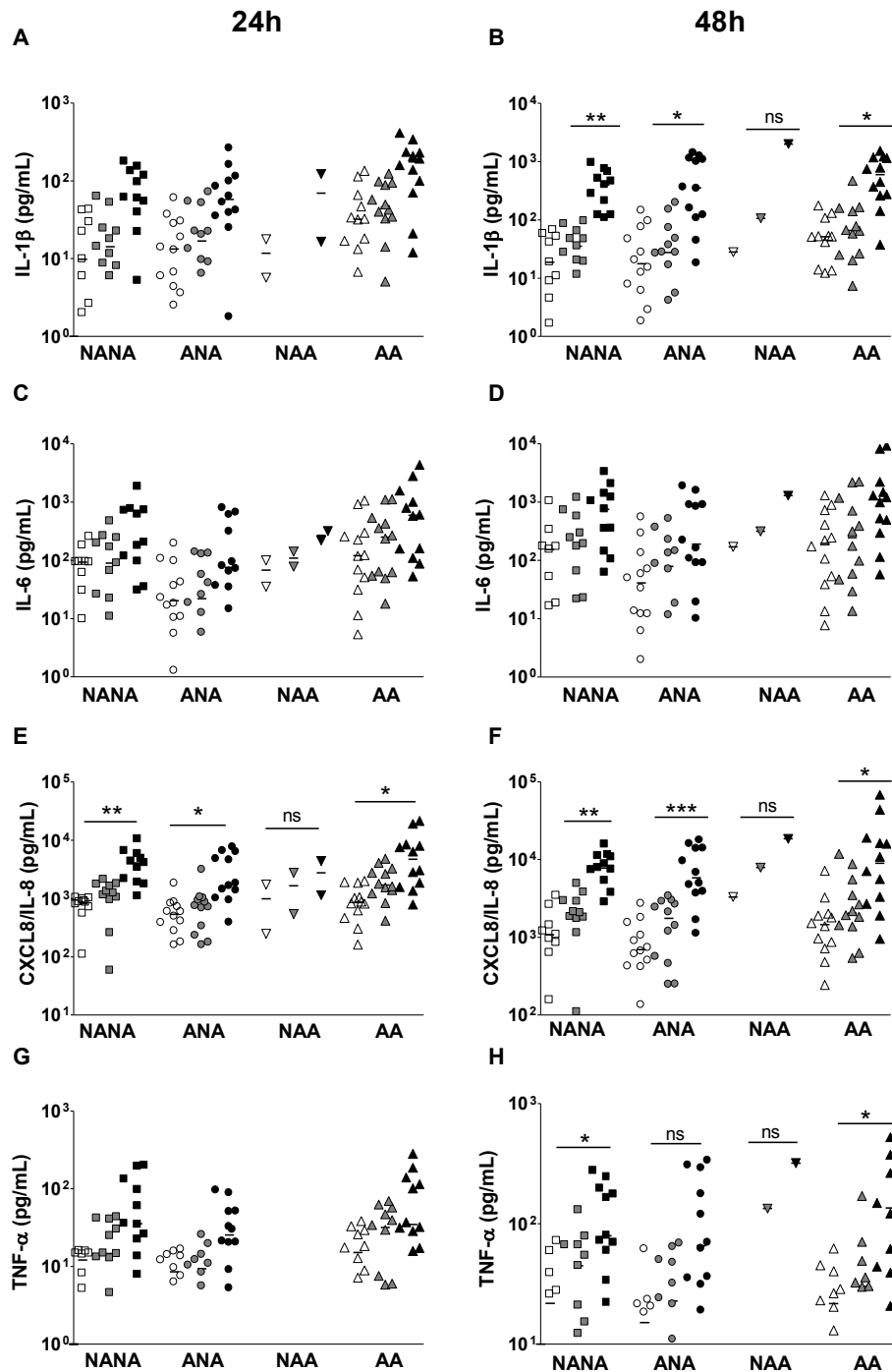


Figure 3.16. Proinflammatory cytokine protein induction in HBECs stimulated with RV16 at 24h (A, C, E and G) and 48h (B, D, F and H). IL-1 β (A and B), IL-6 (C and D), CXCL8/IL-8 (E and F) and TNF- α (G and H). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, grey shapes denote filtered virus, black shapes denote virus; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, ns = non-significant. Proinflammatory cytokine protein increases were observed at both time points with all cytokines in all groups. Significant increases in IL-1 β were observed in NANA, ANA and AA at 48h. Significant increases in CXCL8/IL-8 were observed in NANA, ANA and AA at 24h and 48h. Significant increases in TNF- α were observed in NANA and AA at 48h. No significant differences were observed between groups.

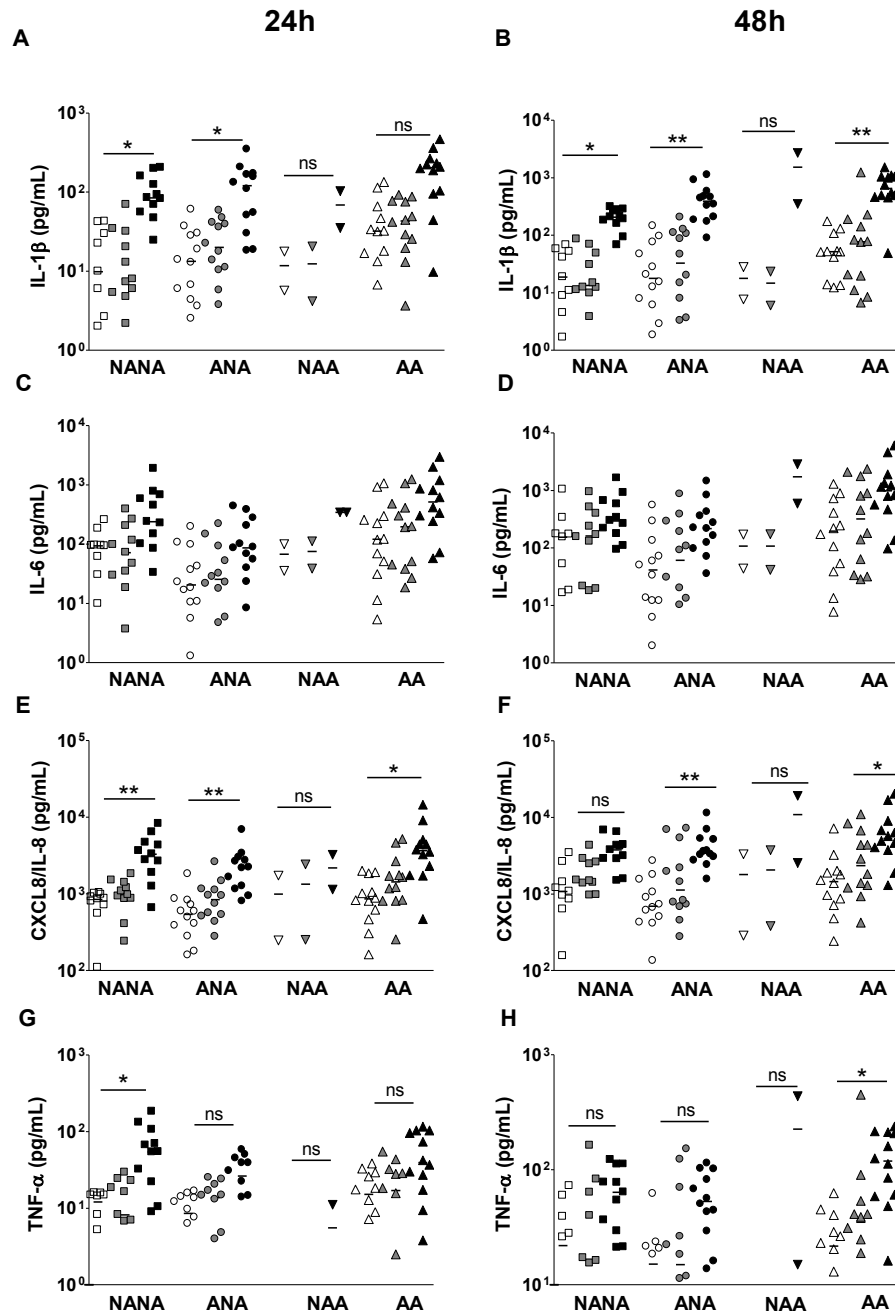


Figure 3.17. Proinflammatory cytokine protein induction in HBECs stimulated with RV1B at 24h (A, C, E and G) and 48h (B, D, F and H). IL-1 β (A and B), IL-6 (C and D), CXCL8/IL-8 (E and F) and TNF- α (G and H). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, grey shapes denote filtered virus, black shapes denote virus; * = $p < 0.05$, ** = $p < 0.01$, ns = non-significant. Proinflammatory cytokine protein increases were observed at both time points with all cytokines in all groups. Significant increases in IL-1 β were observed in NANA and ANA at 24h and in NANA, ANA and AA at 48h. Significant increases in CXCL8/IL-8 were observed in NANA, ANA and AA at 24h and in ANA and AA at 48h. Significant increases in TNF- α were observed in AA at 48h. No significant differences were observed between groups.

Tables 3.5 to 3.8 summarise the IFN and proinflammatory cytokine mRNA and protein induction in HBECS following RV16 and RV1B infection.

Table 3.5 Summary of IFN and proinflammatory cytokine mRNA induction post RV16 infection in HBECS ↑ = significant induction, ns = non-significant increase observed, - = no induction

	8h				24h			
	NANA	ANA	NAA	AA	NANA	ANA	NAA	AA
IFN-β	-	-	-	-	ns	ns	ns	↑
IFNλ2,3/IL-28	↑	ns	ns	↑	↑	↑	ns	↑
IFNλ1/IL-29	ns	↑	ns	↑	↑	↑	ns	↑
IL-6	-	-	-	-	ns	ns	ns	ns
CXCL8/IL-8	-	-	-	-	ns	ns	ns	↑

Table 3.6 Summary of IFN and proinflammatory cytokine protein induction post RV16 infection in HBECS ↑ = significant induction, ns = non-significant increase observed, - = no induction

	24h				48h			
	NANA	ANA	NAA	AA	NANA	ANA	NAA	AA
IFN-β	-	-	-	-	ns	ns	ns	↑
IFNλ1/IL-29	-	-	-	-	↑	↑	ns	↑
IL-1β	ns	ns	ns	ns	↑	↑	ns	↑
IL-6	ns	ns	ns	ns	ns	ns	ns	ns
CXCL8/IL-8	↑	↑	ns	↑	↑	↑	ns	↑
TNF-α	ns	ns	-	ns	↑	ns	ns	↑

Table 3.7 Summary of IFN and proinflammatory cytokine mRNA induction post RV1B infection in HBECs ↑ = significant induction, ns = non-significant increase observed, - = no induction

	8h				24h			
	NANA	ANA	NAA	AA	NANA	ANA	NAA	AA
IFN-β	ns	↑	-	↑	↑	ns	ns	↑
IFNλ2,3/IL-28	↑	ns	ns	↑	↑	↑	ns	↑
IFNλ1/IL-29	↑	↑	ns	↑	↑	↑	ns	↑
IL-6	-	-	-	-	ns	ns	ns	↑
CXCL8/IL-8	-	-	-	-	↑	↑	ns	↑

Table 3.8 Summary of IFN and proinflammatory cytokine protein induction post RV1B infection in HBECs ↑ = significant induction, ns = non-significant increase observed, - = no induction

	24h				48h			
	NANA	ANA	NAA	AA	NANA	ANA	NAA	AA
IFN-β	-	-	-	-	ns	ns	ns	ns
IFNλ1/IL-29	↑	ns	ns	ns	ns	↑	ns	↑
IL-1β	↑	↑	ns	ns	↑	↑	ns	↑
IL-6	ns	ns	ns	ns	ns	ns	ns	ns
CXCL8/IL-8	↑	↑	ns	↑	ns	↑	ns	↑
TNF-α	↑	ns	-	ns	ns	ns	ns	↑

3.2.7 RV infection of PBMCs: induction of type I and III IFN and proinflammatory cytokines protein

Responses to RV infection were assessed in PBMCs from each of the different volunteer groups by measuring type I and III IFN protein, and proinflammatory cytokine protein levels in PBMC supernatants at 24h and 48h. RV16 infection induced type I and III IFN protein production in PBMCs at 24h and 48h (figure 3.18), whereas no such induction was observed by RV1B (figure 3.19).

RV16 stimulated PBMCs produced significant amounts of IFN- α 2a protein at 24h in NANA, ANA and AA (all $p < 0.001$), with non-significant increases observed in NAA (figure 3.18 A). At 48h significant increases were observed in ANA ($p < 0.05$) and AA ($p < 0.001$) with non-significant increases observed in NANA and NAA (figure 3.18 B). No differences were observed between groups.

RV16 stimulated PBMCs produced significant amounts of IFN- β protein at 24h in NANA ($p < 0.01$), ANA ($p < 0.01$) and AA ($p < 0.001$), with non-significant increases observed in NAA (figure 3.18 C). At 48h significant increases were observed in AA ($p < 0.01$) with non-significant increases observed in NANA, ANA and NAA (figure 3.18 D). No differences were observed between groups.

RV16 stimulated PBMCs produced significant amounts of IFN- λ 1/IL-29 protein at 24h in ANA ($p < 0.05$) and AA ($p < 0.001$), with non-significant increases observed in NANA and NAA (figure 3.18 E). At 48h significant increases were observed in AA ($p < 0.001$) with non-significant increases observed in NANA, ANA and NAA (figure 3.18 F). No differences were observed between groups.

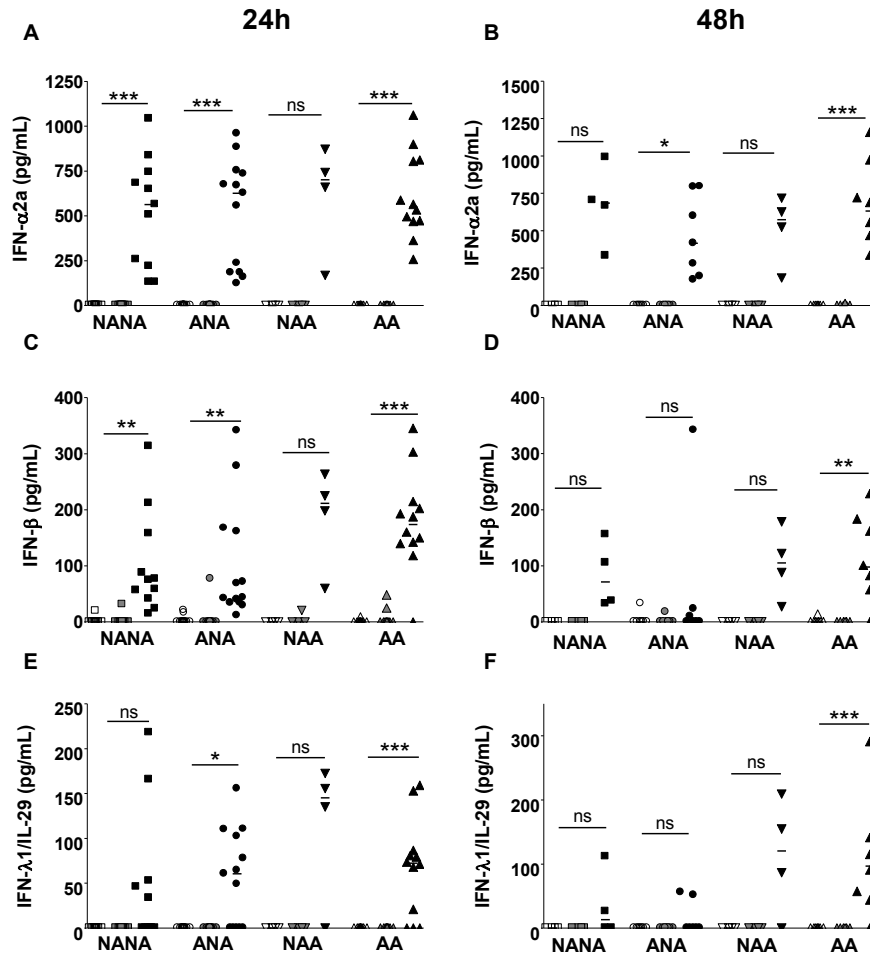


Figure 3.18. Interferon protein induction in PBMCs stimulated with RV16 at 24h (A, C and E) and 48h (B, D and F). IFN-α2a (A and B), IFN-β (C and D) and IFN-λ1/IL-29 (E and F). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, grey shapes denote filtered virus, black shapes denote virus; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, ns = non-significant. RV16 induced type I and III IFN production at 24h and 48h. Significant induction of IFN-α2a was observed in NANA, ANA and AA at 24h, and in ANA and AA at 48h. Significant induction of IFN-β was observed in NANA, ANA and AA at 24h, and in AA at 48h. Significant induction of IFN-λ1/IL-29 was observed in ANA and AA at 24h, and in AA at 48h. No significant differences were observed in type I or III IFN protein production between groups.

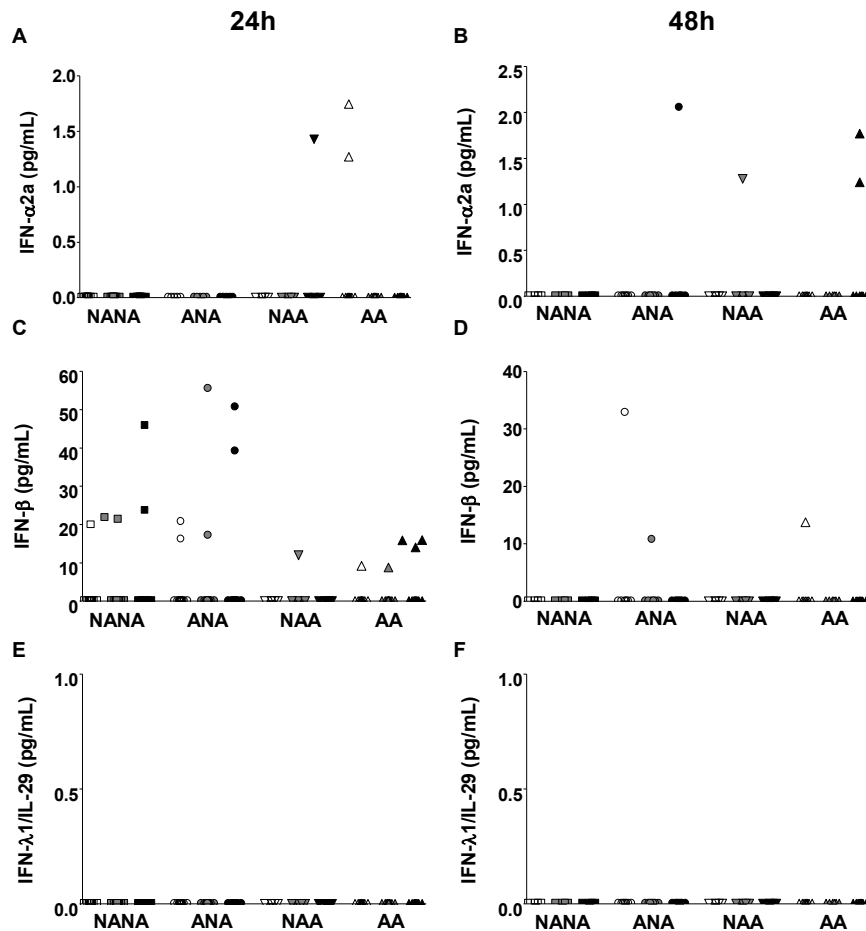


Figure 3.19. Interferon protein induction in PBMCs stimulated with RV1B at 24h (A, C and E) and 48h (B, D and F). IFN-α2a (A and B), IFN-β (C and D) and IFN-λ1/IL-29 (E and F). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, grey shapes denote filtered virus, black shapes denote virus. RV1B did not induce type I or III IFN protein production at 24h or 48h.

To evaluate differences in PBMC proinflammatory cytokine production between NANA, ANA, NAA and AA IL-1β, IL-6, CXCL8/IL-8 and TNF-α protein at 24h and 48h was measured post RV16 and RV1B infection (figure 3.20 and 3.21). Very high baseline levels of all the proinflammatory cytokines measured were observed in medium-treated cells, with the CXCL8/IL-8 data being at the top of the standard curve. There were no significant differences in the baseline levels of cytokines between groups though there was non-significant log difference lower in the virus stimulated cells in the NAA group. Both RV16 (figure 3.20) and RV1B (figure 3.21) failed to significantly induce the production of proinflammatory cytokines at 24h or 48h in PBMCs in all the volunteer groups. No differences were observed between groups.

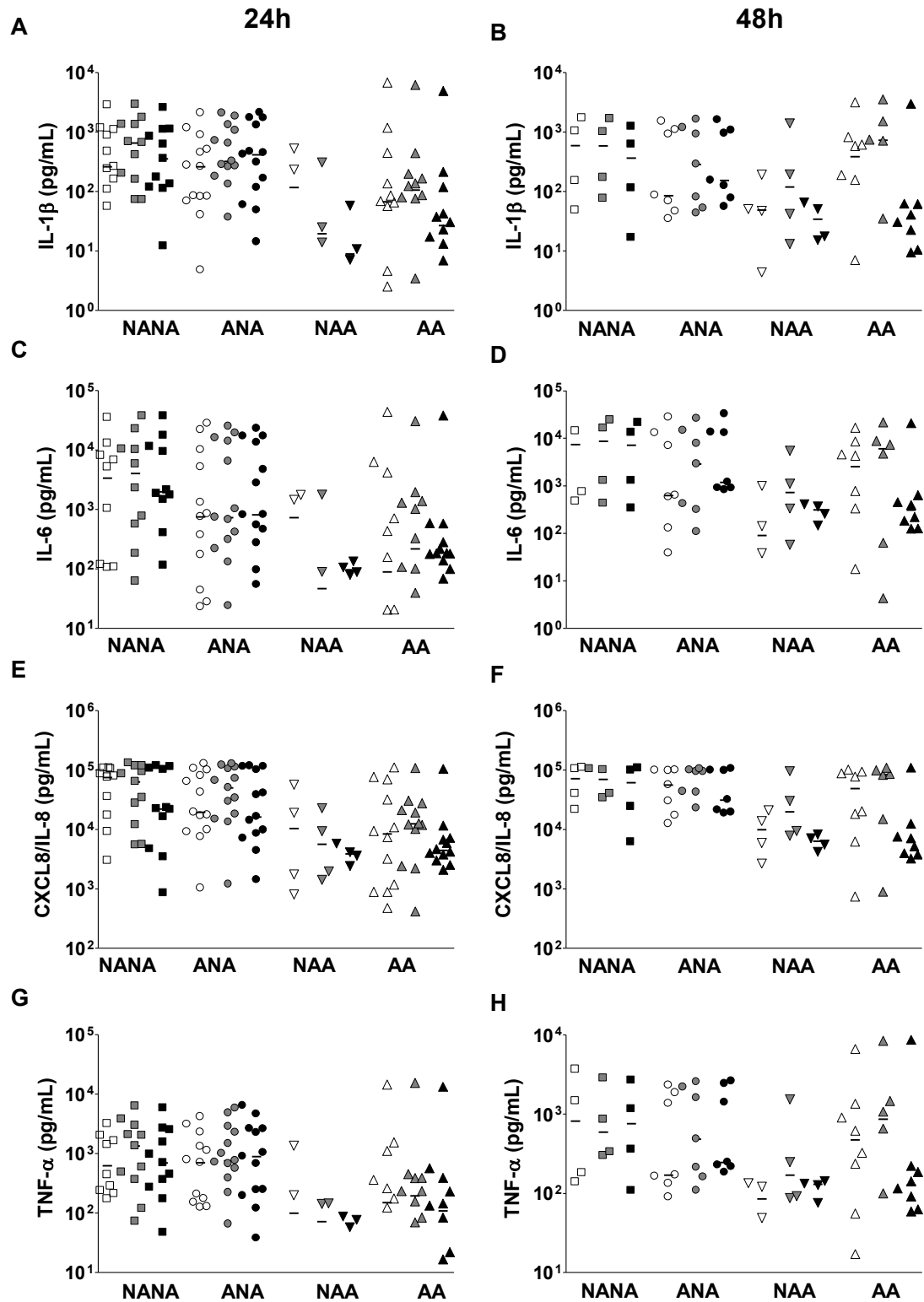


Figure 3.20. Proinflammatory cytokine protein induction in PBMCs stimulated with RV16 at 24h (A, C, E and G) and 48h (B, D, F and H). IL-1 β (A and B), IL-6 (C and D), CXCL8/IL-8 (E and F) and TNF- α (G and H). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, grey shapes denote filtered virus, black shapes denote virus. RV16 did not induce proinflammatory cytokine production in PBMCs at 24h or 48h. High baseline levels of all cytokines were observed.

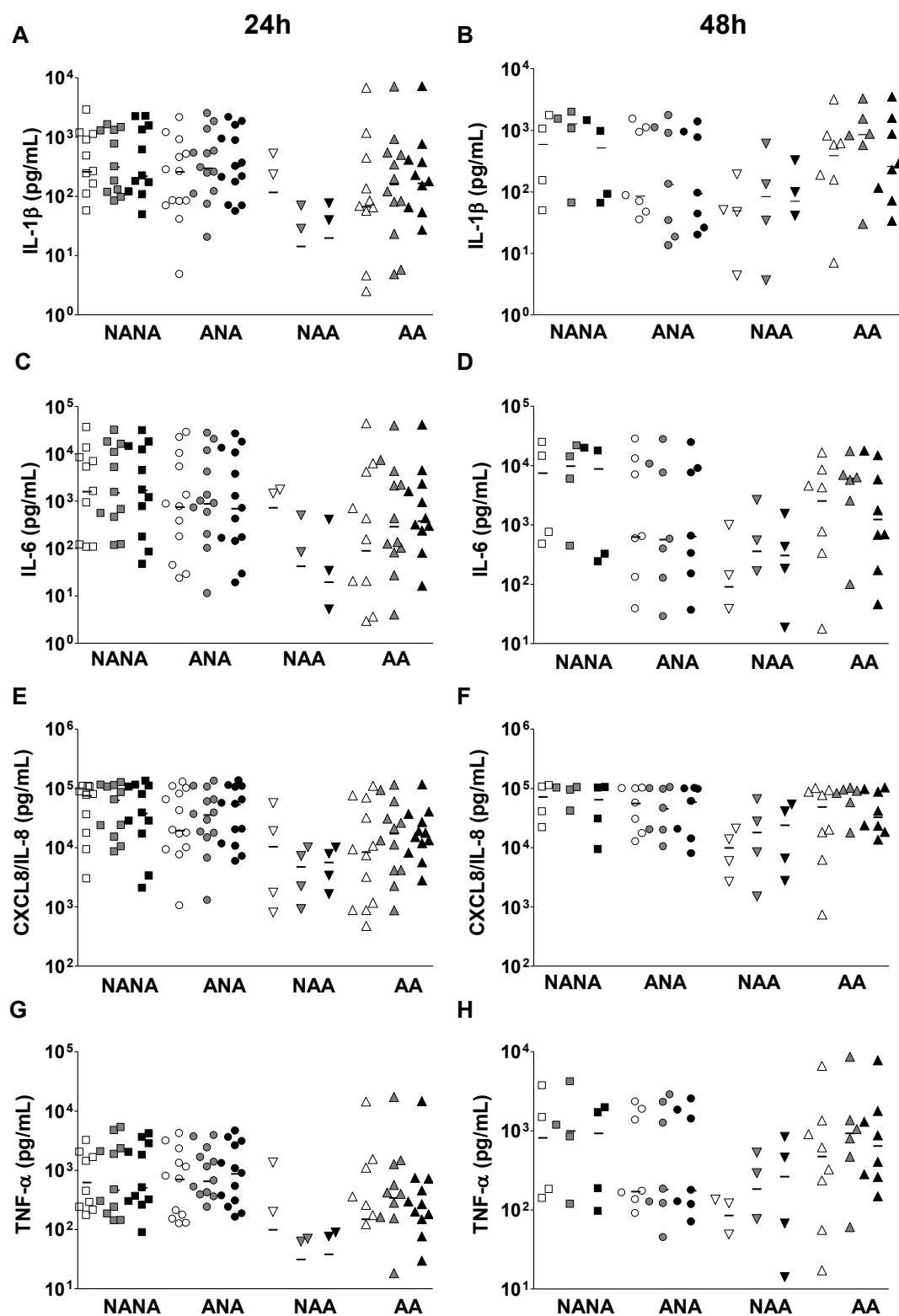


Figure 3.21. Proinflammatory cytokine protein induction in PBMCs stimulated with RV1B at 24h (A, C, E and G) and 48h (B, D, F and H). IL-1 β (A and B), IL-6 (C and D), CXCL8/IL-8 (E and F) and TNF- α (G and H). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, grey shapes denote filtered virus, black shapes denote virus. RV1B did not induce proinflammatory cytokine production in PBMCs at 24h or 48h. High baseline levels of all cytokines were observed.

Tables 3.9 and 3.10 summarise the IFN and proinflammatory cytokine protein induction in PBMCs following RV16 and RV1B infection.

Table 3.9 Summary of IFN and proinflammatory cytokine protein induction post RV16 infection in PBMCs ↑ = significant induction, ns = non-significant increase observed, - = no induction

	24h				48h			
	NANA	ANA	NAA	AA	NANA	ANA	NAA	AA
IFN-α2a	↑	↑	ns	↑	ns	↑	ns	↑
IFN-β	↑	↑	ns	↑	ns	ns	ns	↑
IFNλ1/IL-29	ns	↑	ns	↑	ns	ns	ns	↑
IL-1β	-	-	-	-	-	-	-	-
IL-6	-	-	-	-	-	-	-	-
CXCL8/IL-8	-	-	-	-	-	-	-	-
TNF-α	-	-	-	-	-	-	-	-

Table 3.10 Summary of IFN and proinflammatory cytokine protein induction post RV1B infection in PBMCs - = no induction

	24h				48h			
	NANA	ANA	NAA	AA	NANA	ANA	NAA	AA
IFN-α2a	-	-	-	-	-	-	-	-
IFN-β	-	-	-	-	-	-	-	-
IFNλ1/IL-29	-	-	-	-	-	-	-	-
IL-1β	-	-	-	-	-	-	-	-
IL-6	-	-	-	-	-	-	-	-
CXCL8/IL-8	-	-	-	-	-	-	-	-
TNF-α	-	-	-	-	-	-	-	-

3.2.8 RV infection induced RV replication and viral release

RV infection of HBECs from AA has been reported to result in significantly increased viral RNA (vRNA) production and virus release into cell supernatants when compared to NANA⁶⁰. In order to assess whether cells from all the volunteer groups had increased RV replication and release, and to see whether there was a difference between asthmatic and atopic status, vRNA was measured in cell lysates and live virus release was measured in cell supernatants using a virus titration assay in TCID₅₀/mL.

RV replication at individual time points is demonstrated in figure 3.22, with the time course illustrated in figure 3.23. Although cells were washed following the initial stimulation with virus, high levels of RV vRNA were observed at 0h (figure 3.22 A and B). With RV16, vRNA expression peaked at 24h in NANA, NAA and AA and at 48h in ANA (figure 3.22 C, E and G, and figure 3.23 A). With RV1B, vRNA expression peaked at 48h (figure 3.22 D, F and H, and figure 3.23 B). No significant differences in vRNA expression following stimulation with RV16 or RV1B were observed between all volunteer groups.

Virus release into cell supernatants at individual time points is demonstrated in figure 3.24, with the time course illustrated in figure 3.25. Peak viral release was at 24h with RV16 (figure 3.24 A, C, E and G, and figure 3.25 A) and at 48h with RV1B (figure B, D, F and H, and figure 3.25 B). No significant differences in virus release following stimulation with RV16 or RV1B were observed between all volunteer groups.

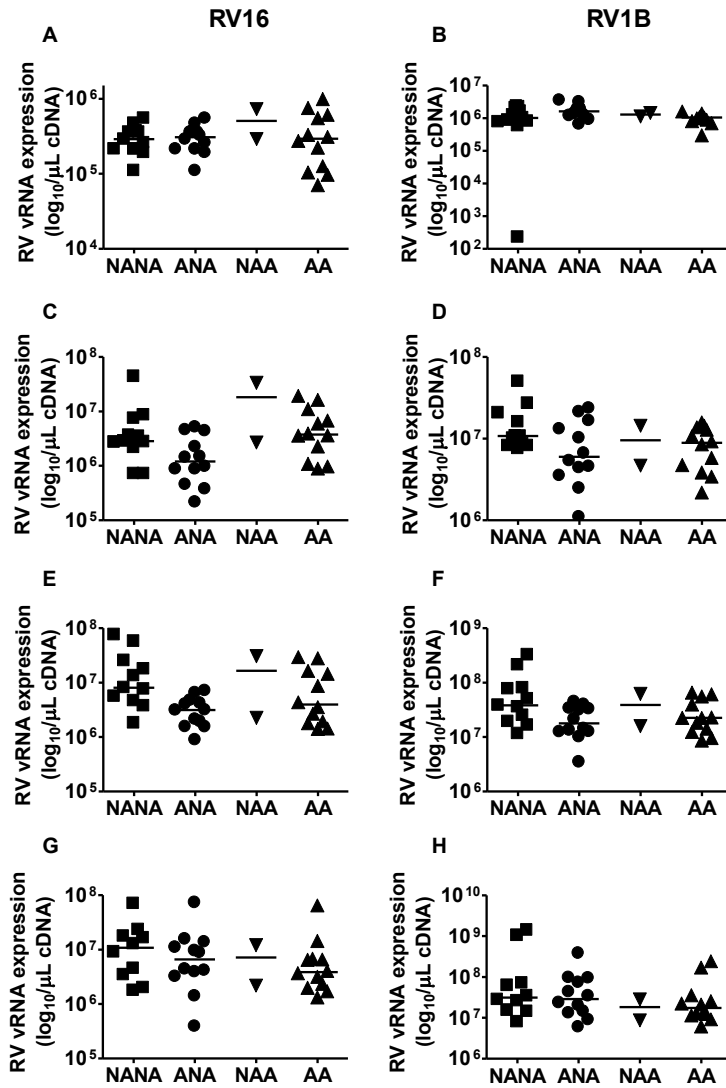


Figure 3.22. RV vRNA induction in HBECs after inoculation with RV16 (A, C, E and G) and RV1B (B, D, F and H) at 0h (A and B), 8h (C and D), 24h (E and F) and 48h (G and H)). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA. No significant differences in vRNA were observed between groups.

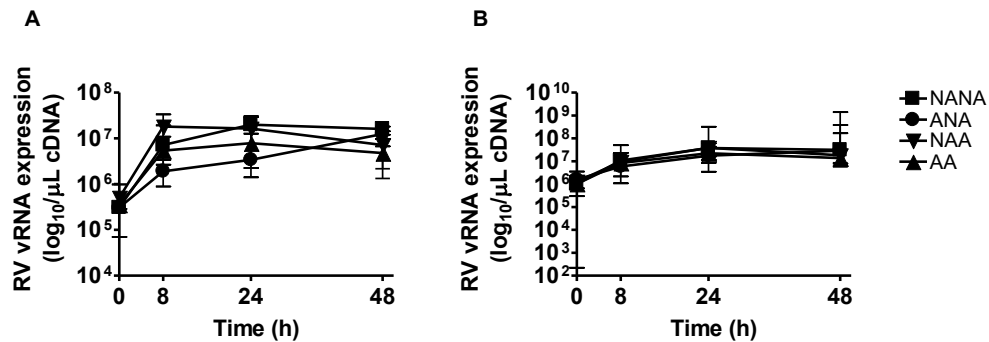


Figure 3.23. Time courses demonstrating RV vRNA induction in HBECs after inoculation with RV16 (A) and RV1B (B). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA. No significant differences in vRNA were observed between groups.

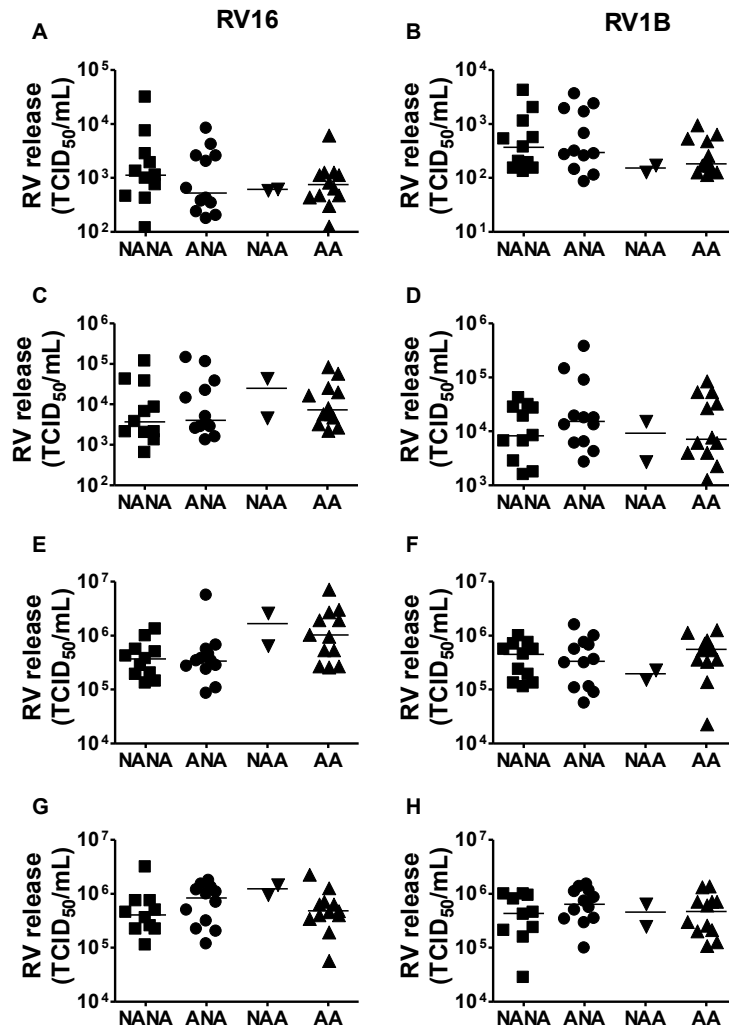


Figure 3.24. RV release in supernatants of HBECs after inoculation with RV16 (A, C, E and G) and RV1B (B, D, F and H) measured by TCID₅₀/mL at 0h (A and B), 8h (C and D), 24h (E and F) and 48h (G and H). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA. No significant differences were observed between groups, though a trend for increased RV release in asthmatics was observed at 24h after inoculation with RV16.

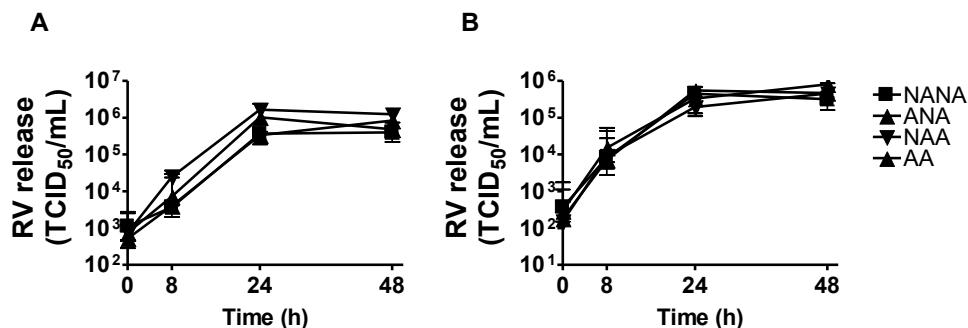


Figure 3.25. Time courses demonstrating RV release in supernatants of HBECs after inoculation with RV16 (A) and RV1B (B) measured by TCID₅₀/mL. Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA. Peak viral release was at 24h with RV16 and 48h with RV1B. No differences were observed between groups.

3.2.9 TLR 3, 7, 8 ligands stimulation of HBECs: induction of type I and III IFN and proinflammatory cytokines mRNA and protein

TLR3 recognises viral dsRNA. TLR7 and TLR8 recognise viral ssRNA. In order to characterise the IFN and proinflammatory cytokine response to “viral TLR” stimulation and to clarify whether the response is different depending on asthmatic and atopic status, HBECs from volunteers were stimulated with poly(IC) (TLR3) and R848 (TLR7/8). Type I and III IFN and IL-6 and CXCL8/IL-8 mRNA was measured at 8h and 24h post stimulation and type I and III IFN and IL-1 β , IL-6, CXCL8/IL-8 and TNF- α protein was measured at 24h post stimulation.

IFN- β mRNA was induced by poly(IC) at 8h in NANA ($p<0.01$), ANA ($p<0.01$) and AA ($p<0.01$) with a non-significant increase observed in NAA (figure 3.26 A). At 24h a significant increase was observed in NANA ($p<0.05$), with non-significant increases observed in ANA, NAA and AA (figure 3.26 B). No significant differences were observed between groups. IFN- β mRNA was not significantly induced by R848 in any group, but small increases were observed (figure 3.27 A and B). No differences were observed between groups.

IFN- λ 2,3/IL-28 mRNA was induced by poly(IC) at 8h in NANA ($p<0.01$), ANA ($p<0.01$) and AA ($p<0.001$) with a non-significant increase observed in NAA (figure 3.26 C). At 24h a significant increase was observed in NANA ($p<0.001$), ANA ($p<0.001$) and AA ($p<0.01$) with non-significant increases observed in NAA (figure 3.26 D). No significant differences were observed between groups. IFN- λ 2,3/IL-28 mRNA was induced by R848 at 8h in ANA and AA (both $p<0.05$), with a non-significant increase in NANA and NAA (figure 3.27 C). At 24h, significant increases were observed in ANA ($p<0.001$) and AA ($p<0.01$), with non-significant increases in NANA and NAA (figure 3.28 D). No significant differences were observed between groups.

IFN- λ 1/IL-29 mRNA was induced by poly(IC) at 8h in NANA ($p<0.01$), ANA ($p<0.01$) and AA ($p<0.001$) with a non-significant increase observed in NAA (figure 3.26 E). At 24h a significant increase was observed in NANA ($p<0.001$), ANA ($p<0.001$) and AA ($p<0.01$) with non-significant increases observed in NAA (figure

3.26 F). No significant differences were observed between groups. IFN- λ 1/IL-29 mRNA was induced by R848 at 8h in AA ($p<0.01$), with a non-significant increase in NANA, ANA and NAA (figure 3.27 E). At 24h, significant increases were observed in NANA, ANA and AA (all $p<0.01$), with non-significant increases in NAA (figure 3.28 F). No significant differences were observed between groups.

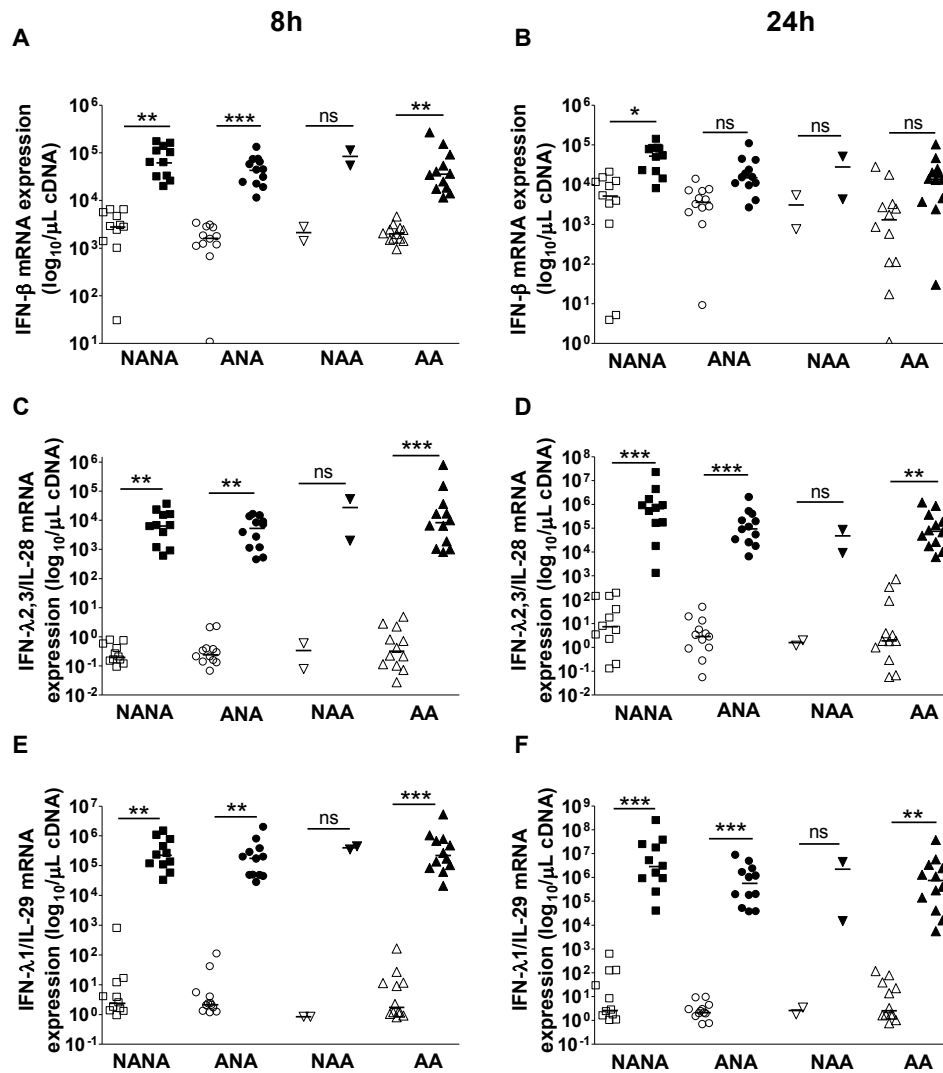


Figure 3.26. Interferon mRNA induction in HBECs post stimulation with poly(IC) at 8h (A, C and E) and 24h (B, D and F). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, black shapes denote poly(IC) ; * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, ns = non-significant. IFN-β (A and B), IFN-λ2,3/IL-28 (C and D), IFN-λ1/IL-29 (E and F). Type I and III IFN mRNA was induced by poly (IC) at both time points. No significant differences were observed between groups.

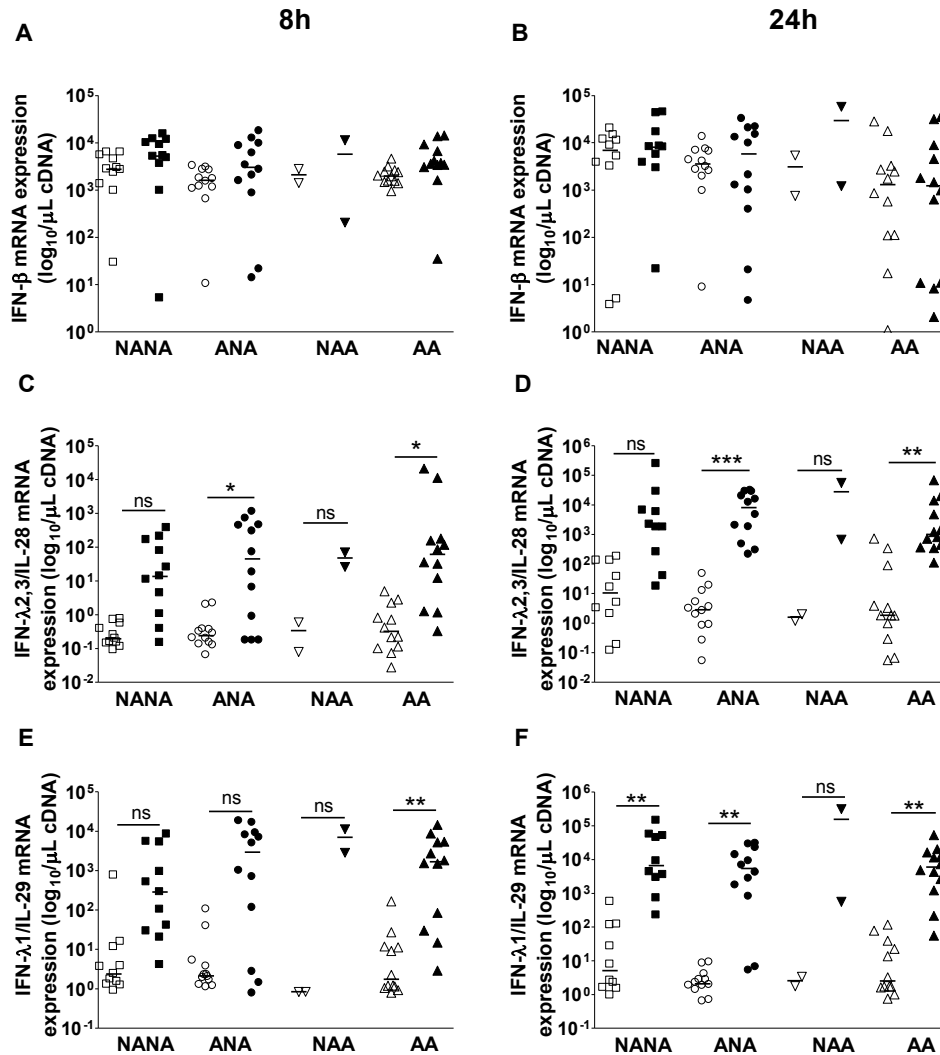


Figure 3.27. Interferon mRNA induction in HBECs post stimulation with R848 at 8h (A, C and E) and 24h (B, D and F). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, black shapes denote R848; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, ns = non-significant. IFN-β (A and B), IFN-λ1/IL-28 (C and D), IFN-λ1/IL-29 (E and F). IFN-β mRNA was not significantly induced by R848. Type III IFN mRNA was induced by R848 at both time points. No significant differences were observed between groups.

Stimulation of HBECs with poly(IC) resulted in non-significant increases in IFN-β protein at 24h in all groups (figure 3.28 A). Increases in IFN-λ1/IL-29 protein were observed at 24h (figure 3.28 B) with significance in NANA ($p < 0.001$), ANA ($p < 0.01$) and AA ($p < 0.001$) and a non-significant increase in NAA. No differences were observed between groups in the poly(IC) induced production of type I or III IFN protein. Stimulation of HBECs with R848 did not induce type I or III IFN protein at

24h (figure 3.28 C and D). An unexplained reduction in IFN- β protein was observed with R848 in AA only, with no significant differences between groups.

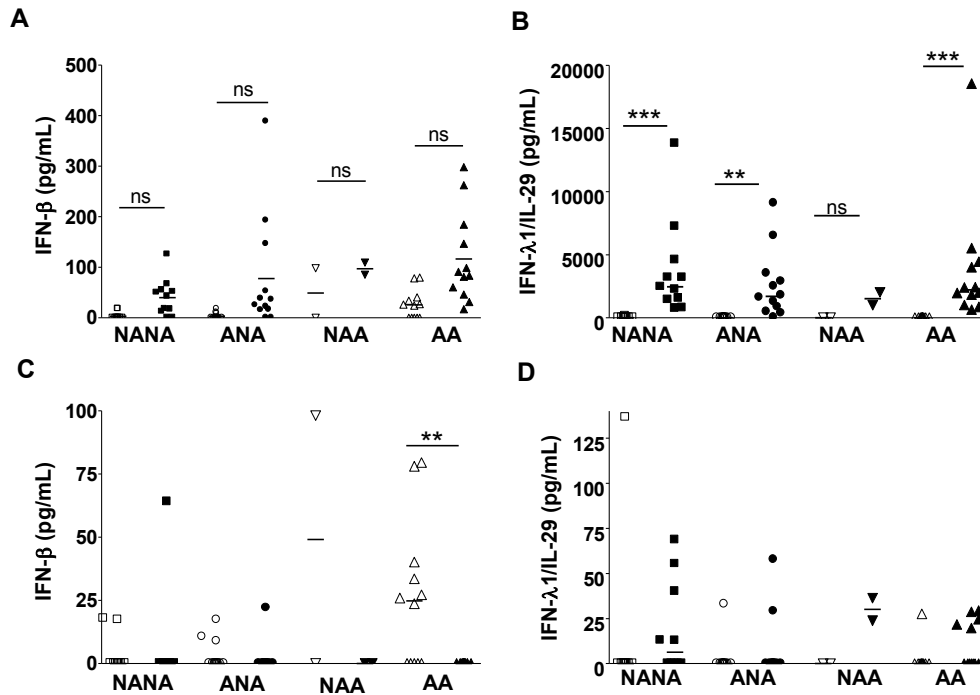


Figure 3.28. Interferon protein induction in HBECs post stimulation with poly(IC) (A and B) and R848 (C and D) at 24h. Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, black shapes denote ligand; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, ns = non-significant. IFN- β (A and C), IFN- λ 1/IL-29 (B and D). Increases in type I and III protein were observed following stimulation with poly(IC), with no differences between groups. No increases were seen following stimulation with R848. An unexplained reduction in IFN- β protein was observed in AA only, with no significant differences between groups.

IL-6 and CXCL8/IL-8 mRNA was measured in HBECs at 8h and 24h post TLR3 and TLR7/8 stimulation. Significant induction of IL-6 mRNA was observed at 8h following stimulation with poly(IC) in NANA ($p < 0.01$), ANA ($p < 0.001$) and AA ($p < 0.01$) with a non-significant increase observed in NAA (figure 3.29 A). At 24h, significant induction was observed in NANA, ANA and AA (all $p < 0.01$) with a non-significant increase observed in NAA (figure 3.29 B). Likewise, at 8h, significant induction of CXCL8/IL-8 mRNA was observed in NANA ($p < 0.05$), ANA ($p < 0.001$) and AA ($p < 0.01$) with a non-significant induction observed in NAA (figure 3.29 C) and at

24h induction was observed in NANA ($p<0.01$), ANA ($p<0.01$) and AA ($p<0.001$), with a non-significant induction observed in NAA (figure 3.29 D). With both IL-6 and CXCL8/IL-8 mRNA no significant differences were observed between groups at both time points.

Following stimulation of HBECs with R848 no induction of IL-6 mRNA was observed at 8h or 24h in all groups (figure 3.29 E and F). Significant induction of CXCL8/IL-8 mRNA was observed at 8h in NANA ($p<0.01$), ANA ($p<0.001$) and AA ($p<0.001$), with a non-significant induction observed in NAA (figure 3.29 G). At 24h, significant induction of CXCL8/IL-8 mRNA was observed in ANA ($p<0.001$) and AA ($p<0.01$), with a non-significant induction observed in NANA and NAA (figure 3.29 H). With both IL-6 and CXCL8/IL-8 mRNA no significant differences were observed between groups at both time points.

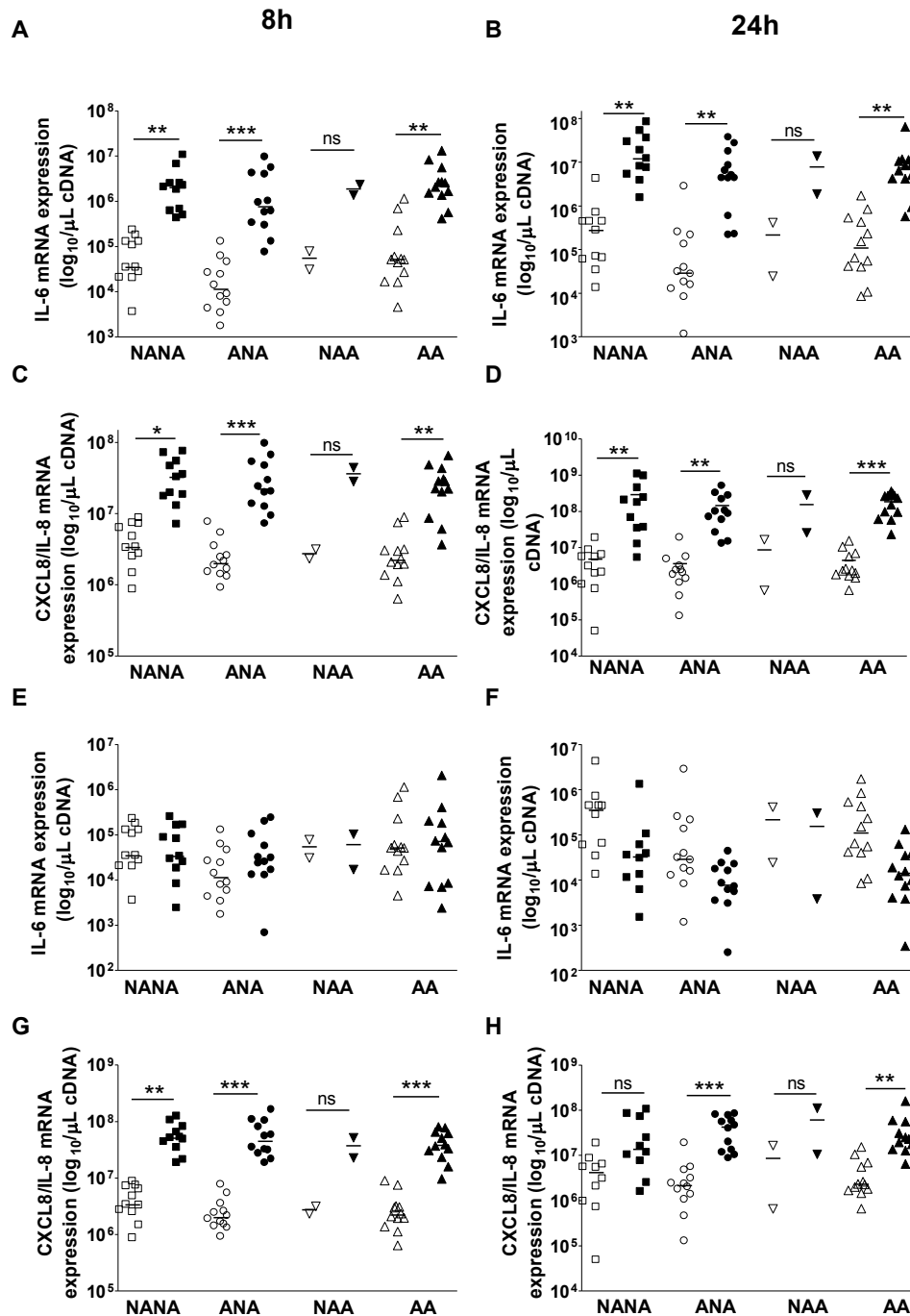


Figure 3.29. IL-6 mRNA induction in HBECs post stimulation with poly(IC) at 8h (A) and 24h (B) and R848 at 8h (E) and 24h (F); CXCL8/IL-8 mRNA induction in HBECs post stimulation with poly(IC) at 8h (C) and 24h (D) and R848 at 8h (G) and 24h (H). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, grey shapes denote filtered virus, black shapes denote virus; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, ns = non-significant. Following stimulation with poly(IC) significant induction of IL-6 and CXCL8/IL-8 mRNA was observed at both 8h and 24h with no significant difference between groups. Following stimulation with R848, no induction of IL-6 mRNA was observed, but significant induction of CXCL8/IL-8 mRNA was observed at both time points, with no significant differences between groups.

Proinflammatory cytokine proteins were measured in HBECs at 24h following stimulation of TLR3 and TLR7/8. Significant production of IL-1 β , IL-6, CXCL8/IL-8 and TNF- α protein from HBECs stimulated with poly(IC) was observed at 24h in NANA, ANA and AA with non-significant increases observed in NAA (figure 3.30 A-D). No differences were observed between the four volunteer groups.

Following stimulation with R848 non-significant weak production of IL-1 β protein was observed in all groups (figure 3.30 E). Production of IL-6 protein was not observed (figure 3.30 F). Non-significant production of CXCL8/IL-8 protein was observed in ANA only (figure 3.30 G). Significant production of TNF- α protein was observed in ANA ($p < 0.05$), with non-significant increases in NAA (figure 3.30 H). Increases were not observed in NANA or AA. With all cytokines measured, no differences were observed between groups.

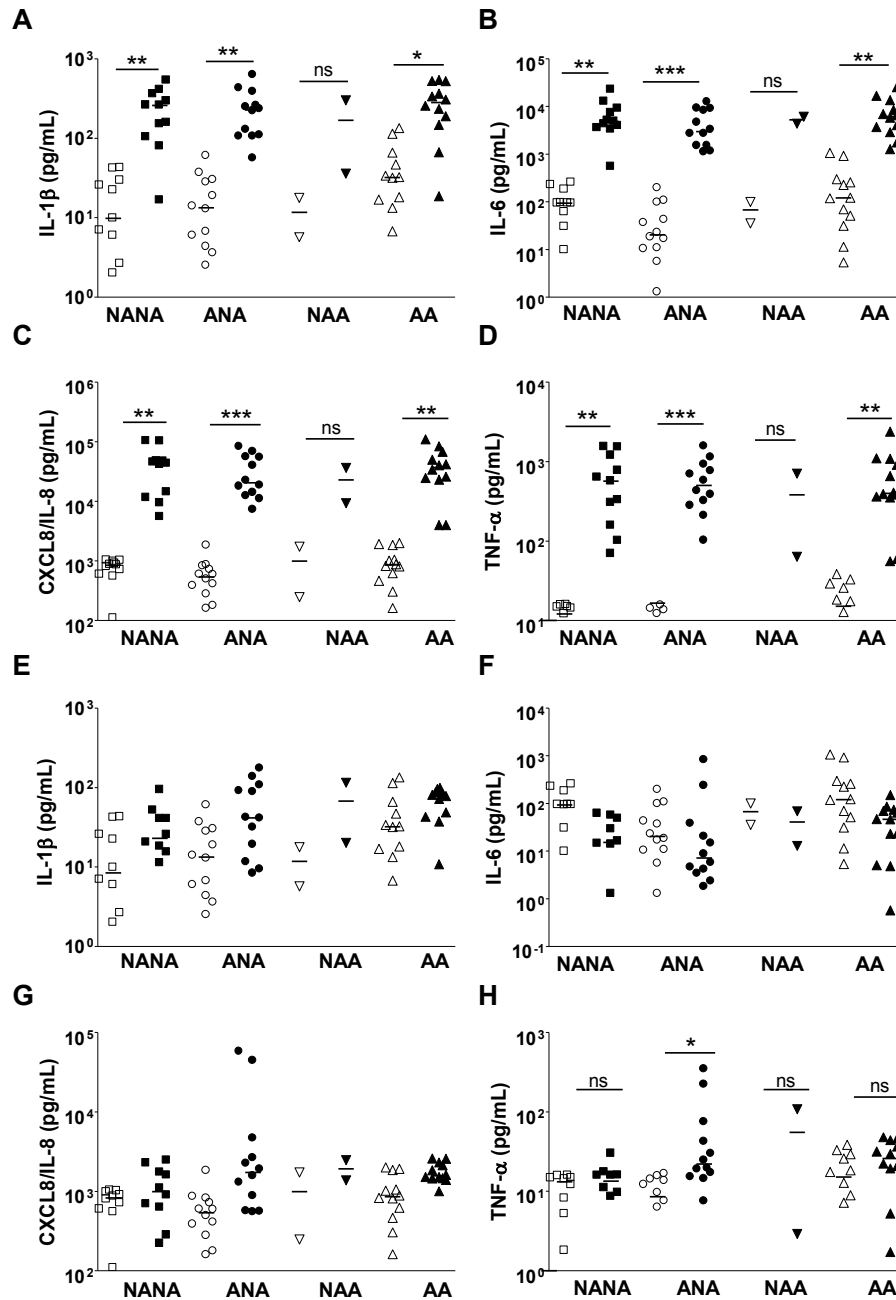


Figure 3.30. Proinflammatory cytokine protein induction in HBECs stimulated with poly(IC) (A, B, C and D) and R848 (E, F, G and H) at 24h. IL-1 β (A and E), IL-6 (B and F), CXCL8/IL-8 (C and G) and TNF- α (D and H). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, grey shapes denote filtered virus, black shapes denote virus; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, ns = non-significant. Significant production of all cytokines was observed following stimulation with poly(IC). At 24h post stimulation with R848, weak production of IL-1 β in all groups, no production of IL-6, production of CXCL8/IL-8 in ANA and of TNF- α in ANA and NAA, was observed. No differences were observed between groups.

Tables 3.11 and 3.12 summarise the IFN and proinflammatory cytokine mRNA and protein induction in HBECs following poly(IC) and R848 stimulation.

Table 3.11 Summary of IFN and proinflammatory cytokine mRNA and protein induction post poly(IC) stimulation in HBECS ↑ = significant induction, ns = non-significant increase observed, black squares = not performed

	8h				24h			
	NANA	ANA	NAA	AA	NANA	ANA	NAA	AA
IFN-β mRNA	↑	↑	ns	↑	↑	ns	ns	ns
IFN-β protein					ns	↑	ns	ns
IFNλ2,3/IL-28 mRNA	↑	↑	ns	↑	↑	↑	ns	↑
IFNλ1/IL-29 mRNA	↑	↑	ns	↑	↑	↑	ns	↑
IFNλ1/IL-29 protein					↑	↑	ns	↑
IL-6 mRNA	↑	↑	ns	↑	↑	↑	ns	↑
CXCL8/IL-8 mRNA	↑	↑	ns	↑	↑	↑	ns	↑
IL-1β protein					↑	↑	ns	↑
IL-6 protein					↑	↑	ns	↑
CXCL8/IL-8 protein					↑	↑	ns	↑
TNF-α protein					↑	↑	ns	↑

Table 3.12 Summary of IFN and proinflammatory cytokine mRNA and protein induction post R848 stimulation in HBECS ↑ = significant induction, ns = non-significant increase observed, - = no induction, ↓ = significant reduction, black squares = not performed

	8h				24h			
	NANA	ANA	NAA	AA	NANA	ANA	NAA	AA
IFN-β mRNA	-	-	-	-	-	-	-	-
IFN-β protein					-	-	-	↓
IFNλ2,3/IL-28 mRNA	ns	↑	ns	↑	ns	↑	ns	↑
IFNλ1/IL-29 mRNA	ns	ns	ns	↑	↑	↑	ns	↑
IFNλ1/IL-29 protein					-	-	-	-
IL-6 mRNA	-	-	-	-	-	-	-	-
CXCL8/IL-8 mRNA	↑	↑	ns	↑	ns	↑	ns	↑
IL-1β protein					ns	ns	ns	ns
IL-6 protein					-	-	-	-
CXCL8/IL-8 protein					-	ns	-	-
TNF-α protein					-	↑	ns	-

3.2.10 TLR 3, 7, 8 ligands stimulation of PBMCs: induction of type I and III IFN and proinflammatory cytokines protein

In order to characterise the IFN and proinflammatory cytokine response to “viral TLR” stimulation, to clarify whether the response is different depending on asthmatic and atopic status and to investigate whether other cell types were involved, PBMCs from volunteers were stimulated with poly(IC) (TLR3) and R848 (TLR7/8). Type I and III IFN and IL-1 β , IL-6, CXCL8/IL-8 and TNF- α protein was measured at 24h and 48h post stimulation (figures 3.31-3.35).

Responses to TLR3 and TLR7/8 stimulation were assessed in PBMCs from each of the different volunteer groups. TLR3 stimulation did not induce type I and III IFN protein production in PBMCs at 24h and 48h (figure 3.31). TLR7/8 stimulated PBMCs produced significant amounts of IFN- α 2a protein at 24h in NANA ($p<0.05$), ANA ($p<0.01$) and AA (all $p<0.01$), with non-significant increases observed in NAA (figure 3.32 A). At 48h non-significant increases were observed in all groups (figure 3.32 B). No differences were observed between groups. Small increases in IFN- β and IFN- λ 1/IL-29 protein at 24h and 48h were observed in all groups but these failed to reach statistical significance (figure 3.32 C-F). No differences were observed between groups.

Production of proinflammatory cytokines in PBMCs following TLR3 and TLR7/8 stimulation was assessed in each of the volunteer groups (figures 3.33-3.35). TLR3 stimulation resulted in significant induction of IL-1 β and IL-6 protein at 24h in AA ($p<0.01$) with non-significant increases in NANA, ANA and NAA (figure 3.33 A and C). In AA significant induction of CXCL8/IL-8 ($p<0.05$) and TNF- α ($p<0.001$) was observed at 24h with non-significant increases observed in NANA, ANA and NAA (figure 3.33 E and G). No significant differences were observed between groups. At 48h non-significant increases were observed in all 4 proinflammatory cytokines measured in all groups (figure 3.33 B, D, F and H). No significant differences were observed between groups. To assess the significance of proinflammatory cytokine production at 48h and having established no significant difference between individual groups, all groups were combined. Significant induction of IL-1 β protein ($p<0.001$), IL-6 protein ($p<0.001$), CXCL8/IL-8 protein

($p < 0.01$) and TNF- α protein ($p < 0.001$) was observed at 48h following TLR3 stimulation of PBMCs from all volunteer groups combined (figure 3.35 A-D).

TLR7/8 stimulation resulted in significant induction of IL-1 β and IL-6 protein at 24h in AA ($p < 0.01$) with non-significant increases in NANA, ANA and NAA (figure 3.34 A and C). Induction of CXCL8/IL-8 protein was not observed (figure 3.34 E). Weak induction of TNF- α protein was observed in AA ($p < 0.01$) at 24h with non-significant increases observed in NANA, ANA and NAA (figure 3.34 G). No significant differences were observed between groups. At 48h non-significant increases were observed in IL-1 β in all groups (figure 3.34 B). Non-significant induction of IL-6 was observed in NANA and NAA (figure 3.34 D). Non-significant induction of CXCL8/IL-8 protein was observed in NAA only (figure 3.34 F). Non-significant induction of TNF- α protein was observed in NANA, ANA and NAA (figure 3.34 H). No significant differences were observed between groups. To assess the significance of proinflammatory cytokine production at 48h and having established no significant difference between individual groups, all groups were combined. Significant induction of IL-1 β protein ($p < 0.001$) and TNF- α protein ($p < 0.05$) was observed at 48h following TLR7/8 stimulation of PBMCs (figure 3.35 E and H). Induction of IL-6 and CXCL8/IL-8 protein was not observed in PBMCs following TLR7/8 stimulation (figure 3.35 F and G).

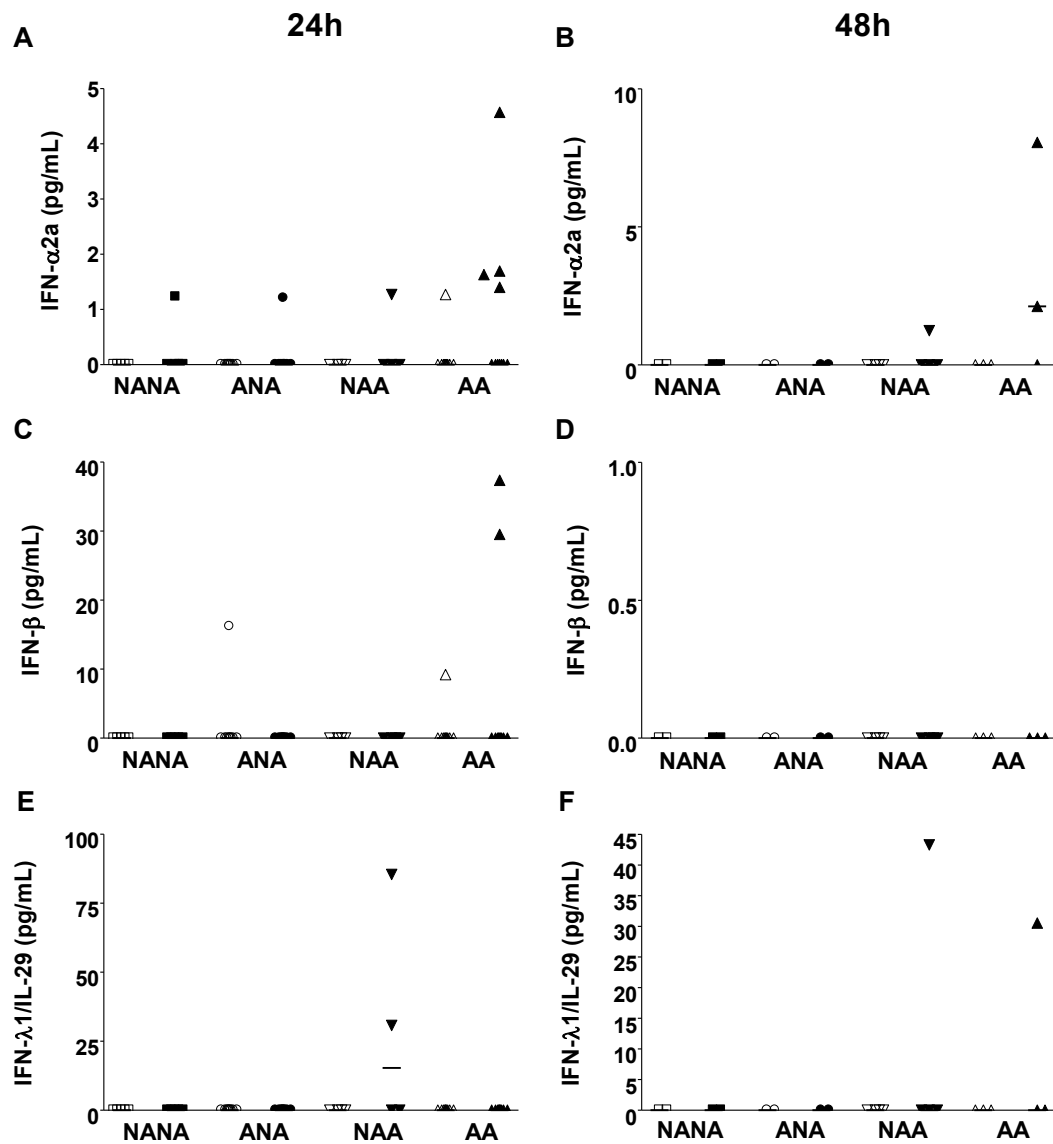


Figure 3.31. Interferon protein induction in PBMCs stimulated with poly(IC) at 24h (A, C and E) and 48h (B, D and F). IFN-α2a (A and B), IFN-β (C and D) and IFN-λ1/IL-29 (E and F). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, black shapes denote poly(IC). No production of type I or III IFN was observed in PBMCs stimulated with poly(IC).

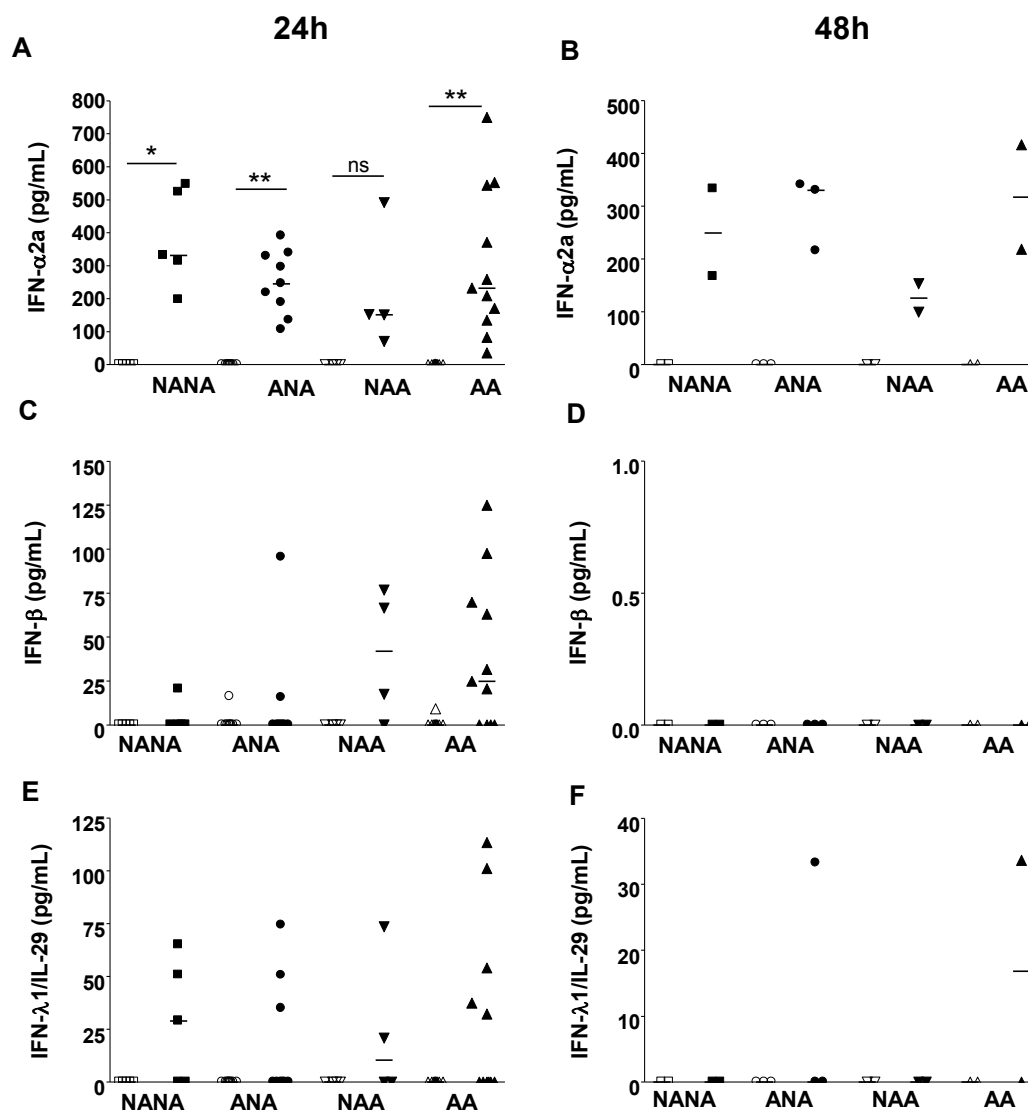


Figure 3.32. Interferon protein induction in PBMCs stimulated with R848 at 24h (A, C and E) and 48h (B, D and F). IFN-α2a (A and B), IFN-β (C and D) and IFN-λ1/IL-29 (E and F). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, black shapes denote R848; * = $p < 0.05$, ** = $p < 0.01$, ns = non-significant. Significant increases in IFN-α2a protein were observed in PBMCs stimulated with R848 at 24h with non-significant increases observed at 48h. Small increases in IFN-β and IFN-λ1/IL-29 protein were observed at 24h and 48h but these did not reach statistical significance. No significant differences were observed between groups.

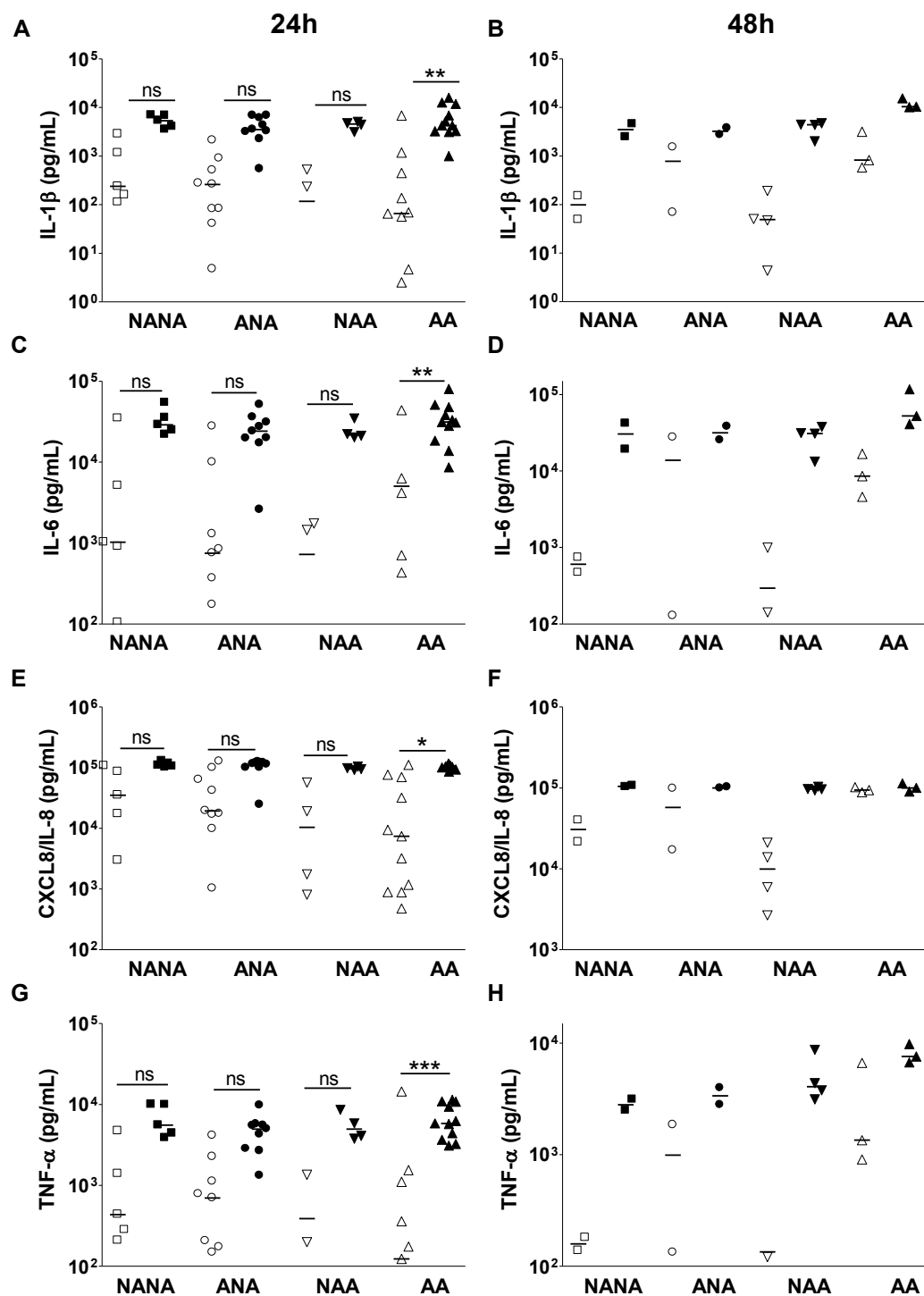


Figure 3.33. Proinflammatory cytokine protein induction in PBMCs stimulated with poly(IC) at 24h (A, C, E and G) and 48h (B, D, F and H). IL-1 β (A and B), IL-6 (C and D), CXCL8/IL-8 (E and F) and TNF- α (G and H). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, black shapes denote poly(IC); * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, ns = non-significant. Induction of proinflammatory cytokine protein was observed in all groups at 24h and 48h, with significant results observed in AA. At 48h increases were observed but these failed to reach statistical significance. No significant differences were observed between groups.

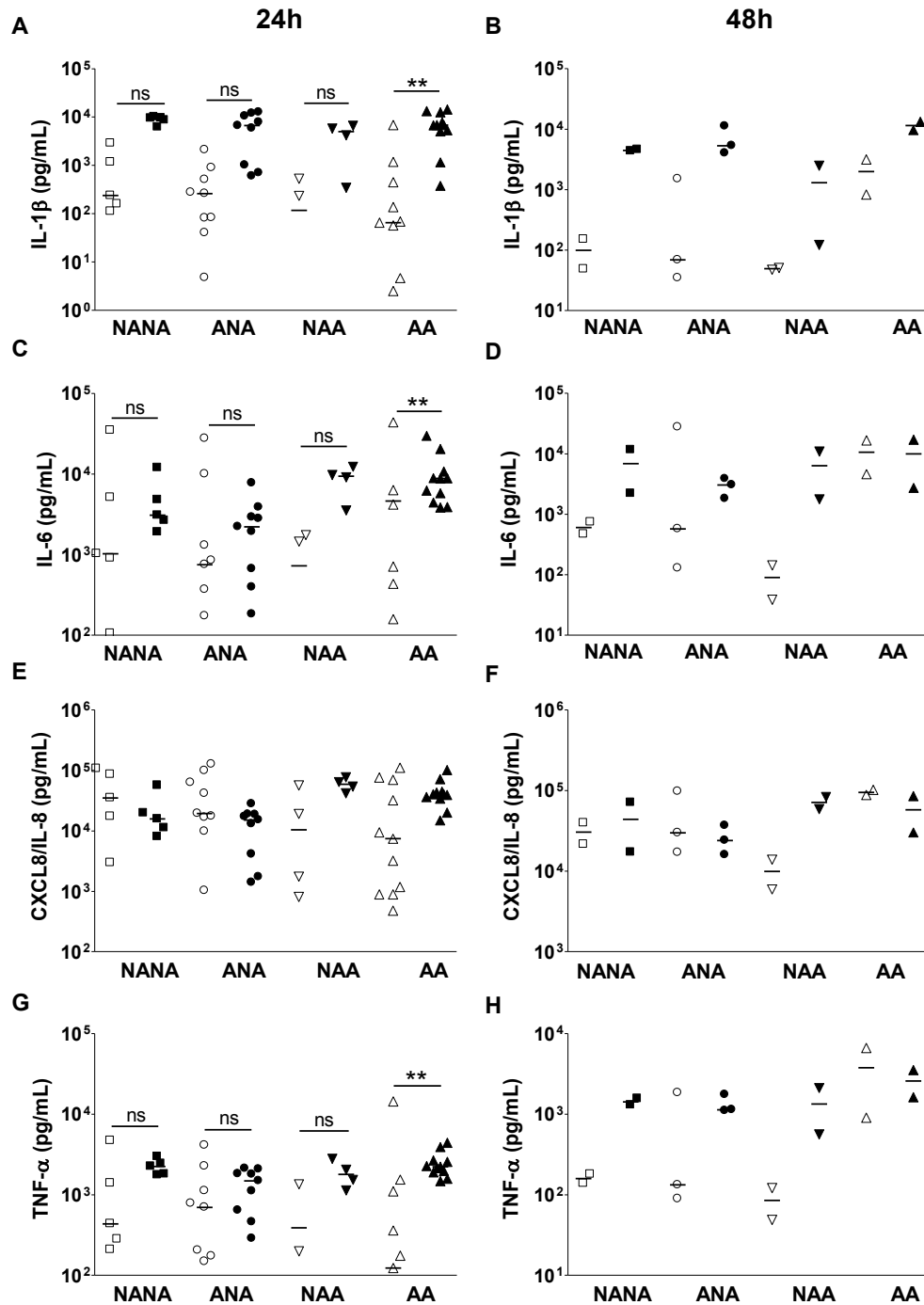


Figure 3.34. Proinflammatory cytokine protein induction in PBMCs stimulated with R848 at 24h (A, C, E and G) and 48h (B, D, F and H). IL-1 β (A and B), IL-6 (C and D), CXCL8/IL-8 (E and F) and TNF- α (G and H). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, black shapes denote R848; ** = $p < 0.01$, ns = non-significant. Significant induction of IL-1 β , IL-6 and TNF- α protein were observed at 24h in AA with non-significant increases observed in other groups. Induction of CXCL8/IL-8 was not observed. At 48h, non-significant increases in IL-1 β and TNF- α were observed in all groups and in IL-6 in NANA and NAA. No significant differences were observed between groups.

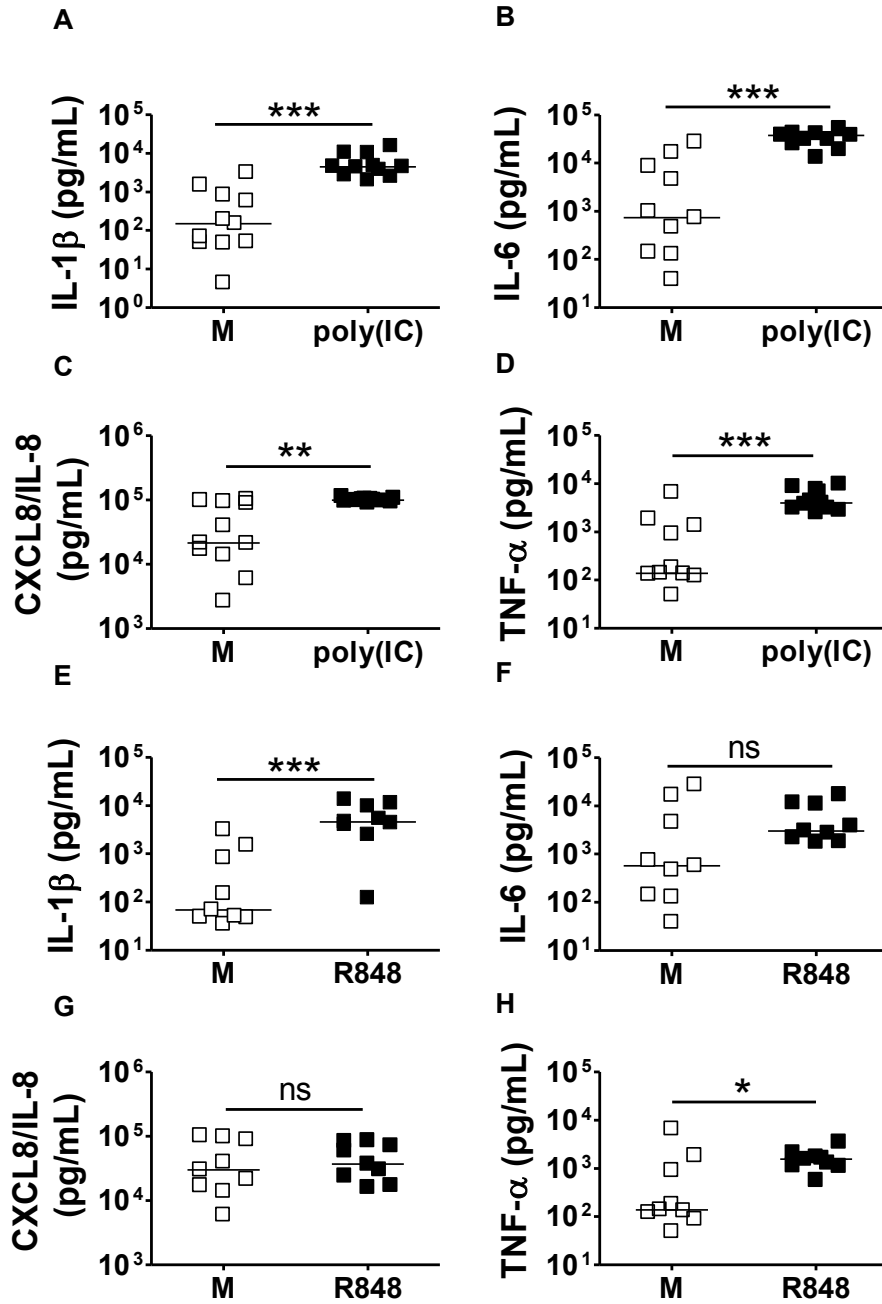


Figure 3.35. Proinflammatory cytokine protein induction at 48h in PBMCs from all groups combined stimulated with poly(IC) (A, B, C and D) and R848 (E, F, G and H). IL-1 β (A and E), IL-6 (B and F), CXCL8/IL-8 (C and G) and TNF- α (D and H). Open squares denote medium (M); black squares denote poly(IC) or R848; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, ns = non-significant. Non-significant induction of proinflammatory cytokine protein had been observed in all groups, with no apparent difference between groups. Groups were therefore combined. Significant induction of all proinflammatory cytokines measured was observed when PBMCs from all groups stimulated with poly(IC) were combined. Likewise, significant induction of IL-1 β and TNF- α protein but not IL-6 and CXCL8/IL-8 was observed in PBMCs from all groups stimulated with R848.

Tables 3.13 and 3.14 summarise the IFN and proinflammatory cytokine protein induction in PBMCs following poly(IC) and R848 stimulation.

Table 3.13 Summary of IFN and proinflammatory cytokine protein induction post poly(IC) stimulation in PBMCs ↑ = significant induction, ns = non-significant increase observed, - = no induction

	24h				48h			
	NANA	ANA	NAA	AA	NANA	ANA	NAA	AA
IFN-α2a	-	-	-	-	-	-	-	-
IFN-β	-	-	-	-	-	-	-	-
IFNλ1/IL-29	-	-	-	-	-	-	-	-
IL-1β	ns	ns	ns	↑	ns	ns	ns	ns
IL-6	ns	ns	ns	↑	ns	ns	ns	ns
CXCL8/IL-8	ns	ns	ns	↑	ns	ns	ns	ns
TNF-α	ns	ns	ns	↑	ns	ns	ns	ns

Table 3.14 Summary of IFN and proinflammatory cytokine protein induction post R848 stimulation in PBMCs ↑ = significant induction, ns = non-significant increase observed, - = no induction

	24h				48h			
	NANA	ANA	NAA	AA	NANA	ANA	NAA	AA
IFN-α2a	↑	↑	ns	↑	ns	ns	ns	ns
IFN-β	ns	ns	ns	ns	ns	ns	ns	ns
IFNλ1/IL-29	ns	ns	ns	ns	ns	ns	ns	ns
IL-1β	ns	ns	ns	↑	ns	ns	ns	ns
IL-6	ns	ns	ns	↑	ns	-	ns	-
CXCL8/IL-8	-	-	-	-	-	-	ns	-
TNF-α	ns	ns	ns	↑	ns	ns	ns	-

3.3 Discussion

Previous studies have identified defective type I and III IFN production in HBECs and type III IFN in BAL cells^{60, 61}. A more recent study has identified deficient type I IFN production in BAL cells from AA and this deficiency may be related to asthma severity²⁰⁸. Defective IFN- α 2 production has been observed in PBMCs from AA following RSV and NDV infection^{82, 206}. The only report of the role of atopy in deficient IFN production in asthmatics prior to initiation of this study was that of Bufe et al. which demonstrated significantly lower amounts of virus-induced IFN- α production in AA compared to NANA and NAA and that the number of eosinophils (a marker for allergic inflammation) negatively correlated with IFN- α levels²⁰⁶. While this study was ongoing, a recent study reported deficient IFN- β and - λ responses to rhinovirus infection in BECs in childhood in both atopic and non-atopic asthmatic subjects and surprisingly, in atopic children without asthma²⁰⁹. These atopic children without asthma, however, had similar degrees of IL-4 staining and epithelial damage in their bronchial biopsies as did the children with asthma, thus the roles of atopy and asthma in IFN deficiency remain uncertain. Another recent study examined innate immune responses in plasmacytoid dendritic cells (pDCs) to RV in allergic asthmatic children finding significant differences between allergic non-asthmatic and non-allergic non-asthmatic children³¹⁵. Another study identified deficient IFN- α secretion from pDCs stimulated with influenza viruses from allergic asthmatics which was inversely correlated with serum IgE levels²⁰⁵.

The experiments reported in this chapter aimed to investigate type I and III IFN and proinflammatory cytokine responses in atopics and non-atopics, asthmatics and non-asthmatics to determine whether the previously reported IFN deficiency observed in atopic asthmatic subjects is asthma- or atopy-specific. It also aimed to investigate whether deficiency was observed to similar degrees in several different cell types – BAL cells, HBECs and PBMCs sampled simultaneously from the same subjects, as with the exception of Contoli et al, who reported on IFN- λ deficiency in both BAL cells and BECs, but did not report PBMCs⁶¹, and Sykes et al who reported type I IFN deficiency in BAL cells and PBMCs, but who did not report BECs²⁰⁸, previous reports of IFN deficiency in asthma have only reported results in single cell types.

3.3.1 Rhinoviruses

3.3.1.1 BAL cells & RV

This study demonstrated differences between major and minor group RVs in their abilities to induce type I and III IFNs in BAL cells (tables 3.3 and 3.4), with both type I and III IFNs being induced by RV16 and only type III IFN being induced by RV1B. Indeed neither type I or III IFN protein was produced at 8h following stimulation with RV1B, although this may have been observed at a later time point, if sufficient cells had been acquired at bronchoscopy. The reason for these differences is unclear. Although titres of RV16 and RV1B stocks were not identical, as measured by TCID₅₀ assays (RV16 2×10^7 , RV1B 8.43×10^6), RV1B is a more potent virus, as determined by the fact that more diluted virus was required to produce similar effects in PBMCs, which was translated to experiments with BAL cells (figure 3.2).

Deficiencies in IFN production, both mRNA and protein were not observed in asthmatics (figures 3.7 and 3.8), meaning the question of whether asthma or atopy is important in IFN deficiency remains unanswered. The lack of observed deficiency differs from another study where type I IFN production was reduced in atopic asthmatics, compared to non-atopic non-asthmatics²⁰⁸. The differences between the two studies may be a result of this study having a group of less severe asthmatics; as although ACQ score for the asthmatic subjects reported in this study and that of Sykes et al were similar (0.917 vs 0.8), the subjects in Sykes et al. had more exacerbations/year (0.0 vs 1.5), and more severe airway hyperresponsiveness (PC₂₀ 3.0 vs 2.0), i.e. this study's group of asthmatics, had well controlled disease, were not exacerbation prone and had normal lung function. In analysis of the severity of asthmatics, it would have been of interest to determine the median dose of inhaled corticosteroid and the peripheral eosinophil and neutrophil counts (data not available).

It is possible that the lack of identified IFN- λ deficiency was due to only an 8h time point being used, due to the limited number of cells obtained at bronchoscopy and the desire to investigate responses to *Spn* also. The previous studies in BAL

cells observed deficient IFN- λ protein production at 48h after RV16 infection ⁶¹ and type I IFN deficiency at both 8 and 24hrs ²⁰⁸.

It is of interest, and perhaps surprise that RV failed to induce production of proinflammatory cytokine mRNA and protein. High baseline levels of these cytokines was observed in media treated cells, suggesting the acquisition of BAL cells at bronchoscopy itself resulted in the production of proinflammatory cytokines, an inherent problem with an *ex vivo* model of infection.

3.3.1.2 HBECs & RV

IFN production in HBECs was different for major and minor group RVs. IFN- β and type III IFN mRNA was observed for both groups, though protein production differed. With RV16 both type I and III IFN protein was produced at 48h, but with RV1B only type III IFN protein was observed. This is in agreement with previous studies suggesting diversity in IFN responses to different RV strains ²⁰¹. This previous study however noted different HBEC responses to different RV infections in that major group RVs produce a more intense inflammatory mediator release and minor group RVs produce a marked IFN- β response. Proinflammatory cytokine release, both mRNA and protein, was observed in all groups with both RV16 and RV1B, reaching significance with certain cytokines and certain groups, but with no significant difference between the different volunteer groups (tables 3.5 - 3.8).

The lack of difference in IFN production from HBECs between asthmatics and non-asthmatics is somewhat surprising given the previous studies identifying IFN deficiencies in cells stimulated with different RV strains ^{60, 61}. Given the lack of difference in BAL cells in this study, however, it may not be surprising that differences were not observed in HBECs, perhaps due to the mild nature of the disease in atopic asthmatics. Other studies failed to reproduce the findings of deficient IFN production by Wark et al. and Contoli et al. ^{203, 204}. The Lopez-Souza et al. study differed however in that the HBECs were cultured using an air liquid interface (ALI) and the sample size (5 NANA and 6 AA) was small. ALI cultures allow the development of tight junctions and cilia, which may resemble the *in vivo* appearance of the airway more closely ²⁰³. The Bochkov et al. study used

microarrays and qPCR and discovered no difference in type I or III IFN expression between NANA and AA ²⁰⁴. These other studies support the finding from the results reported in this chapter in that an IFN deficiency was not identified in asthmatics. More recent studies have identified deficient IFN responses in children with asthma, both atopic and non-atopic, and in atopic patients without asthma, with airway Th₂ inflammatory profile and epithelial damage correlating with decreased IFN production ²⁰⁹. Similarly, a study in severe therapy resistant asthmatic children had impaired type I and III IFN mRNA and protein in response to RV ³¹⁶. These studies support the conclusion that the reason why there was no IFN deficiency identified in this study was that the asthmatic subjects had too mild disease. Due to problems of NAA volunteer recruitment, and that only 2 volunteers' HBECs were experimented on (the other 2 being lost to contamination), sample size was insufficient to determine whether a difference might be seen in NAA. Certainly the small numbers of NAA recruited would have had an impact on the robustness of the data, had a difference been identified. Another potential flaw in the study design was that more severe asthmatics were not sought out, but the study used a similar template and inclusion/exclusion criteria to other studies which identified IFN deficiency ²⁰⁸. A potential explanation for the lack deficient IFN production in asthmatics observed in this study and other studies ^{203, 204} may be that this group of asthmatics had a more mild form of the disease and that there are subgroups of asthmatics which have IFN deficiency.

Why might there have been a difference between the different cells experimented upon? The nature of the experiments in HBECs is very different compared to BAL cells and PBMCs, in that HBECs undergo several weeks of growth whereas BAL cells and PBMCs were stimulated immediately after being obtained. If any difference in IFN expression had been observed, given the different experimental conditions, it may have been difficult to extrapolate findings between the different cell types. The different cells received the same viruses as virus was produced in one batch, confirmed by neutralisation assay and titrated, so the cells received the same multiplicity of infection (MOI=1.0).

Previous studies have identified increased RV replication in AA compared to NANA ^{60, 61}. In this study, differences were observed between the 2 viruses. vRNA and RV release (measured by TCID₅₀/mL) peaked at 24h with RV16 and at 48h with

RV1B (figures 3.22-3.25). More vRNA was produced by RV1B which is of interest in the degree of potency of the virus, given a 1 in 10 dilution of RV1B was used. These findings again highlight the differences between major and minor group viruses. RV replication studies did not identify differences between asthmatics and non-asthmatics, atopics and non-atopics. This may not be surprising given no differences in IFN production had been observed and previous studies reported the increase viral replication in AA correlating with IFN- β mRNA levels at 8h and protein at 48h ⁶⁰.

3.3.1.3 PBMCs & RV

PBMCs were studied as previous studies have identified deficient IFN- α in asthmatics with viruses other than RV ^{82, 206}. The aim was to identify whether IFN deficiencies were observed following stimulation by RV and whether these deficiencies were also observed in HBECs and BAL cells from the same individual. This would allow a less invasive way to identify deficiencies in IFN production, thereby avoiding the need for bronchoscopy. However this was not found to be the case.

These experiments identified that major group RV (RV16) but not minor group (RV1B) resulted in the production of type I and III IFN. It is of interest that RV1B induced IFNs in BAL cells, but not PBMCs. The different responses may be due to the different cell types having differing levels receptor expression, different efficiencies of viral replication or different intracellular responses. No significant differences were observed between asthmatics or non-asthmatics, atopics or non-atopics. Indeed the only group where significant induction of type I and III IFNs was observed at both 24h and 48h after RV stimulation was the AA. This differs from a recent study where asthmatic youths aged 7-19 were identified as having impaired IFN- α production in PBMCs stimulated with RV14, though this was not observed in adults, as in this study ⁸¹.

As with BAL cells, induction of proinflammatory cytokines was not observed in PBMCs stimulated with RV16 or RV1B, hence comments regarding differences between the different groups could not be made. This is in contrast to the study

where IL-6 and TNF- α was induced in PBMCs and was significantly lower in an asthmatic youth (but not adult) group compared to non-asthmatics⁸¹.

3.3.2 “Viral” TLR ligands

In order to attempt to explain the signalling pathways for viral stimulation of various cell types the experiments reported in this chapter used various TLR ligands to determine whether type I and III IFNs were induced and whether differences were observed between the various volunteer groups. In the experiments where IFN was expressed no differences were observed between asthmatics or non-asthmatics, atopics or non-atopics. This may not be surprising in that differences were not observed in cells stimulated with rhinoviruses. Due to the limited numbers of BAL cells obtained at bronchoscopy only HBECs and PBMCs were experimented upon. It would be of interest to observe whether IFNs were expressed in BAL cells following stimulation with these TLR ligands.

3.3.2.1 TLR3 stimulation

Induction of type I and III IFN mRNA and protein was observed in HBECs stimulated with poly(IC) in all groups, with significance in NANA, ANA and AA, and trends for induction in NAA (table 3.11). Significant induction of proinflammatory cytokine mRNA and protein was also observed in the same groups. No significant difference was observed between any groups. Induction of both type I and III IFNs was not observed in PBMCs stimulated with poly(IC) (table 3.13). Induction of proinflammatory cytokine protein was observed at 24h and 48h, reaching significance only in AA. At 48h, in all groups combined highly significant expression of proinflammatory cytokine protein was observed. The experiments reported in this chapter suggest there may be a difference in the expression/function of TLR3 in different cell types. TLR3 has variable expression *in vivo*. CNS cells from humans with a dominant-negative allele of TLR3 which predisposes to HSV encephalitis have been shown to have a deficient TLR3 response whereas epithelial cells and PBMCs from the same individuals have normal type I and II IFN responses²⁶¹.

3.3.2.2 TLR7/8 stimulation

Induction of type III IFN mRNA only, and not type I IFNs, was observed in HBECs stimulated with R848. No differences were observed between groups. Induction of CXCL8/IL-8 and not IL-6 mRNA was observed. Only non-significant increases in IL-1 β and TNF- α protein were observed at 24h (table 3.12). The findings of induction of IFNs and proinflammatory cytokines in HBECs by TLR7/8 stimulation supports the previously conflicting reports for TLR7/8 expression in HBECs^{317, 318} in favour of the presence of TLR7/8 in HBECs.

In PBMCs stimulated with R848 significant induction of IFN- α 2a protein was observed at 24h, with non-significant increases at 48h and in the other IFNs measured at 24h and 48h (table 3.14). No differences were observed between groups. This is in contrast to the study which identified reduced TLR7 function in PBMCs from asthmatic adolescents with mild-moderate disease³¹¹. Increases in proinflammatory cytokine protein (other than CXCL8/IL-8) were observed in all groups, with significance only in AA. However no significant differences were observed between groups. Having identified no difference, in order to determine significance of the 48h time point all groups were combined. Significant IL-1 β and TNF- α protein was observed (figure 3.35).

3.4 Conclusions

This is the first report of type I and III IFN and proinflammatory cytokine responses to stimulation with rhinoviruses and TLR3 and TLR7/8 ligands in cells from adult asthmatics and non-asthmatics, atopics and non-atopics. The hypothesised deficiencies in IFN production in asthmatic subjects were not observed in BAL cells, HBECs or PBMCs stimulated with RV, or HBECs and PBMCs stimulated with TLR3 and TLR7/8. The most likely cause for this deficiency not being identified is the mild nature of the disease in the asthmatics recruited. Indeed, it may be the propensity to exacerbate which is a key factor in IFN deficiency. Due to difficulties in recruitment of NAA volunteers it is impossible to say whether

deficiencies would have been identified, but there were no trends to suggest a deficiency. The study hypothesis should therefore be rejected.

3.5 Summary

IFN was produced in BAL cells, HBECs or PBMCs following stimulation with rhinoviruses, TLR3 and TLR7/8 but no difference in IFN production was observed between asthmatics or non-asthmatics, or atopics or non-atopics.

Chapter 4: *Streptococcus pneumoniae* infection of BAL cells, HBECS and PBMCs

4.1 Introduction

There is increasing evidence for the role of bacteria, particularly atypical bacteria such as *Cpn* and mycoplasma in asthma, both in the development of asthma and in exacerbations. Lately the role for typical bacteria such as *Spn* has been questioned. Neonates colonised by *Spn* have an increased risk of later development of wheeze and asthma ⁸⁸. Pneumococcal colonisation of the oropharynx is more common in asthmatics ⁹³. Asthma is a risk factor for invasive pneumococcal disease ^{104, 319}. The mechanism for the increased susceptibility to *Spn* in asthmatics is unknown, but asthmatics have lower pneumococcal antibodies than non-asthmatics ¹⁰⁵. Studies have identified deficiencies in the innate immune response in asthmatics, particularly in response to viral infection, and in particular deficiencies in type I and III IFN production. BAL macrophages from asthmatics have deficient IFN- λ production in response to the gram negative bacterial cell wall product LPS ⁶¹.

IFNs have classically been considered antiviral cytokines. Studies have identified extracellular bacteria inducing IFNs. In particular, *Spn* has been shown to activate type I IFNs in airway epithelial cells ²²³ and *Spn* colonisation of mice leads to type I IFN mRNA induction ²²². Human BAL macrophages have been identified as producers of type I IFN upon infection with *Spn* ²²⁴. Whether the previously identified IFN deficiency in asthmatics in response to viruses can be extrapolated to *Spn* infection has not been investigated. If a deficiency were identified the role of atopy in this deficiency could be evaluated further. Similarly, investigation of anti-bacterial cytokines such as IL-1 β , IL-6, CXCL8/IL-8 and TNF- α may identify deficiency in asthmatics, a finding that has only been recently observed, and published after the commencement of the project ³²⁰.

4.1.1 Hypothesis

- *Spn* infection will induce type I and III IFNs and proinflammatory cytokines in BAL cells, HBECs and PBMCs and will result in deficient type I and III IFN production in AA and NAA, compared to NANA and ANA.

4.1.2 Aims

- To establish whether *Spn* infection of BAL cells, HBECs and PBMCs results in type I and III IFN production.
- To further characterise whether *Spn* infection of BAL cells, HBECs and PBMCs results in production of proinflammatory cytokines important in host defence against bacterial infections.
- To establish whether *Spn* infection of BAL cells, HBECs and PBMCs results in deficient type I and III IFN production in AA and NAA, compared to NANA and ANA.
- To establish whether *Spn* infection of BAL cells, HBECs and PBMCs results in deficient anti-bacterial cytokine production in AA and NAA, compared to NANA and ANA.

4.2 Results

4.2.1 Pre-study investigation of *Spn*

Prior to the assessment of the effects of *Spn* in interferon and proinflammatory cytokine production in cells acquired from volunteers, and to the investigation as to whether there were any differences depending on the atopic and/or asthmatic status of volunteers, the effects of *Spn* was analysed in BEAS-2B cells, primary HBECs and PBMCs. Concentration-time experiments were performed in these cell types and type I and III IFN and IL-6 and CXCL8/IL-8 mRNA was measured in cell lysates (figure 4.1). Serial 10-fold dilutions from 1×10^6 colony forming units/mL (cfu/mL) to 1×10^0 cfu/mL were made. Cells were stimulated with varying concentrations of *Spn* and were harvested at various times following stimulation.

In BEAS-2B cells a non-significant induction of IL-6 and CXCL8/IL-8 mRNA was observed at 24h post stimulation with *Spn* (figure 4.1 A and B). Weak induction of IFN- β mRNA was observed at 4h post stimulation with a concentration of 1×10^4 cfu/mL but this failed to reach statistical significance (figure 4.1 C). At other time points no induction was observed. No induction of type III IFN mRNA was observed in BEAS-2B cells following stimulation with *Spn* (data not shown).

In primary HBECs induction of IL-6 and CXCL8/IL-8 mRNA was not observed (figure 4.1 D and E). A non-significant weak induction of IFN- β mRNA was observed at 48h with *Spn* at 1×10^4 cfu/mL (figure 4.1 F). No induction of type III IFN mRNA was observed in primary HBECs following stimulation with *Spn* (data not shown).

In PBMCs a non-significant induction of IL-6 and CXCL8/IL-8 was seen with all 3 concentrations of *Spn* at 8h, 24h and 48h (figure 4.1 G and H). A non-significant weak induction of IFN- β mRNA was observed at 24 and 48h (figure 4.1 I). No induction of type III IFN mRNA was observed in PBMCs following stimulation with *Spn* (data not shown). For all cell types the concentration of *Spn* chosen for subsequent experiments on samples from volunteers was 1×10^4 cfu/mL (in view of there being no marked difference between this concentration and the higher

concentrations, and the need to re-test any further *Spn* stocks prior to use in volunteers' samples).

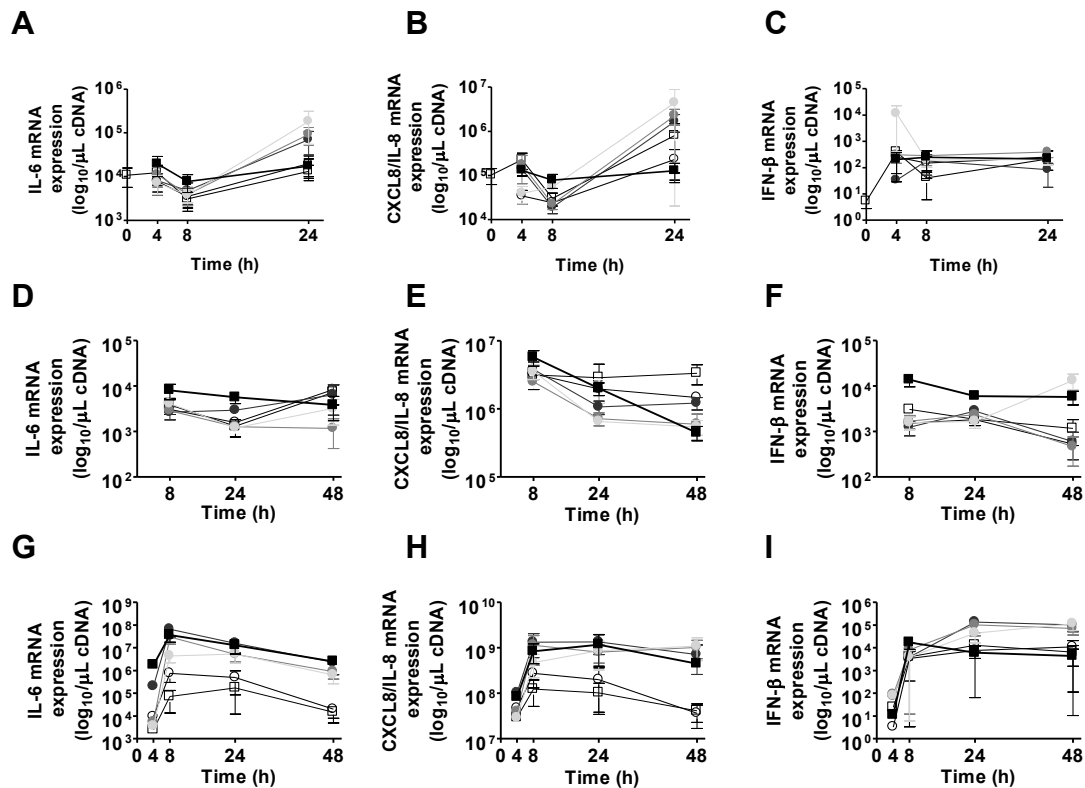


Figure 4.1. Concentration and time course response of *S. pneumoniae* (*Spn*) in BEAS-2B cells (A, B and C, N=3), primary HBECS (D, E and F, N=3) and PBMCs (G, H and I, N=3). Concentrations from 1×10^6 cfu/mL to 1×10^0 cfu/mL were used. Only the highest 3 dilutions are shown. Poly(IC) was used as a positive control. □ = medium, ○ = filtered *Spn*, ● = *Spn* 1×10^6 cfu/mL, ◐ = *Spn* 1×10^5 cfu/mL, ◑ = *Spn* 1×10^4 cfu/mL, ■ = poly(IC) 10μg/mL for epithelial cells, 100μg/mL for PBMCs. In BEAS-2B cells a non-significant induction of IL-6 and CXCL8/IL-8 mRNA at 24h was observed (A and B). Weak induction of IFN-β mRNA at 4h with *Spn* at 1×10^4 cfu/mL was observed, but this was non-significant (C). In primary HBECS induction of IL-6 and CXCL8/IL-8 was not observed (D and E). A non-significant weak induction of IFN-β mRNA was observed at 48h with *Spn* at 1×10^4 cfu/mL (F). In PBMCs a non-significant induction of IL-6 and CXCL8/IL-8 was seen in all 3 doses of *Spn* at 8h, 24h and 48h (G and H). A non-significant weak induction of IFN-β mRNA was observed in IFN-β mRNA induction at 24 and 48h (I). Induction of IFN-λ2,3/IL-28 or IFN-λ1/IL-29 mRNA was not observed (not shown).

4.2.2 *Spn* infection of BAL cells: induction of type I and III IFN and proinflammatory cytokine mRNA and protein

In order to assess whether IFN production was a characteristic of the innate immune response to infection with *Spn* and to observe whether there is a difference between asthmatic/atopic and non-asthmatic/atopic groups, IFN- α , IFN- β and IFN- λ mRNA and protein production by BAL cells was measured at 8h following infection with *Spn*. Only an 8h time point was chosen due to other aims of the project, particularly the investigation of rhinoviruses and the limited number of BAL cells obtained. The BAL cell composition and clinical characteristics of volunteers are described in chapter 3. No significant induction of type I or III IFN mRNA or protein was observed at 8h in BAL cells (figures 4.2 and 4.3). No differences were observed between groups.

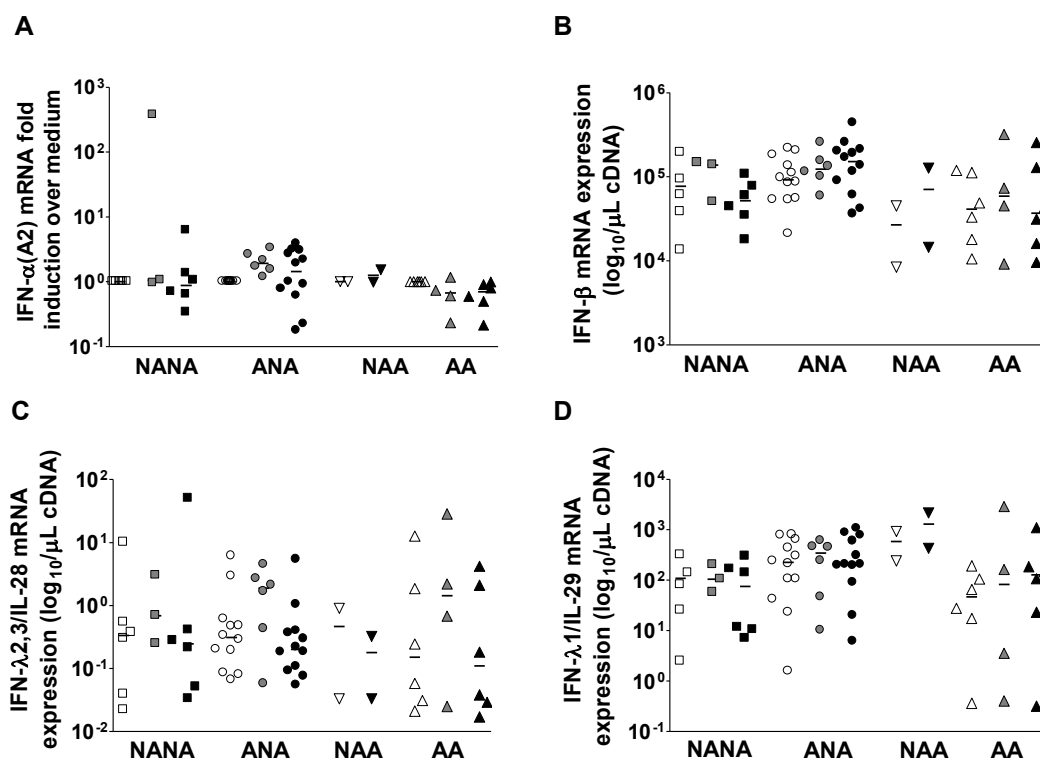


Figure 4.2. Interferon mRNA induction in BAL macrophages at 8h post stimulation with *Spn*. Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, grey shapes denote filtered *Spn*, black shapes denote *Spn*. IFN- α (A2) (A), IFN- β (B), IFN- λ 2,3/IL-28 (C), IFN- λ 1/IL-29 (D). No significant induction of type I or III IFN mRNA was observed in BAL cells stimulated with *Spn*.

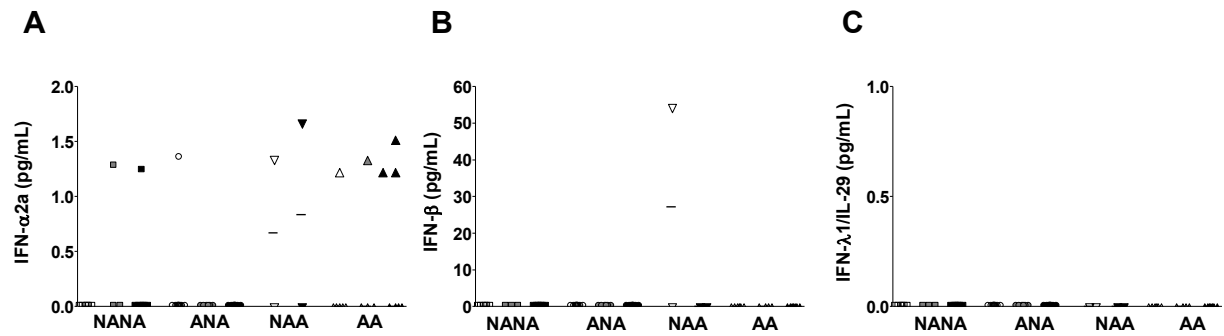


Figure 4.3. Interferon protein induction in BAL macrophages at 8h post stimulation with *Spn*. Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, grey shapes denote filtered *Spn*, black shapes denote *Spn*. IFN-α2a (A), IFN-β (B), IFN-λ1/IL-29 (C). No IFN protein production at 8h was observed.

In order to assess the model of *Spn* infection of BAL cells proinflammatory cytokine production was measured. Following 8h incubation the BAL cells stimulated with media alone (containing no supplementary antibiotics) was visibly cloudy, suggestive of a bacterial contamination, possibly from the upper airway. Proinflammatory cytokine production in BAL cells stimulated with *Spn* was therefore examined and compared to cells treated to media alone, with and without antibiotic supplementation. There were no differences in induction of IL-6 mRNA in cells stimulated with *Spn* compared to cells treated with media without antibiotics (figure 4.4 A). When compared to media which was supplemented with antibiotics a weak induction of IL-6 mRNA was observed but this failed to reach statistical significance (figure 4.4 B). No induction of CXCL8/IL-8 mRNA was observed in BAL cells when compared to either type of medium (figure 4.4 C and D). No differences were observed between the individual volunteer groups.

Proinflammatory cytokine protein production was also measured in BAL cell supernatants stimulated with *Spn* and media alone (with and without supplementary antibiotics, due to the observed visible contamination of the media without supplementary antibiotics-treated BAL cells). When comparing cells stimulated with *Spn* to those stimulated with media without supplementary antibiotics no production of IL-1β, IL-6, CXCL8/IL-8 or TNF-α protein was observed (figure 4.5 A, C, E and G). Weak induction of IL-1β was observed in cells stimulated with *Spn* compared to cells stimulated with media with supplementary antibiotics, but this failed to reach

statistical significance (figure 4.5 B). There was a suggestion for lower amounts of IL-1 β protein produced in asthmatics compared to non-asthmatics, but this was not statistically different. A non-significant induction of TNF- α protein was observed in NAA, though sample size was small. Induction was not observed in other groups (figure 4.5 H). No induction of IL-6 or CXCL8/IL-8 protein was observed in any group (figure 4.5 D and F).

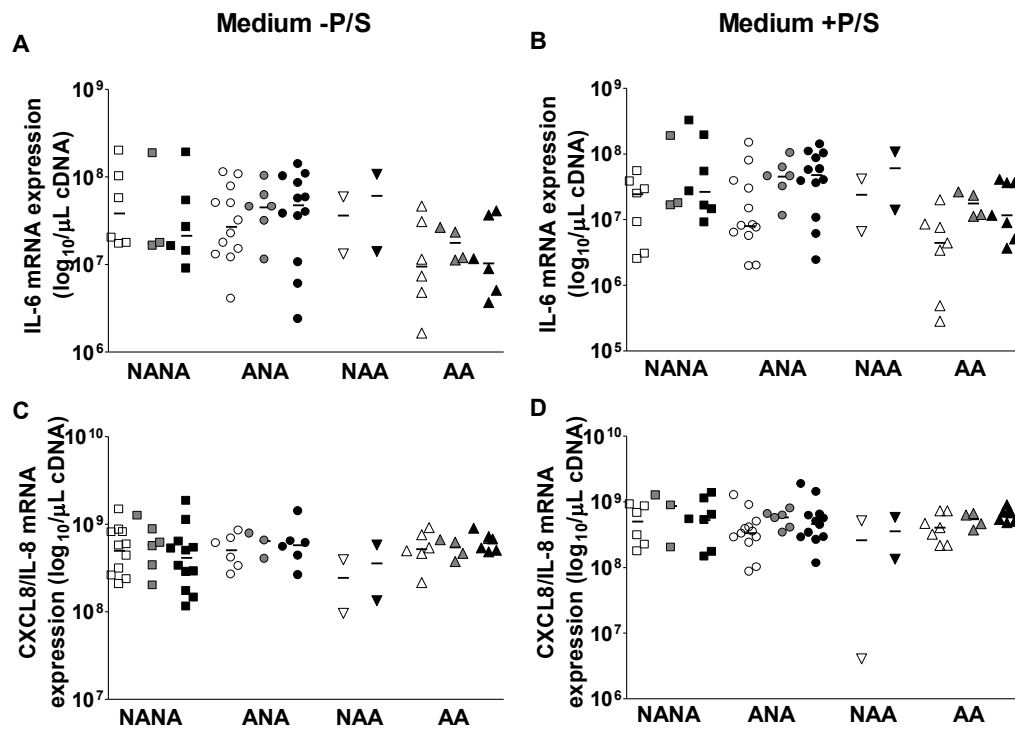


Figure 4.4. Proinflammatory cytokine mRNA induction in BAL macrophages at 8h post stimulation with *Spn*. Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, grey shapes denote filtered *Spn*, black shapes denote *Spn*. IL-6 (A and B), CXCL8/IL-8 (C and D). A and C compared to medium without supplementary antibiotics (medium -P/S); B and D compared to medium with supplementary antibiotics (medium +P/S). No induction of IL-6 or CXCL8/IL-8 mRNA was observed. After 8h the medium was visibly cloudy in all conditions. When compared to medium with supplementary antibiotics a non-significant induction of IL-6 mRNA was observed. This was not observed for CXCL8/IL-8 mRNA.

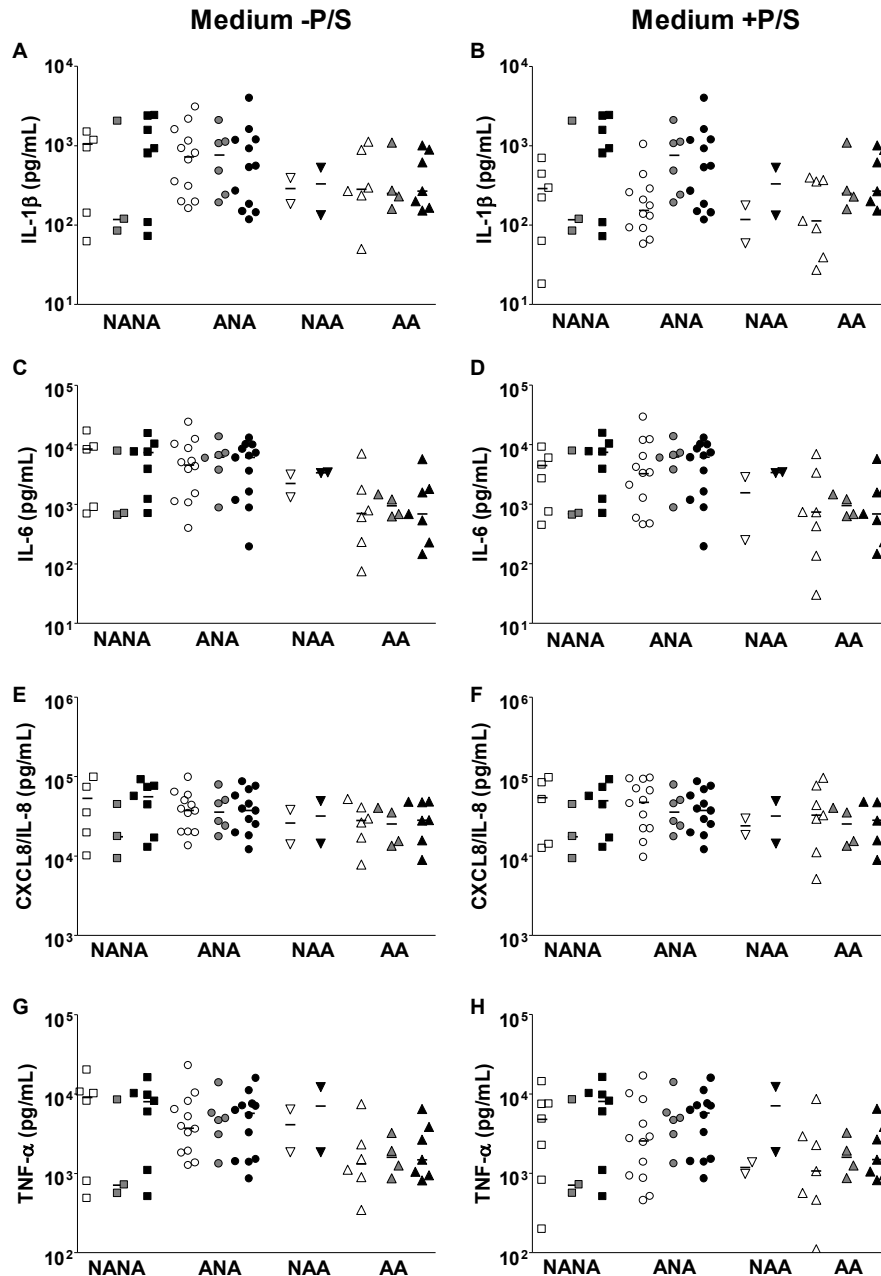


Figure 4.5. Proinflammatory cytokine protein induction in BAL macrophages at 8h post stimulation with *Spn*. Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, grey shapes denote filtered *Spn*, black shapes denote *Spn*. IL-1 β (A and B), IL-6 (C and D), CXCL8/IL-8 (E and F), TNF- α (G and H). A, C, E and G compared to medium without supplementary antibiotics; B, D, F and H compared to medium with supplementary antibiotics. At 8h cells treated to medium without supplementary antibiotics alone had evidence of bacterial contamination, presumably from the upper airway at bronchoscopy. No induction of IL-1 β , IL-6, CXCL8/IL-8 and TNF- α was observed when comparing to medium without supplementary antibiotics (A, C, E and G). When comparing to medium with supplementary antibiotics cells stimulated with *Spn* had a non-significant production of IL-1 β protein. Although no significant difference was observed between groups there was a suggestion for reduced production in asthmatics compared to non-asthmatics. No production of IL-6 or CXCL8/IL-8 protein was observed (D and F). In the small sample size of NAA a non-significant production of TNF- α was observed with no production observed in other groups (H).

Table 4.1 summarises IFN and proinflammatory cytokine protein and mRNA induction in BAL cells at 8h following infection with *Spn*.

Table 4.1 Summary of IFN and proinflammatory cytokine mRNA and protein induction post *Spn* infection in BAL cells - = no induction

	8h			
	NANA	ANA	NAA	AA
IFN- α mRNA	-	-	-	-
IFN- α protein	-	-	-	-
IFN- β mRNA	-	-	-	-
IFN- β protein	-	-	-	-
IFN λ 1/IL-29 mRNA	-	-	-	-
IFN λ 1/IL-29 protein	-	-	-	-
IFN λ 2,3/IL-28 mRNA	-	-	-	-
IL-6 mRNA	-	-	-	-
CXCL8/IL-8 mRNA	-	-	-	-
IL-1 β protein	-	-	-	-
IL-6 protein	-	-	-	-
CXCL8/IL-8 protein	-	-	-	-
TNF- α protein	-	-	-	-

4.2.3 *Spn* infection of HBECs: induction of type I and III IFN and proinflammatory cytokine mRNA and protein

To establish whether the previously reported defective IFN- β and IFN- λ production in AAs following infection with RV was also observed following infection with *Spn*, HBECs from the four patient groups were stimulated with *Spn* and type I and III IFN mRNA was measured at 8h and 24h, and protein at 24h and 48h. Induction of IFN- β , IFN- λ 2,3/IL-28 and IFN- λ 1/IL-29 mRNA was not observed at 8h or 24h in HBECs stimulated with *Spn* (figure 4.6). A high baseline level of IFN- β mRNA was observed. Production of IFN- β and IFN- λ 1/IL-29 protein was not

observed in HBEC supernatants at 24h or 48h post stimulation with *Spn* (figure 4.7). No differences were observed between groups.

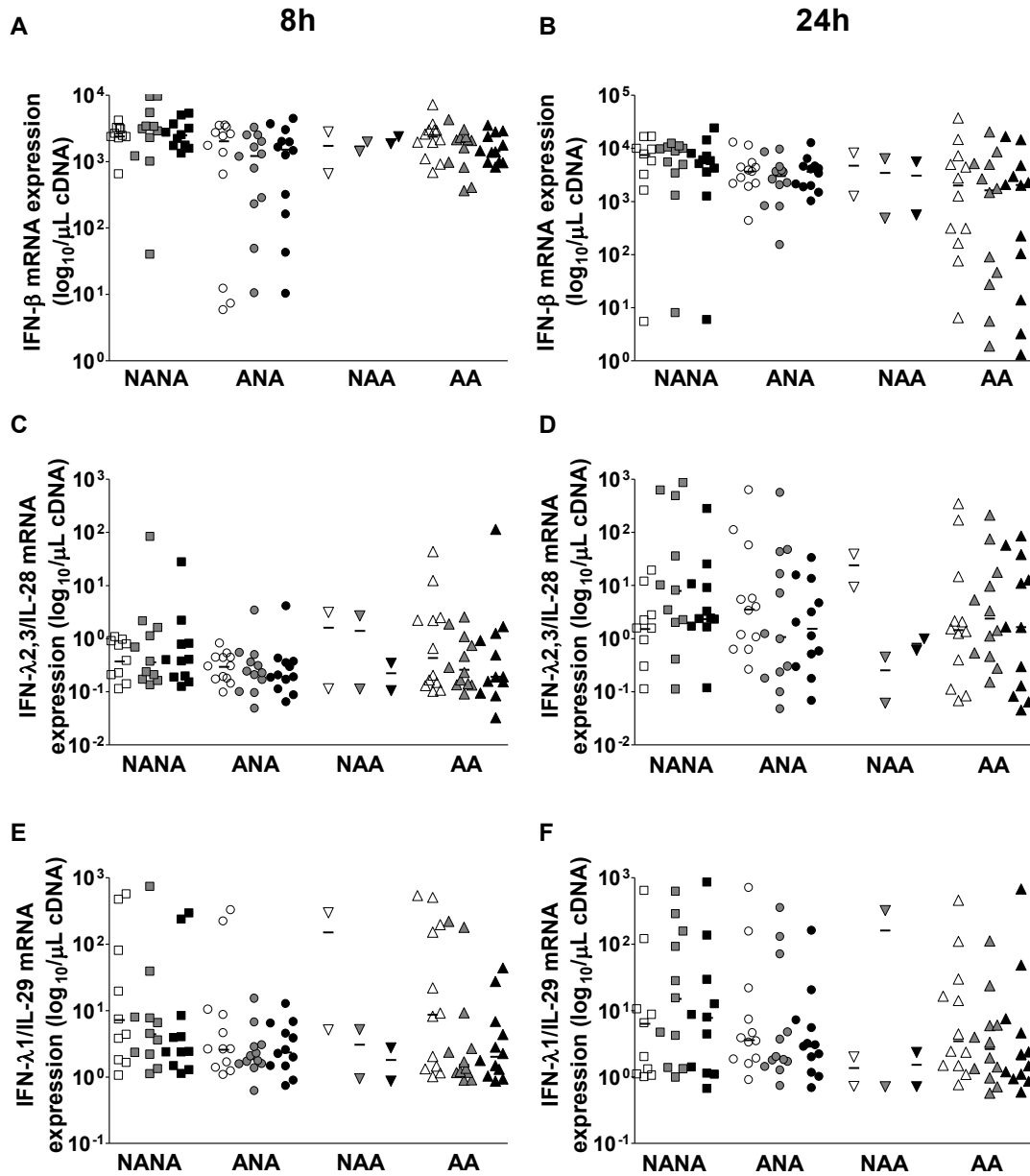


Figure 4.6. Interferon mRNA induction in HBECs at 8h (A, C and E) and 24h (B, D and F) post stimulation with *Spn*. Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, grey shapes denote filtered *Spn*, black shapes denote *Spn*. IFN-β (A and B), IFN-λ2,3/IL-28 (C and D), IFN-λ1/IL-29 (E and F). No significant IFN mRNA induction was observed at 8 or 24h. High levels of IFN-β mRNA were observed in medium treated cells, with no increased induction in *Spn* treated cells.

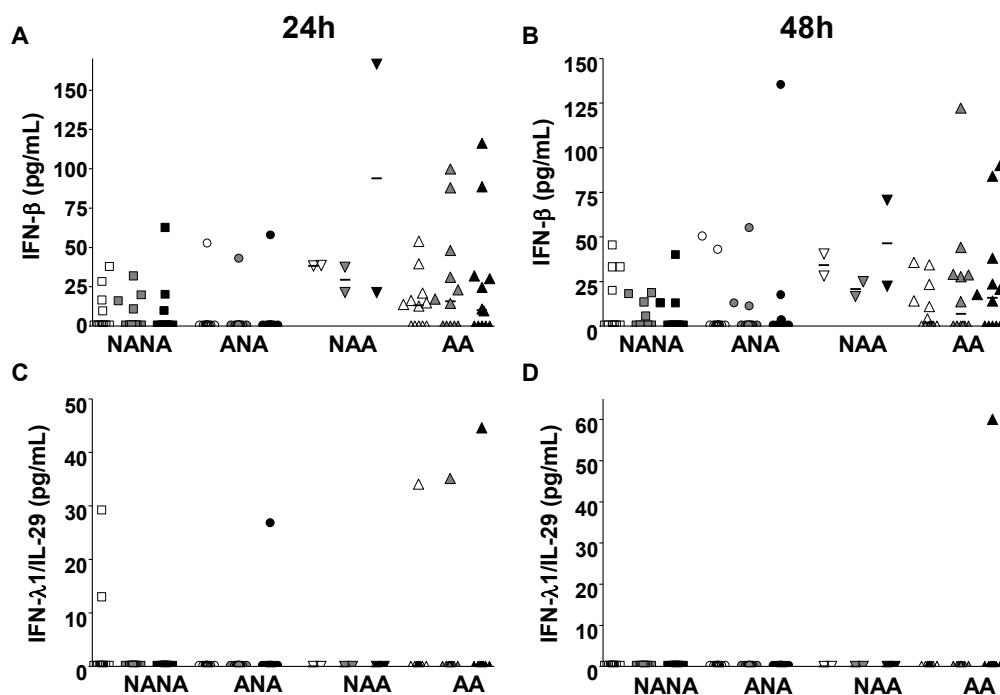


Figure 4.7 Interferon protein induction in HBECs at 24h (A and C) and 48h (B and D) post stimulation with *Spn*. Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, grey shapes denote filtered *Spn*, black shapes denote *Spn*. IFN- β (A and B), IFN- λ 1/IL-29 (C and D). No significant IFN protein production was observed.

In order to examine whether stimulation of HBECs with *Spn* resulted in induction of proinflammatory cytokines and to observe whether there were differences between the asthmatic or atopic status of the volunteers from whom the cells were obtained, IL-6 and CXCL8/IL-8 mRNA was measured at 8h and 24h, and IL-1 β , IL-6, CXCL8/IL-8 and TNF- α protein was measured at 24h and 48h. Induction of IL-6 and CXCL8/IL-8 mRNA was not observed at either time point in HBECs stimulated with *Spn* (figure 4.8). Induction of IL-1 β , IL-6, CXCL8/IL-8 and TNF- α protein from HBECs stimulated with *Spn* was not observed at 24 or 48h (figure 4.9). No differences were observed between the four volunteer groups.

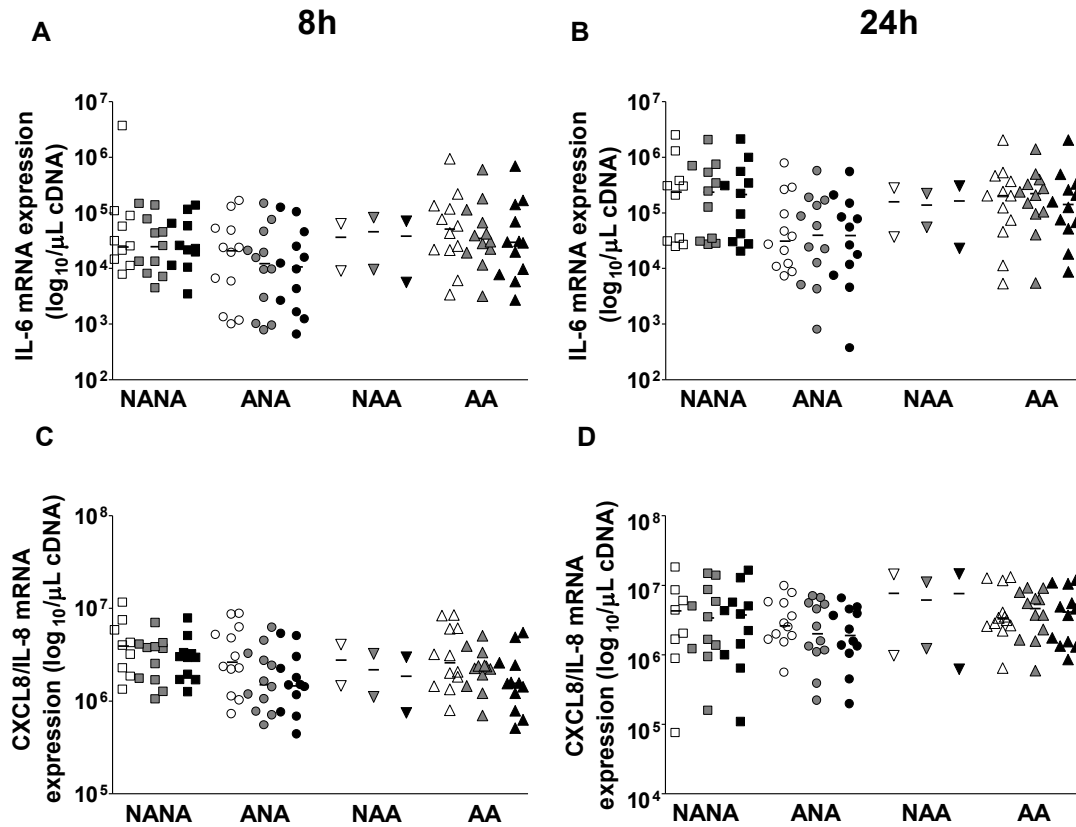


Figure 4.8. Proinflammatory cytokine mRNA induction in HBECs at 8h (A and C) and 24h (B and D) post stimulation with *Spn*. Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, grey shapes denote filtered *Spn*, black shapes denote *Spn*. IL-6 (A and B), CXCL8/IL-8 (C and D). No significant induction of proinflammatory cytokine mRNA was observed at 8 or 24h.

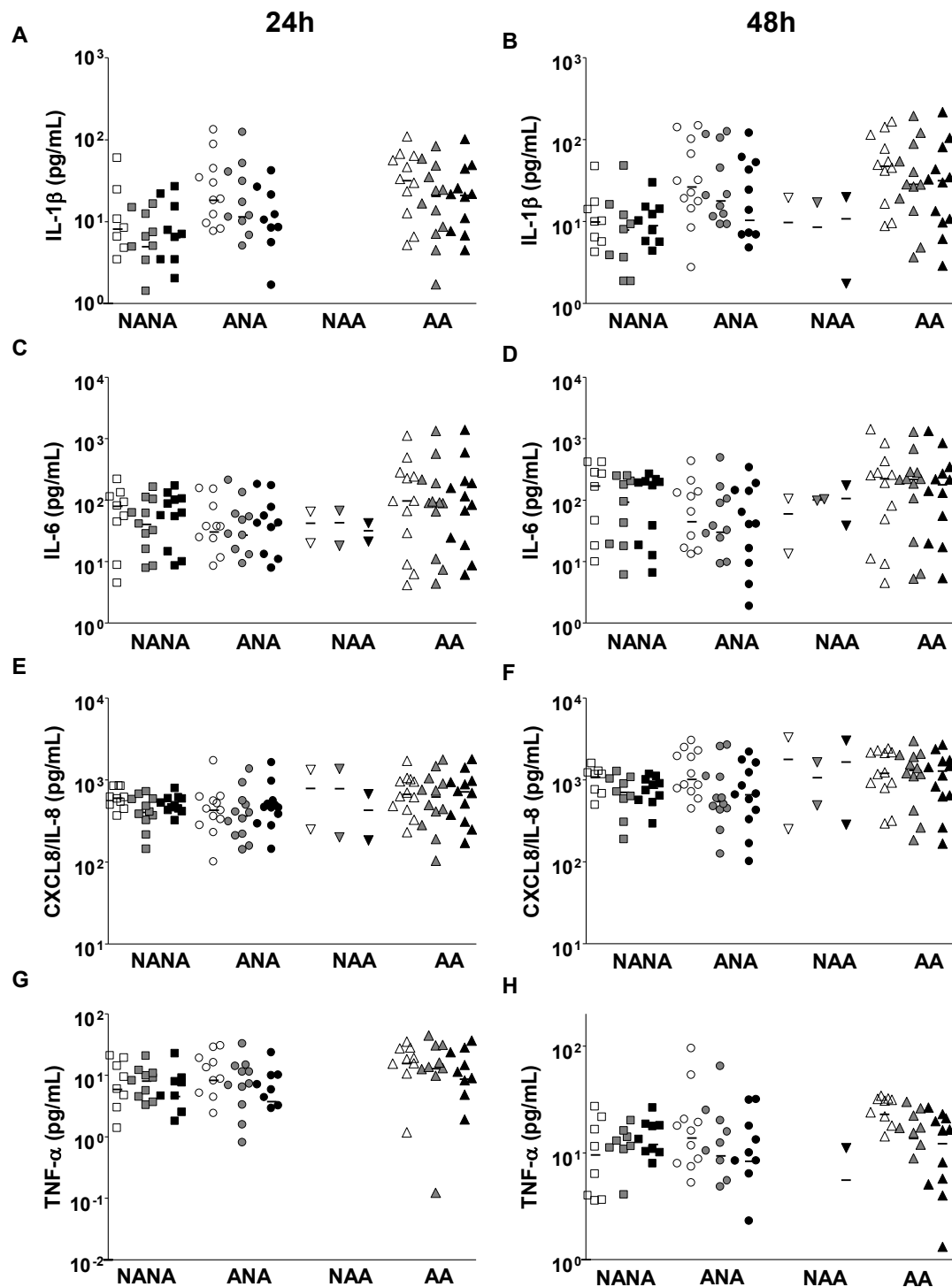


Figure 4.9. Proinflammatory cytokine protein induction in HBECs at 24h (A, C, E and G) and 48h (B, D, F, H) post stimulation with *Spn*. Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, grey shapes denote filtered *Spn*, black shapes denote *Spn*. IL-1 β (A and B), IL-6 (C and D), CXCL8/IL-8 (E and F), TNF- α (G and H). No significant production of proinflammatory cytokines was observed.

Tables 4.2 and 4.3 summarise IFN and proinflammatory cytokine protein and mRNA induction in HBECs at 8h following infection with *Spn*.

Table 4.2 Summary of IFN and proinflammatory cytokine mRNA induction post *Spn* infection in HBECs - = no induction

	8h				24h			
	NANA	ANA	NAA	AA	NANA	ANA	NAA	AA
IFN- β	-	-	-	-	-	-	-	-
IFN λ 2,3/IL-28	-	-	-	-	-	-	-	-
IFN λ 1/IL-29	-	-	-	-	-	-	-	-
IL-6	-	-	-	-	-	-	-	-
CXCL8/IL-8	-	-	-	-	-	-	-	-

Table 4.3 Summary of IFN and proinflammatory cytokine protein induction post *Spn* infection in HBECs - = no induction

	24h				48h			
	NANA	ANA	NAA	AA	NANA	ANA	NAA	AA
IFN- β	-	-	-	-	-	-	-	-
IFN λ 1/IL-29	-	-	-	-	-	-	-	-
IL-1 β	-	-	-	-	-	-	-	-
IL-6	-	-	-	-	-	-	-	-
CXCL8/IL-8	-	-	-	-	-	-	-	-
TNF- α	-	-	-	-	-	-	-	-

4.2.4 *Spn* infection of PBMCs: induction of type I and III IFN and proinflammatory cytokine protein

The effect of *Spn* stimulation on PBMCs, as a further cell type, was examined in order to establish whether the IFNs were produced following infection with *Spn* and if they were produced, whether the asthmatic or atopic status of volunteers was important. The production of type I and III IFN protein was assessed at 24h and 48h post stimulation. As previously discussed, unlike HBECs, PBMCs

produce IFN- α as well as the other type I and III IFNs. IFN- α , IFN- β and IFN- λ 1/IL-29 protein production was not observed in PBMCs stimulated with *Spn* at 24h and 48h with no differences between the volunteer groups (figure 4.10).

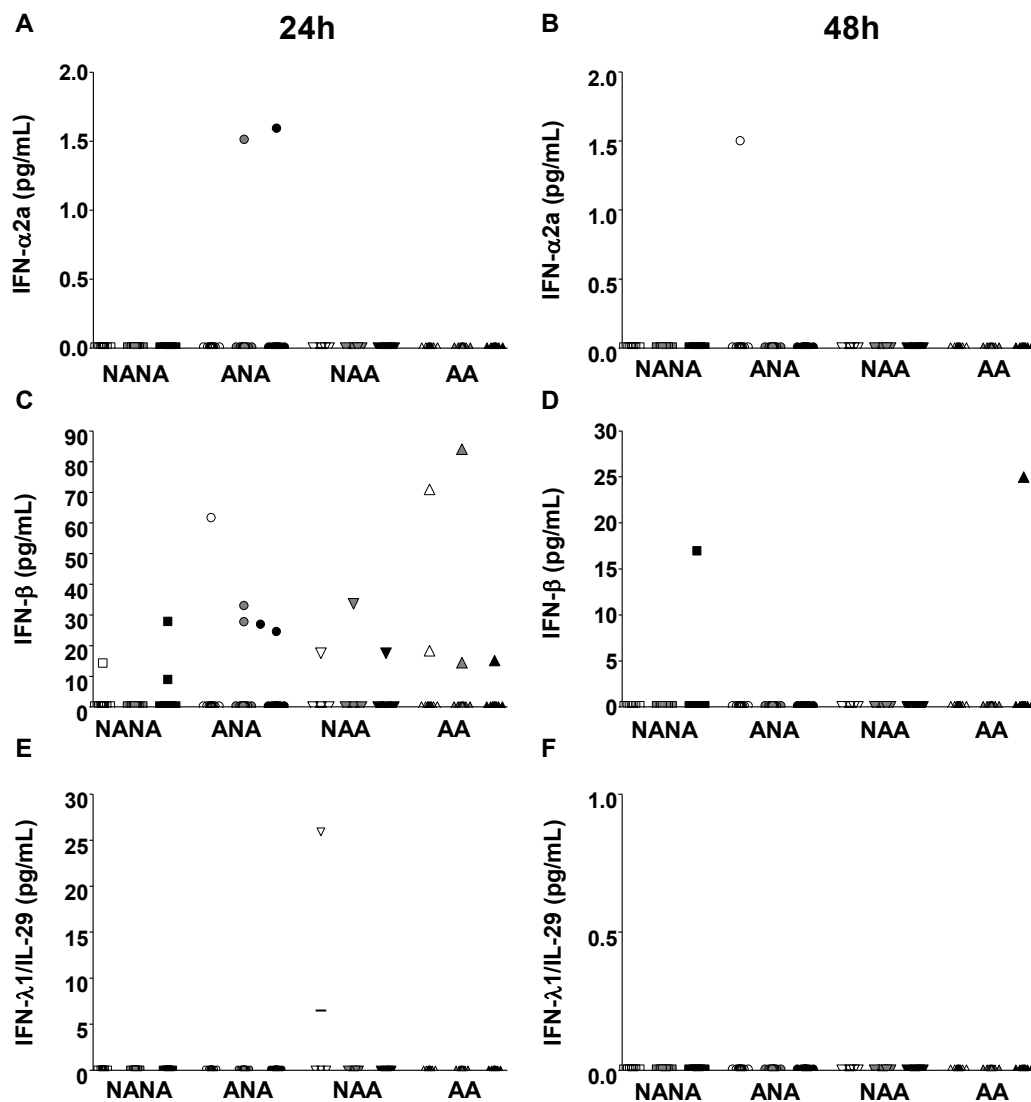


Figure 4.10. Interferon protein induction in PBMCs at 24h (A, C and E) and 48h (B, D and F) post stimulation with *Spn*. Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, grey shapes denote filtered *Spn*, black shapes denote *Spn*. IFN- α 2a (A and B), IFN- β (C and D), IFN- λ 1/IL-29 (E and F). No significant IFN protein production was observed.

In order to establish whether *Spn* results in production of proinflammatory cytokines from PBMCs and to examine whether the different asthmatic and atopic

phenotype of volunteers resulted in different proinflammatory cytokine expression from PBMCs simulated with *Spn*. IL-1 β , IL-6, CXCL8/IL-8 and TNF- α protein was measured in PBMC supernatants at 24h and 48h.

IL-1 β was produced at 24h and 48h in PBMCs stimulated with *Spn*. At 24h significant production was observed in all 4 groups of volunteers (figure 4.11 A); NANA ($p<0.01$), ANA ($p<0.05$), NAA ($p<0.05$) and AA ($p<0.001$). No significant difference was observed between groups. At 48h significant production of IL-1 β was observed in asthmatic subjects; NAA ($p<0.05$) and AA ($p<0.001$). Non-significant increases in IL-1 β protein were observed in NANA and ANA (figure 4.11 B). No differences were observed between groups.

Increases in IL-6 and CXCL8/IL-8 protein were not observed in PBMCs stimulated with *Spn* at either 24h or 48h other than in the small sample of NAA (figure 4.11 C-F).

TNF- α protein was produced at 24h and 48h in PBMCs stimulated with *Spn*. At 24h significant production was observed in AA only ($p<0.01$) with non-significant increases observed in NANA, ANA and NAA (figure 4.11 G). At 48h significant production was observed in AA only ($p<0.001$) with non-significant increases observed in NANA, ANA and NAA (figure 4.11 H). No significant differences were observed between groups at either time point.

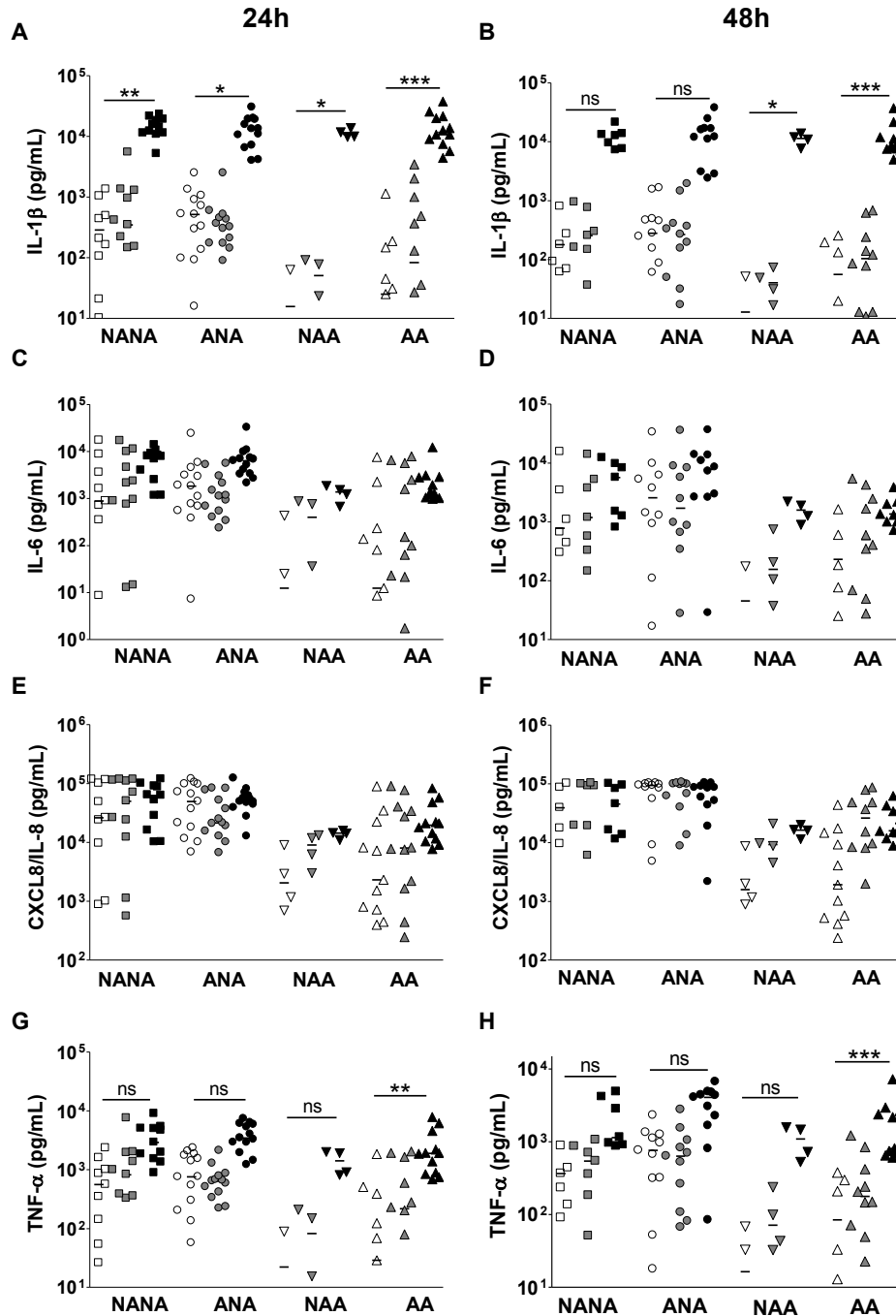


Figure 4.11. Proinflammatory cytokine protein induction in PBMCs at 24h (A, C, E and G) and 48h (B, D, F, H) post stimulation with *Spn*. Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, grey shapes denote filtered *Spn*, black shapes denote *Spn*; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, ns = non-significant. IL-1 β (A and B), IL-6 (C and D), CXCL8/IL-8 (E and F), TNF- α (G and H). Significant increases in IL-1 β protein were observed in all groups at 24h. At 48h increases were observed which reached statistical significance in asthmatic volunteers. No significant increases were observed in IL-6 or CXCL8/IL-8 protein at either 24h or 48h. Increases in TNF- α protein were observed at 24h and 48h, with statistical significance reached in AA only. No differences were observed between in any of the cytokines measured at either time point.

Table 4.4 summarises the IFN and proinflammatory cytokine protein induction in PBMCs following *Spn* infection.

Table 4.4 Summary of IFN and proinflammatory cytokine protein induction post *Spn* infection in PBMCs ↑ = significant induction, ns = non-significant increase observed, - = no induction

	24h				48h			
	NANA	ANA	NAA	AA	NANA	ANA	NAA	AA
IFN-α2a	-	-	-	-	-	-	-	-
IFN-β	-	-	-	-	-	-	-	-
IFNλ1/IL-29	-	-	-	-	-	-	-	-
IL-1β	↑	↑	↑	↑	ns	ns	↑	↑
IL-6	-	-	ns	-	-	-	ns	-
CXCL8/IL-8	-	-	ns	-	-	-	ns	-
TNF-α	ns	ns	ns	↑	ns	ns	ns	↑

4.3 Discussion

There is increasing evidence to suggest a role for bacteria in asthma. Asthma is a risk factor for invasive pneumococcal disease^{104, 319} and *Spn* colonisation has been reported more common in asthmatics⁹³. The aim of the experiments performed in this chapter were to examine whether *Spn* infection of BAL cells, HBECs and PBMCs resulted in production of type I and III IFNs and whether there were any differences observed between asthmatics and non-asthmatics, atopics and non-atopics.

In the experiments reported in this chapter *Spn* failed to induce type I or III IFN mRNA or protein in any of the cell types examined. In BAL cells no induction was observed at 8h. It could be that 8h was too early a time point to detect IFN mRNA expression, especially in view of the suggestion from the preliminary experiments in PBMCs that IFN-β mRNA was detected at 24h and 48h. Previous studies have identified type I IFN expression in human alveolar macrophages co-

cultured with alveolar epithelial cells infected with *Spn* for 16h ²²⁴. This induction of type I IFN was mediated by a cytosolic DNA-sensing pathway involving the adaptor molecule STING and IRF-3 and was dependent on the pore-forming toxin pneumolysin. The production of proinflammatory cytokine mRNA and protein was also examined in BAL cells, again using 8h as a time point, to be in line with experiments performed on RV. Protocols developed in the department have routinely used medium supplemented with antibiotics for experiments with RV in BAL cells and PBMCs. This seemed counterintuitive for experiments with bacteria, so medium without supplementary antibiotics was used. However, visible contamination of BAL cell samples was observed in medium-only treated cells, presumably from airway commensal bacteria acquired at bronchoscopy. Hence no difference in proinflammatory cytokine production was observed between *Spn*- and medium-treated BAL cells. In an attempt to clarify proinflammatory cytokine induction, *Spn*-treated cells were compared to medium with supplementary antibiotics. Only non-significant induction of IL-1 β was observed, with no differences between groups. If a later time point had been used, more significant results may have been found. Previous studies have identified the alveolar macrophage as important sources for early proinflammatory cytokine release in response to *Spn* infection ¹³², which was not replicated in this chapter.

Although a high level of baseline IFN- β mRNA was observed in HBECs, no increased induction of type I or III IFNs was observed in HBECs treated with *Spn* at any of the time points examined. Some studies have not identified alveolar epithelial cells infected with *Spn* as a source of type I IFN production ²²⁴, whereas other studies using mouse models of *Spn* infection have identified type I IFN induction from the airway epithelium ²²³. In the initial dose-time experiments weak induction of IL-6 and CXCL8/IL-8 mRNA was observed at 24h in BEAS-2B cells, but not primary HBECs. This disparity between different types of BECs made further examination in the volunteer groups of interest. Experimental conditions were identical, yet no significant induction of proinflammatory cytokine mRNA or protein was observed at any time point. The reason for this may have been insufficient stimulation time for the *Spn*. There is variation in other studies with the stimulation time of *Spn* – 2h ¹²³, 6h ³²¹ and up to 16h ²⁸⁷ with the latter two studies observing production of CXCL8/IL-8 from BECs stimulated with *Spn*. In the preliminary experiments performed in this

chapter, 24h stimulation with *Spn* lead to marked cell death (observed with light microscopy). In this study, with the experiments on RV being performed at the same time as those reported in this chapter and the initial experiments suggesting induction after 1h of stimulation, this timing was thought sufficient. There was insufficient evidence obtained from the experiments reported in this chapter to suggest *Spn* induces type I or III IFNs from HBECs, meaning observations between asthmatics and non-asthmatics, atopics and non-atopics could not be made.

PBMCs were the only cell type stimulated with *Spn* in which clear inflammatory responses were observed. Significant production of IL-1 β protein was observed at 24h in all groups and at 48h in AA and NAA, with non-significant increases observed in NANA and ANA. No differences were observed between groups. With TNF- α protein, significant increases were observed in AA at 24h and 48h, with non-significant production in the other groups at both time points. Less convincing production of IL-6 and CXCL8/IL-8 protein was observed. Several other anti-bacterial cytokines could also be involved in host defence against bacteria, particularly IFN- γ . This has a role in anti-bacterial immunity, though it appears to be microbe-specific³²². Due to time and financial constraints of the project and the multiplex used not having IFN- γ as a cytokine to measure, the production of IFN- γ was not analysed.

Production of proinflammatory cytokines from PBMCs stimulated by *Spn* has been known for some years³²³, and the data presented in this chapter support this. However no difference was observed between asthmatic and/or atopic nature of the cells. As with the other cell types experimented upon in this chapter, no induction of type I or III IFN mRNA or protein was observed, suggesting *Spn* leads to an inflammatory response and not IFN production.

It may be that IFN signalling is not an important part of the innate immune response to *Spn* infection. Other components of the innate immune system may be important, and, in future, deficiencies could be identified in asthmatics. One such system could be the anti-microbial peptides such as defensins. These are present in airway secretions and expressed in the airway epithelium, and have direct antimicrobial action, modulate innate and adaptive immunity and play a role in wound repair³²⁴. Recently *Spn* has been identified as an inducer of β -defensins in

lung epithelium³²⁵. Although beyond the scope of this study, it would be interesting to examine whether *Spn* resulted in the expression of such a system in the cells obtained in this study, and to determine whether a difference between asthmatic and non-asthmatics, atopics and non-atopics was observed. It was for this reason that, despite *Spn* not clearly inducing IFNs in any cell type investigated in the preliminary investigations, a decision was made to continue with the investigation of *Spn* in the volunteers' samples, and to investigate the proinflammatory responses to *Spn* in the various cell types.

4.4 Conclusions

This is the first report of the investigation of type I and III and proinflammatory cytokine responses to stimulation *Spn* in cells from adult asthmatics and non-asthmatics, atopics and non-atopics. Induction of type I and III IFN mRNA and protein was not observed in BAL cells, HBECs or PBMCs stimulated with *Spn*. Induction of proinflammatory cytokines was observed in PBMCs stimulated with *Spn*, but there was no difference identified between asthmatics and non-asthmatics, atopics and non-atopics. The study hypothesis should therefore be rejected.

4.5 Summary

Type I and III IFN was not produced from BAL cells, HBECs or PBMCs stimulated with *Spn*. Induction of anti-bacterial cytokines was observed in PBMCs stimulated with *Spn* but no differences were observed between asthmatics or control groups.

Chapter 5: TLR 1/2/6/4/5/9 stimulation of HBECs and PBMCs

5.1 Introduction

TLRs 1, 2, 6, 4, 5 and 9 recognise products of bacterial infection. Asthmatics have an increased susceptibility to atypical bacterial infection ³²⁶ and invasive pneumococcal disease ^{104, 319}. Recent data suggest these “bacterial” TLRs may be involved in IFN signalling pathways. With studies demonstrating IFN deficiency in asthmatics, further clarification of TLR stimulation may highlight potential therapeutic targets.

TLR2 forms heterodimers with TLR1 or TLR6 to recognise bacterial triacyl or diacyl lipoproteins. Administration of the TLR1/2 ligand Pam₃CSK₄ reduces total inflammatory cell infiltrates and BAL eosinophilia in a mouse model of allergic airways disease ³²⁷. Upregulation of TLR2 has been identified in the nasal mucosa of humans with allergic rhinitis ³²⁸ and a polymorphism of TLR2 has been associated with allergic asthma ³²⁹. TLR1, 2 and 6 signalling is largely understood to result in the induction of proinflammatory cytokines, through the MyD88 dependent pathway. Recent data, however, suggests TLR2 activation resulting in the induction of type I IFNs ^{257, 258}. Further investigation is required to characterise the role of TLR1, 2 and 6 in IFN induction and to determine whether deficiencies in asthma or atopy are identified.

TLR4 recognises the bacterial ligands LPS and pneumolysin. For this study LPS was used as a previous study identified macrophages from asthmatics stimulated with LPS *ex-vivo* having a deficient IFN- λ response which correlated with exacerbation severity *in vivo* ⁶¹. TLR4 polymorphisms have been associated with asthma and atopy in humans, with decreased LPS-induced IL-12 and IL-10 in PBMCs ³³⁰. TLR4 is also important for IFN signalling ²⁶⁴. Further study therefore needs to be done on the role of TLR4 and IFN deficiency in asthma and/or atopy.

TLR5 is expressed by epithelial cells, monocytes and immature dendritic cells and recognises the flagellar element of motile bacteria. A recent study has suggested that the TLR5 ligand flagellin promotes the development of allergic asthma by priming allergic responses to indoor allergens ³³¹. Data is limited, but

TLR5 stimulation had been shown to stimulate IFN expression in mouse macrophages²⁶⁸. Whether TLR5 is important in IFN signalling in asthma and atopy has not yet been elucidated.

TLR9 recognises CpG motifs found in bacterial DNA and is important for the clearance of bacteria from the lower respiratory tract of mice²⁷⁰. Administration of CpG-ODN in mouse models of asthma and allergy results in reduction of Th₂ responses, eosinophilic inflammation and airway hyperreactivity³³² and is effective in treatment of ragweed rhinitis in humans³³³. TLR9 stimulation results in induction of type I IFNs in human primary cells via the MyD88 dependent pathway²⁷² and TLR9 is known to be expressed in bronchial epithelial cells³¹⁷. TLR9 therefore may be of relevance in the further characterisation of IFN deficiency in asthma.

In this chapter type I and III IFNs and proinflammatory cytokines were investigated in HBECs and PBMCs from NANA, ANA, NAA and AA. Experiments were not carried out in BAL cells due to the limited number of cells acquired at bronchoscopy.

5.1.1 Hypothesis

- TLRs sensing products of bacterial infection (TLR1, TLR2, TLR4, TLR5, TLR6 and TLR9) will induce type I and III IFNs and proinflammatory cytokines in HBECs and PBMCs and will result in deficient type I and III IFN induction in AA and NAA, compared to NANA and ANA.

5.1.2 Aims

- To establish whether TLR1, TLR2, TLR4, TLR5, TLR6 and TLR9 stimulation of HBECs and PBMCs results in type I and III IFN and proinflammatory cytokine induction.
- To determine whether this response is deficient in AA and NAA compared to NANA and ANA.

5.2 Results

5.2.1 Preliminary experiments: time course and concentration responses to TLR ligands in BEAS-2B cells, primary HBECs and PBMCs

Prior to the assessment of the effects of different TLR ligands in interferon and proinflammatory cytokine induction in cells acquired from volunteers, and to the investigation as to whether there were any differences depending on the atopic and/or asthmatic status of volunteers, the effects of different TLR ligands was analysed in BEAS-2B cells, primary HBECs and PBMCs. Dose-time experiments were performed in these cell types and type I and III IFN and IL-6 and CXCL8/IL-8 mRNA was measured in cell lysates (figures 5.1-5.5). 10-fold dilutions of each ligand were made. Cells were stimulated with varying concentrations of TLR ligand and were harvested at various times following stimulation.

5.2.1.1 Pam₃CSK₄ (PAM) (TLR1/2 ligand)

In BEAS-2B cells a concentration dependent induction of IL-6 mRNA at 8h, 24h and 48h, and CXCL8/IL-8 mRNA at 8h and 24h was observed following stimulation with Pam₃CSK₄ (figure 5.1 A and B). This failed to reach statistical significance. A non-significant weak induction of IFN- β mRNA was observed at 24h with a concentration of 10 μ g/mL (figure 5.1 C). Induction of IFN- λ _{2,3}/IL-28 or IFN- λ ₁/IL-29 mRNA was not observed (not shown). In primary HBECs a non-significant induction of IL-6 and CXCL8/IL-8 mRNA was observed at 8h and 24h (figure 5.1 D and E). A non-significant induction IFN- β mRNA was observed at 8h and 24h with a concentration of 10 μ g/mL and at 24h with a concentration of 1 μ g/mL (figure 5.1 F). Induction of IFN- λ _{2,3}/IL-28 or IFN- λ ₁/IL-29 mRNA was not observed (not shown). In PBMCs induction of IL-6 and IFN- β mRNA was not observed (figure 5.1 G and I). Non-significant induction of CXCL8/IL-8 mRNA was observed at 48h (figure 5.1 H). Induction of IFN- λ _{2,3}/IL-28 or IFN- λ ₁/IL-29 mRNA was not observed (not shown). In each cell type, Pam₃CSK₄ at a concentration of 10 μ g/mL was used for subsequent experiments in participants' samples.

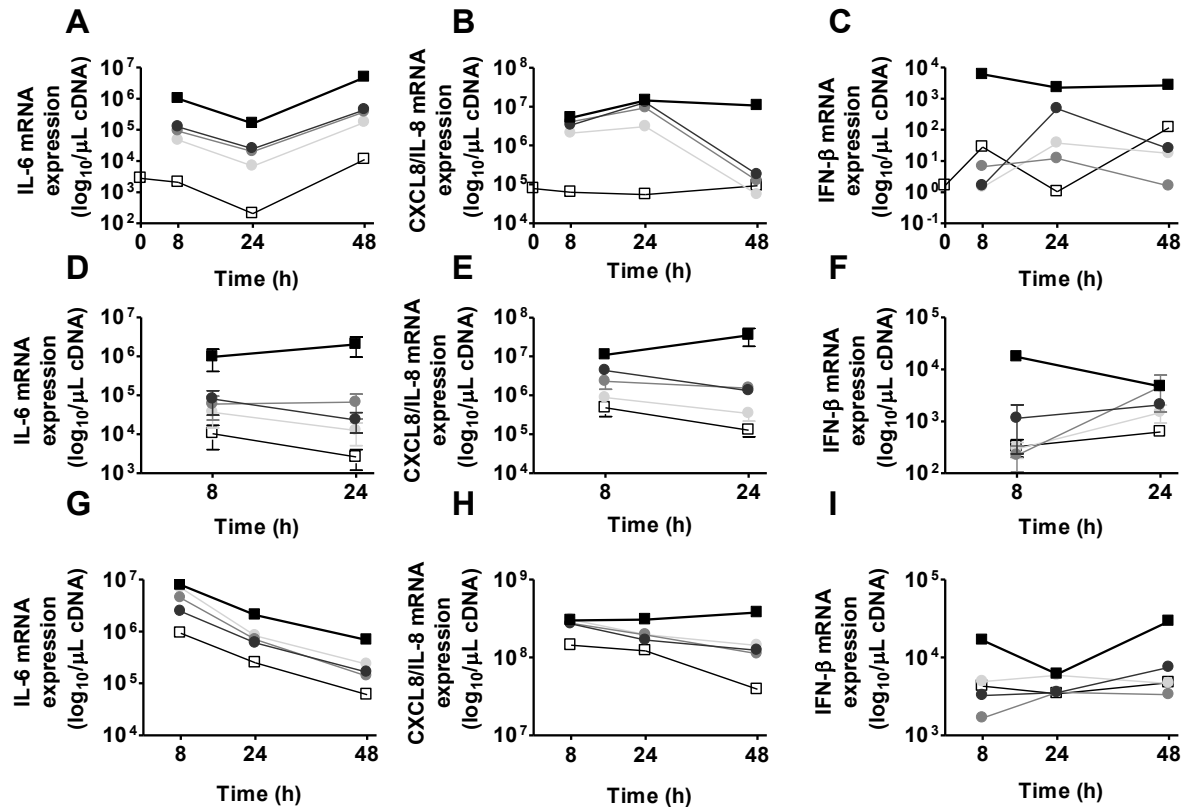


Figure 5.1. Concentration and time course response of Pam₃CSK₄ (PAM) in BEAS-2B cells (A, B and C, N=2), primary HBECs (D, E and F, N=3) and PBMCs (G, H and I, N=2) (□ = medium, ● = PAM 10μg/mL, ● = PAM 1μg/mL, ● = PAM 0.1μg/mL, ■ = poly(IC) 10μg/mL for epithelial cells, 100μg/mL for PBMCs). Poly(IC) was used as a positive control. In BEAS-2B cells non-significant concentration dependent induction of IL-6 mRNA at all time points (A) and CXCL8/IL-8 mRNA at 8h and 24h was observed (B). Non-significant weak induction of IFN-β mRNA was observed at 24h for 10μg/mL(C). In primary HBECs a non-significant induction of IL-6 mRNA and CXCL8/IL-8 mRNA at 8h and 24h was observed (D and E). Non-significant weak induction of IFN-β mRNA at 8 and 24h for 10μg/mL and 24h for 1μg/mL was observed (F). In PBMCs induction of IL-6 mRNA was not observed (G). Non-significant induction of CXCL8/IL-8 mRNA at 48h was observed (H). Induction of IFN-β mRNA was not observed (I). Induction of IFN-λ2,3/IL-28 or IFN-λ1/IL-29 mRNA was not observed in any cell type (not shown).

5.2.1.2 Lipoteichoic acid (LTA) (TLR2/6 ligand)

In BEAS-2B cells a concentration dependent induction of IL-6 and CXCL8/IL-8 mRNA was observed following stimulation with LTA (figure 5.2 A and B). This failed to reach statistical significance. Induction of IFN-β mRNA was not observed (figure 5.2 C). Induction of IFN-λ2,3/IL-28 or IFN-λ1/IL-29 mRNA was not observed (not shown). In primary HBECs induction of IL-6, CXCL8/IL-8 and IFN-β mRNA was not observed (figure 5.2 D-F). Likewise, induction of IFN-λ2,3/IL-28 or

IFN- λ 1/IL-29 mRNA was not observed (not shown). In PBMCs induction of IL-6 and CXCL8/IL-8 mRNA was observed in a concentration dependent manner following stimulation with LTA (figure 5.2 G and H). This failed to reach statistical significance. A non-significant induction of IFN- β mRNA was observed at 8h (figure 5.2 I). Induction of IFN- λ 2,3/IL-28 or IFN- λ 1/IL-29 mRNA was not observed (not shown). A dose of 25 μ g/mL was found to be optimal in BEAS-2Bs and PBMCs and was used for subsequent experiments in participants' samples.

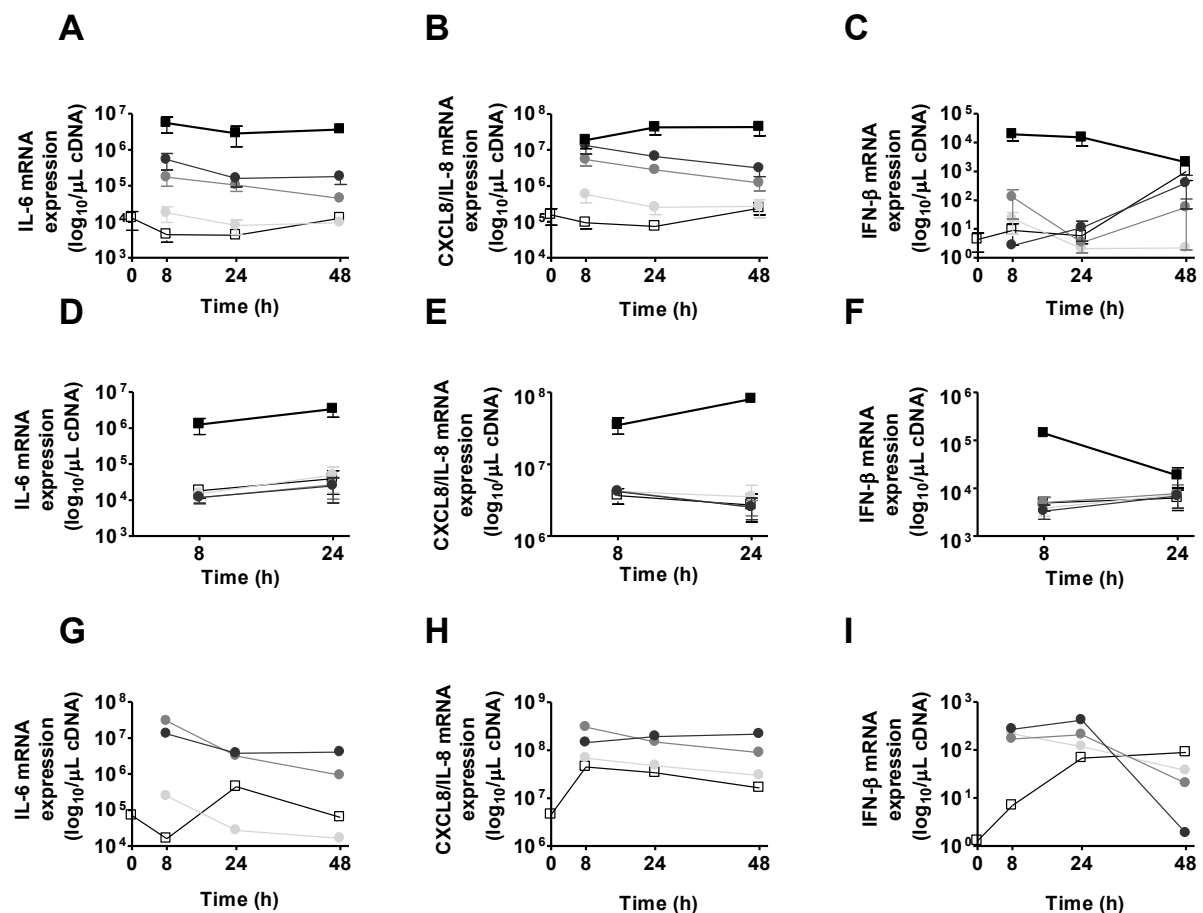


Figure 5.2. Concentration and time course response of Lipoteichoic acid (LTA) in BEAS-2B cells (A, B and C, N=3), primary HBECs (D, E and F, N=3) and PBMCs (G, H and I, N=2) (□ = medium, ● = LTA 25 μ g/mL, ● = LTA 10 μ g/mL, ● = LTA 1 μ g/mL, ■ = poly(IC) 10 μ g/mL). In epithelial cells poly(IC) was used as a positive control. In BEAS-2B cells non-significant concentration dependent induction of IL-6 and CXCL8/IL-8 mRNA was observed (A and B); induction of IFN- β mRNA was not observed (C). In primary HBECs induction of IL-6 mRNA (D), CXCL8/IL-8 mRNA (E) and IFN- β mRNA (F) was not observed. In PBMCs a non-significant induction of IL-6 and CXCL8/IL-8 mRNA at 8, 24 and 48h for the higher concentrations of LTA was observed (G and H). A non-significant weak induction of IFN- β mRNA was observed at 8h (I). Induction of IFN- λ 2,3/IL-28 or IFN- λ 1/IL-29 mRNA was not observed in any cell type (not shown).

5.2.1.3 Synthetic lipoprotein (FSL) (TLR2/6 ligand)

In BEAS-2B cells a concentration dependent induction of IL-6 and CXCL8/IL-8 mRNA was observed following stimulation with FSL (figure 5.3 A and B). This failed to reach statistical significance. Induction of IFN- β mRNA was not observed (figure 5.3 C). Induction of IFN- λ 2,3/IL-28 or IFN- λ 1/IL-29 mRNA was not observed (not shown). In primary HBECs induction of IL-6 and CXCL8/IL-8 mRNA was observed at 8h (figure 5.3 D and E). Induction of IFN- β mRNA was not observed (figure 5.3 F). Likewise, induction of IFN- λ 2,3/IL-28 or IFN- λ 1/IL-29 mRNA was not observed (not shown). In PBMCs weak induction of IL-6 mRNA at all time points, and CXCL8/IL-8 mRNA at 48h was observed following stimulation with FSL (figure 5.3 G and H). This failed to reach statistical significance. Induction of IFN- β mRNA was not observed (figure 5.3 I). Induction of IFN- λ 2,3/IL-28 or IFN- λ 1/IL-29 mRNA was not observed (not shown). A concentration of 1 μ g/mL was found to be optimal in BEAS-2Bs, HBECs and PBMCs and was used for subsequent experiments in participants' samples.

5.2.1.4 Peptidoglycan (PGN) (TLR2/6 ligand)

In BEAS-2B cells induction of IL-6 and IFN- β mRNA was not observed following stimulation with PGN (figure 5.4 A and C). Non-significant weak induction of CXCL8/IL-8 mRNA was observed at 8h and 24h (figure 5.4 B). In primary HBECs non-significant induction of IL-6 mRNA was observed (figure 5.4 D). Induction of CXCL8/IL-8 and IFN- β mRNA was not observed (figure 5.4 E and F). In PBMCs a non-significant induction of IL-6 mRNA at 8, 24 and 48h (figure 5.4 G), and CXCL8/IL-8 mRNA at 24 and 48h was observed (figure 5.4 H). A non-significant induction of IFN- β mRNA was observed at 8h (figure 5.4 I). Induction of IFN- λ 2,3/IL-28 or IFN- λ 1/IL-29 mRNA was not observed in any cell type (data not shown). A concentration of 25 μ g/mL was found to be optimal in HBECs and PBMCs and was used for subsequent experiments in participants' samples.

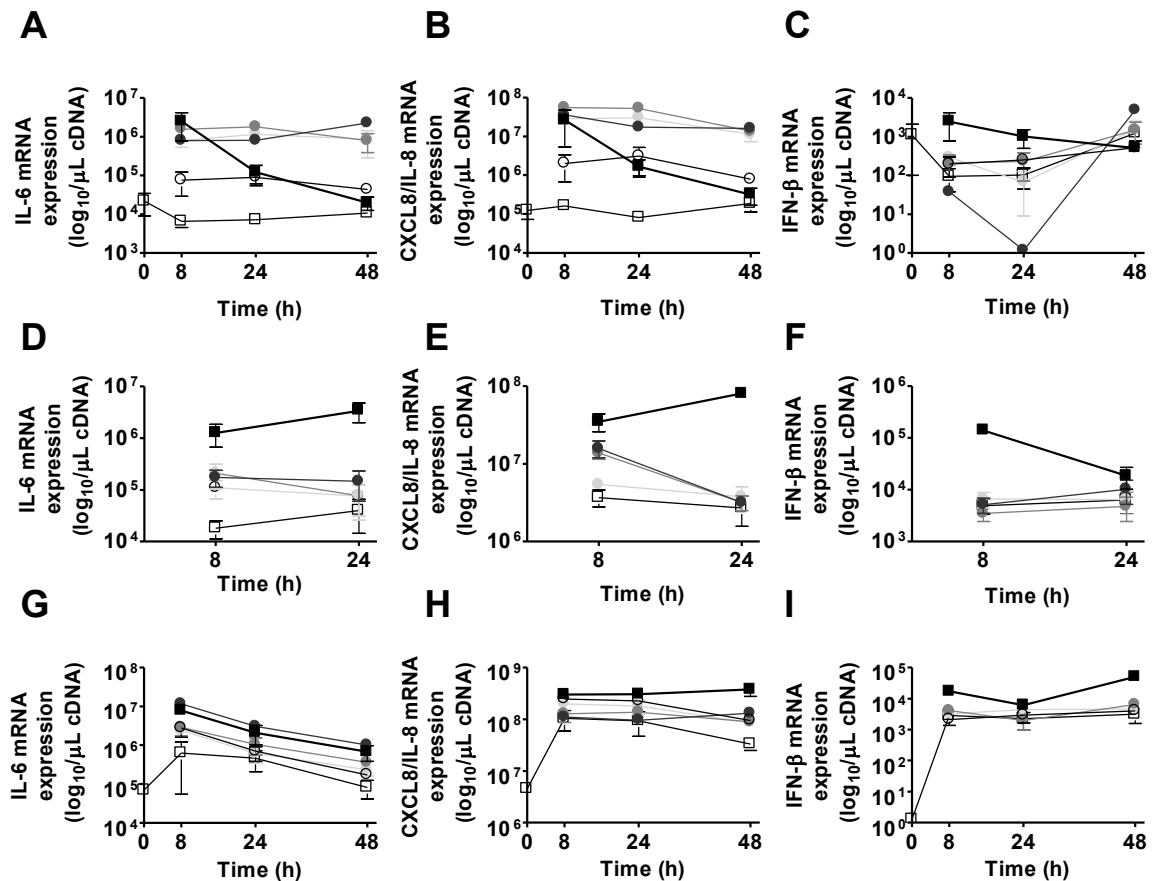


Figure 5.3. Concentration and time course response of Synthetic lipoprotein (FSL) in BEAS-2B cells (A, B and C, N=3), primary HBECs (D, E and F, N=3) and PBMCs (G, H and I, N=3) (□ = medium, ● = FSL 10μg/mL, ● = FSL 1μg/mL, ● = FSL 0.1μg/mL, ○ = FSL 0.01μg/mL, ■ = poly(IC) 10μg/mL for epithelial cells, 100μg/mL for PBMCs). Poly(IC) was used as a positive control. In BEAS-2B cells a non-significant induction of IL-6 mRNA and CXCL8/IL-8 mRNA at 8, 24 and 48h was observed (A and B) with the optimal concentration being 1μg/mL. IFN-β mRNA induction not observed (C). In primary HBECs induction of IL-6 mRNA at 8h (D), and CXCL8/IL-8 mRNA at 8h with the higher 2 concentrations (E) was observed. Induction of IFN-β mRNA (F) was not observed. In PBMCs a non-significant weak induction of IL-6 mRNA at 8, 24 and 48h, and CXCL8/IL-8 mRNA at 48h for the higher concentrations of FSL was observed (G and H). IFN-β mRNA induction in FSL treated cells over that of medium treated cells was not observed (I). Induction of IFN-λ2,3/IL-28 or IFN-λ1/IL-29 mRNA was not observed (not shown).

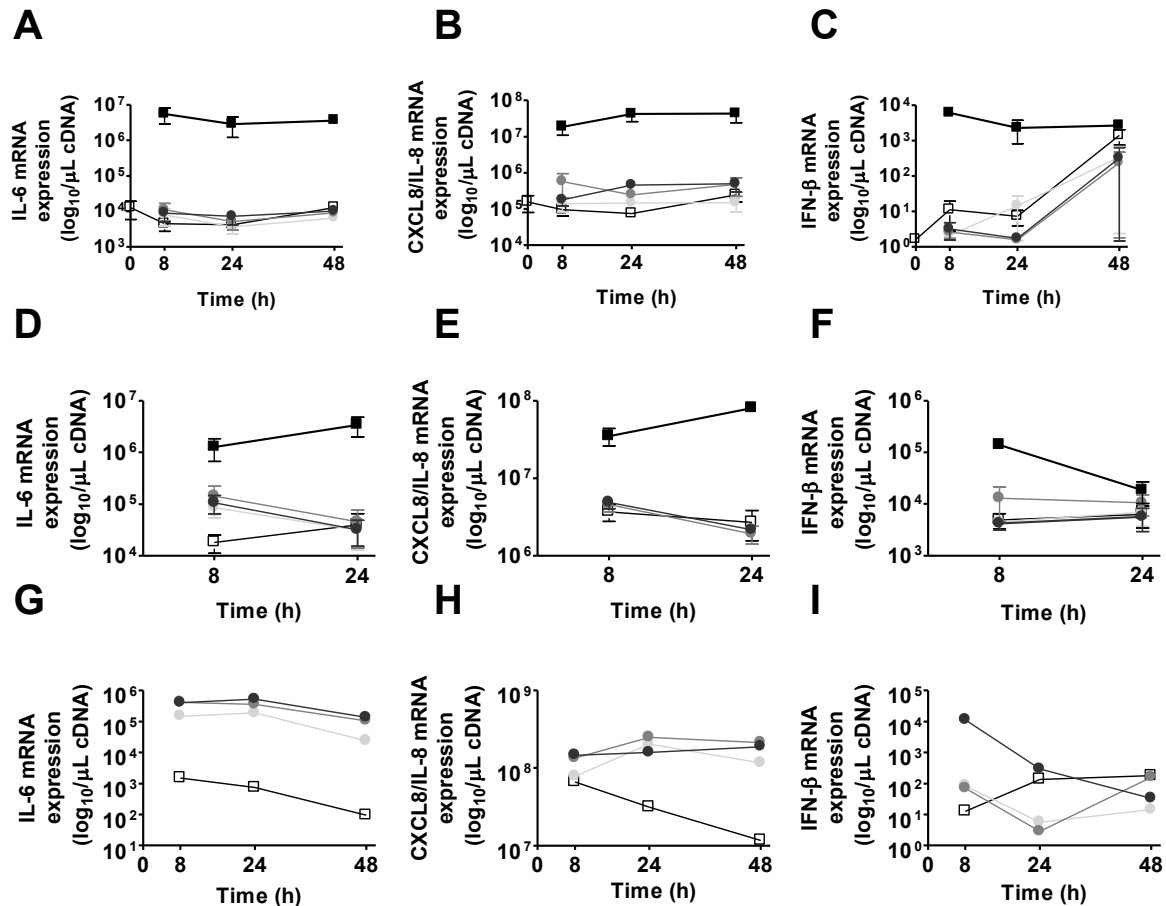


Figure 5.4. Concentration and time course response of Peptidoglycan (PGN) in BEAS-2B cells (A, B and C, N=3), primary HBECs (D, E and F, N=3) and PBMCs (G, H and I, N=2) (□ = medium, ● = PGN 25μg/mL, ● = PGN 10μg/mL, ● = PGN 1μg/mL, ■ = poly(IC) 10μg/mL). In epithelial cells poly(IC) was used as a positive control. In BEAS-2B cells induction of IL-6 mRNA was not observed (A). Weak induction of CXCL8/IL-8 mRNA, at 8h with PGN 10μg/mL and at 24h with PGN 25μg/mL, was observed (B). Induction of IFN-β mRNA was not observed (C). In primary HBECs a non-significant induction of IL-6 mRNA at 8h was observed (D). Induction of CXCL8/IL-8 mRNA and IFN-β mRNA was not observed (E and F). In PBMCs a non-significant induction of IL-6 mRNA at 8, 24 and 48h (G), and CXCL8/IL-8 mRNA at 24 and 48h (H). A non-significant induction of IFN-β mRNA was observed at 8h (I). Induction of IFN-λ2,3/IL-28 or IFN-λ1/IL-29 mRNA was not observed in any cell type (not shown).

5.2.1.5 Flagellin (FLA) (TLR5 ligand)

In BEAS-2B cells a concentration dependent induction of IL-6 and CXCL8/IL-8 mRNA was observed following stimulation with FLA. This failed to reach statistical significance (figure 5.5 A and B). Induction of IFN-β mRNA was not observed (figure 5.5 C). In primary HBECs induction of IL-6 and IFN-β mRNA was not observed (figure 5.5 D and F). A non-significant weak induction of CXCL8/IL-8

mRNA was observed at 8h (figure 5.5 E). In PBMCs a non-significant induction of IL-6 mRNA was observed for 100ng/mL FLA and of CXCL8/IL-8 mRNA for each concentration of FLA at 8h (figure 5.5G and H). Induction of IFN- β mRNA was not observed (figure 5.5 I). Induction of IFN- λ 2,3/IL-28 or IFN- λ 1/IL-29 mRNA was not observed in any cell type (data not shown). A concentration of 100ng/mL was found to be optimal in BEAS-2Bs, HBECs and PBMCs and was used for subsequent experiments in participants' samples.

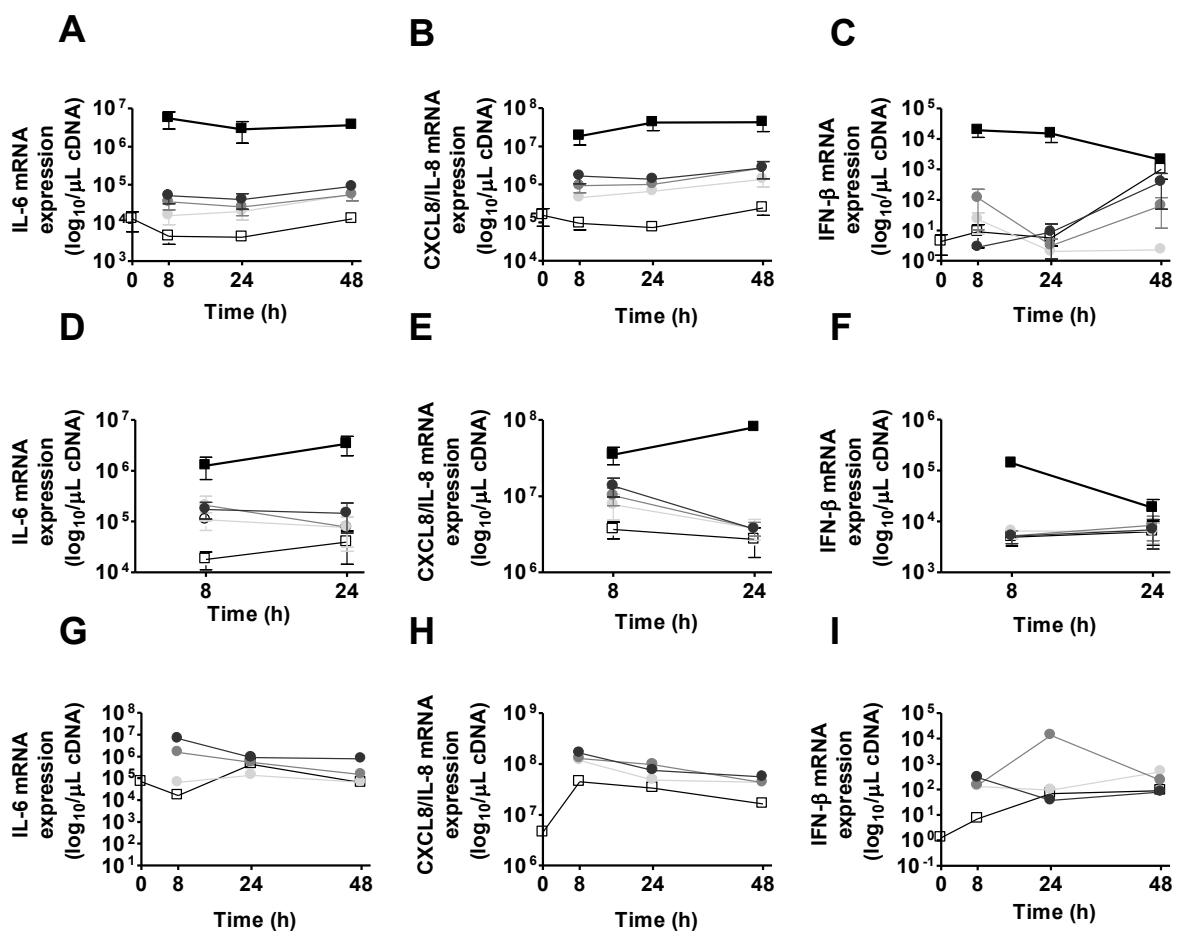


Figure 5.5. Concentration and time course response of Flagellin (FLA) in BEAS-2B cells (A, B and C, N=3), primary HBECs (D, E and F, N=3) and PBMCs (G, H and I, N=2) (□ = medium, ● = FLA 100ng/mL, ● = FLA 25ng/mL, ● = FLA 10ng/mL, ■ = poly(IC) 10μg/mL). In epithelial cells poly(IC) was used as a positive control. In BEAS-2B cells non-significant concentration dependent induction of IL-6 and CXCL8/IL-8 mRNA was observed (A and B); induction of IFN- β mRNA was not observed (C). In primary HBECs induction of IL-6 mRNA (D) and IFN- β mRNA (F) was not observed. A non-significant concentration dependent weak induction of CXCL8/IL-8 mRNA was observed at 8h (E). In PBMCs a non-significant induction of IL-6 mRNA with 100ng/mL at 8 and 48h and a half log increase in CXCL8/IL-8 mRNA at 8h for each concentration of FLA was observed (G and H). Induction of IFN- β mRNA was not observed at (I). Induction of IFN- λ 2,3/IL-28 or IFN- λ 1/IL-29 mRNA was not observed in any cell type (not shown).

5.2.1.6 Lipopolysaccharide (LPS) (TLR4) and CpG-C (TLR9)

The concentrations of LPS and CpG-C were used from already established protocols within the department (A. Sykes, unpublished data/thesis ³³⁴). No preliminary experiments were performed with LPS or CpG-C.

5.2.2 TLR1, 2 and 6 stimulation of HBECs: type I and III IFN and proinflammatory cytokine mRNA and protein induction

As discussed in chapter 3, stimulation of TLR3 leads to the induction of type I and III IFNs in HBECs. In order to establish whether other TLRs were involved in type I and III IFN signalling, particularly those TLRs stimulated by ligands which are bacterial components, each TLR was evaluated using different TLR ligands. For the purposes of discussion they were grouped into TLR1, 2 and 6, TLR4, TLR5 and TLR9. Whether there was a difference between the asthmatic or atopic nature of volunteers' samples was investigated. Cells were stimulated with different TLR ligands and type I and III IFN and proinflammatory cytokines were measured; mRNA at 8h and 24h, and protein at 24h post stimulation.

5.2.2.1 Pam₃CSK₄

IFN- β mRNA was not induced by Pam₃CSK₄ at 8h or 24h (figure 5.6 A and B). At 8h IFN- λ 2,3/IL-28 mRNA was not significantly induced by Pam₃CSK₄. There was a suggestion of induction in the NAA group but sample size was small (figure 5.6 C). At 24h a weak increase was observed in NANA and ANA but this failed to reach statistical significance (figure 5.6 D). There was a suggestion that IFN- λ 1/IL-29 mRNA was induced by Pam₃CSK₄ at 8h in NAA (the sample size was small), but not in the other groups (figure 5.6 E). At 24h a non-significant increase was observed in NANA only (figure 5.6 F). With type I and III IFN mRNA induction there were no significant differences were observed between groups.

No significant induction of IFN- β protein was observed at 24h post stimulation with Pam₃CSK₄ in any group, with a significant difference observed between ANA

and AA (figure 5.7 A). A non-significant weak increase in IFN- λ 1/IL-29 protein induction in NANA was observed, with no induction in other groups (figure 5.7 B).

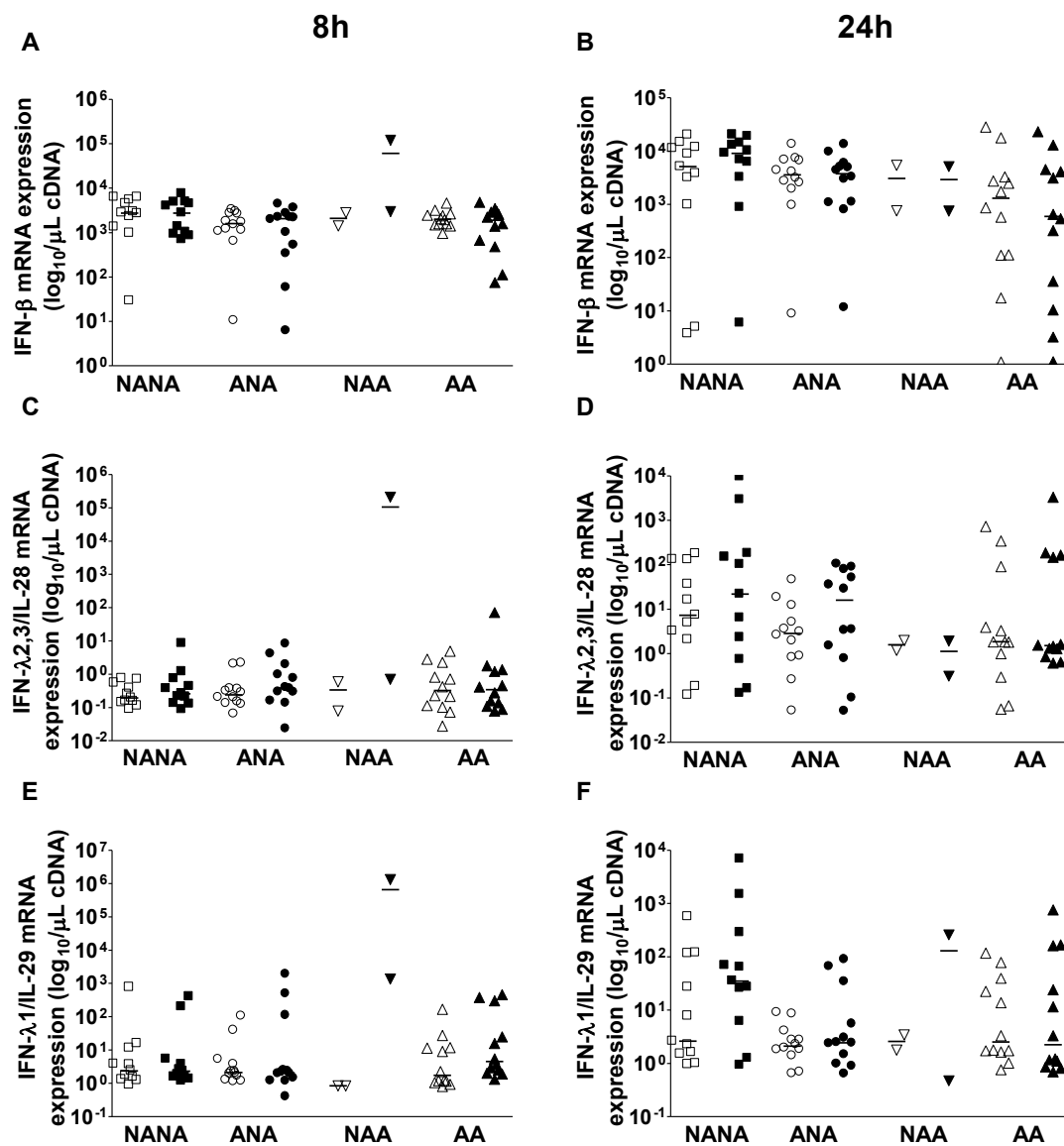


Figure 5.6. Interferon mRNA induction in HBECs post stimulation with Pam₃CSK₄ at 8h (A, C and E) and 24h (B, D and F). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, black shapes denote Pam₃CSK₄. IFN-β (A and B), IFN-λ2,3/IL-28 (C and D), IFN-λ1/IL-29 (E and F). No significant induction of IFN-β mRNA was observed in any group. In NAA induction of IFN-λ2,3/IL-28 and IFN-λ1/IL-29 mRNA was observed at 8h. A non-significant induction of IFN-λ2,3/IL-28 and IFN-λ1/IL-29 mRNA was observed in NANA at 24h. No significant differences were observed between groups.

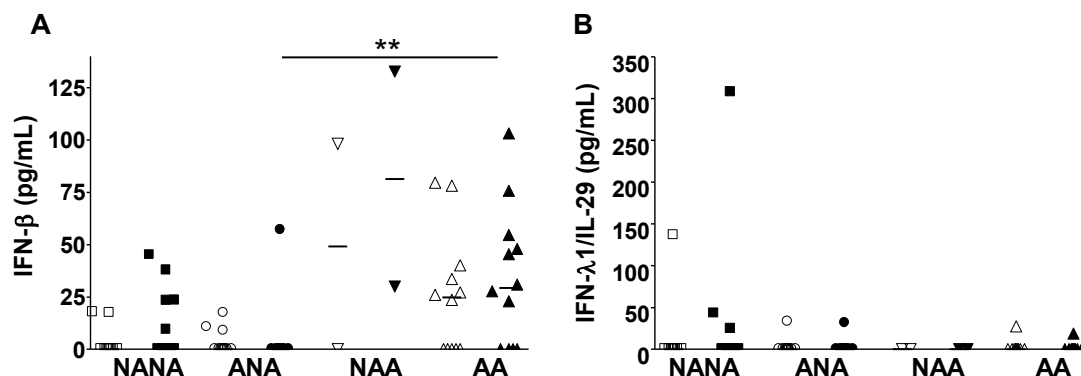


Figure 5.7. Interferon protein induction in HBECs post stimulation with Pam₃CSK₄ at 24h. Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, black shapes denote Pam₃CSK₄; ** = $p < 0.01$. IFN- β (A), IFN- λ 1/IL-29 (B). A trend for IFN- β protein induction was observed in NANA, NAA and AA, with a significant difference between ANA and AA. A non-significant weak increase in IFN- λ 1/IL-29 protein induction in NANA was observed, with no induction in other groups.

In order to examine whether stimulation of HBECs with Pam₃CSK₄ resulted in induction of proinflammatory cytokines and to observe whether there were differences between the asthmatic or atopic status of the volunteers from whom the cells were obtained, IL-6 and CXCL8/IL-8 mRNA was measured at 8h and 24h, and IL-1 β , IL-6, CXCL8/IL-8 and TNF- α protein was measured at 24h. Non-significant induction of IL-6 mRNA was observed at 8h in NAA, with no induction observed in other groups (figure 5.8 A). Induction was not observed at 24h (figure 5.8 B). At 8h, significant induction of CXCL8/IL-8 mRNA was observed in NANA ($p < 0.05$), with a non-significant induction observed in ANA, NAA and AA (figure 5.8 C). Induction was not observed at 24h (figure 5.8 D). With both IL-6 and CXCL8/IL-8 mRNA no significant differences were observed between groups at both time points.

Weak induction of CXCL8/IL-8 protein from HBECs stimulated with Pam₃CSK₄ was observed at 24h but this failed to reach statistical significance (figure 5.9 C). Induction of IL-1 β , IL-6 and TNF- α protein was not observed (figure 5.9 A, B and D). No differences were observed between the four volunteer groups.

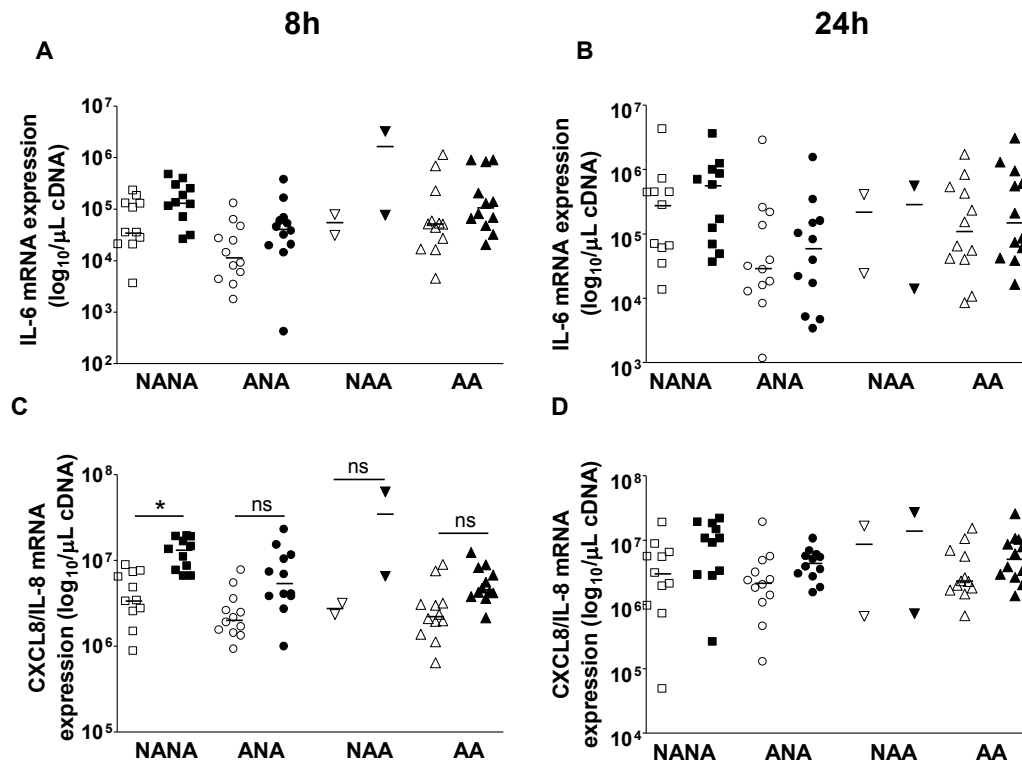


Figure 5.8. IL-6 and CXCL8/IL-8 mRNA induction in HBECs post stimulation with Pam₃CSK₄ at 8h (A and C) and 24h (B and D). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, black shapes denote Pam₃CSK₄; * = p<0.05, ns = non-significant. IL-6 (A and B), CXCL8/IL-8 (C and D). Non-significant induction of IL-6 mRNA at 8h was observed in NAA only, with no induction at 24h. Significant induction of CXCL8/IL-8 mRNA was observed in NANA at 8h with non-significant increases in other groups. No induction was observed at 24h. No significant differences were observed between groups.

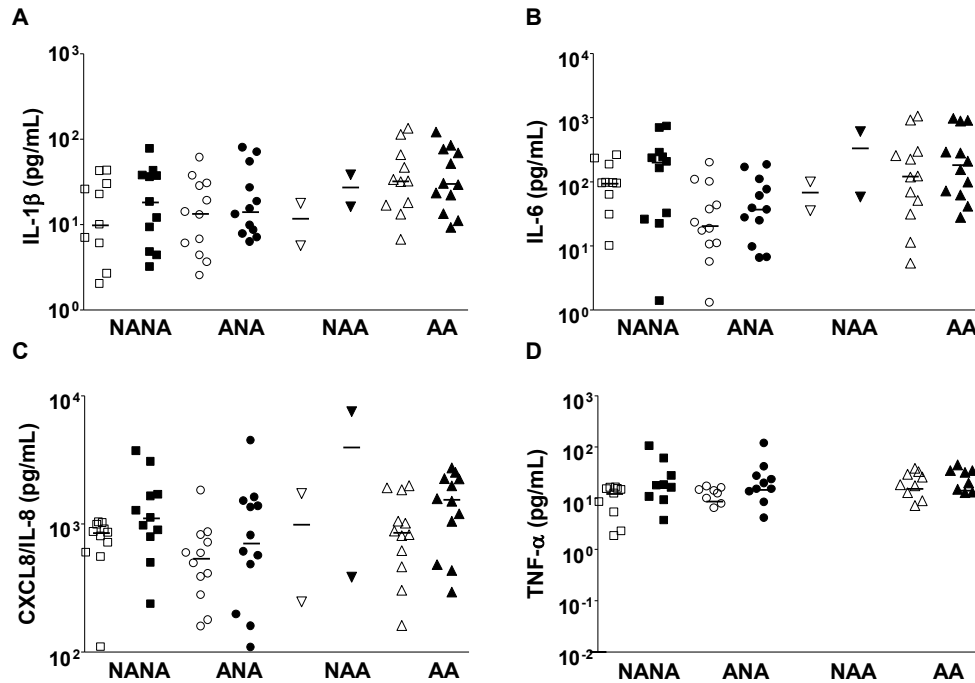


Figure 5.9. Proinflammatory cytokine protein induction in HBECs post stimulation with Pam₃CSK₄ at 24h. Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, black shapes denote Pam₃CSK₄. IL-1 β (A), IL-6 (B), CXCL8/IL-8 (C), TNF- α (D). Non-significant weak induction of CXCL8/IL-8 protein was observed in all groups. Induction of IL-1 β , IL-6 and TNF- α was not observed. There were no differences between groups.

5.2.2.2 Lipoteichoic Acid (LTA)

As with Pam₃CSK₄, the type I and III IFN and proinflammatory cytokine response to LTA was assessed in HBECs. IFN- β mRNA was not induced by LTA at 8h or 24h but high basal levels of IFN- β mRNA were observed (figure 5.10 A and B). At 8h and 24h IFN- λ 2,3/IL-28 mRNA was weakly induced by LTA in NAA but sample size was small (figure 5.10 C and D). At 24h a weak increase was observed in NANA, ANA and NAA but this failed to reach statistical significance (figure 5.10 D). IFN- λ 1/IL-29 mRNA was not induced by LTA at 8h in any group (figure 5.10 E). At 24h a non-significant increase was observed in all groups (figure 5.10 F). With type I and III IFN mRNA induction there were no significant differences were observed between groups.

No significant induction of IFN- β or IFN- λ 1/IL-29 protein was observed at 24h post stimulation with LTA in any group (appendix A, figure A1).

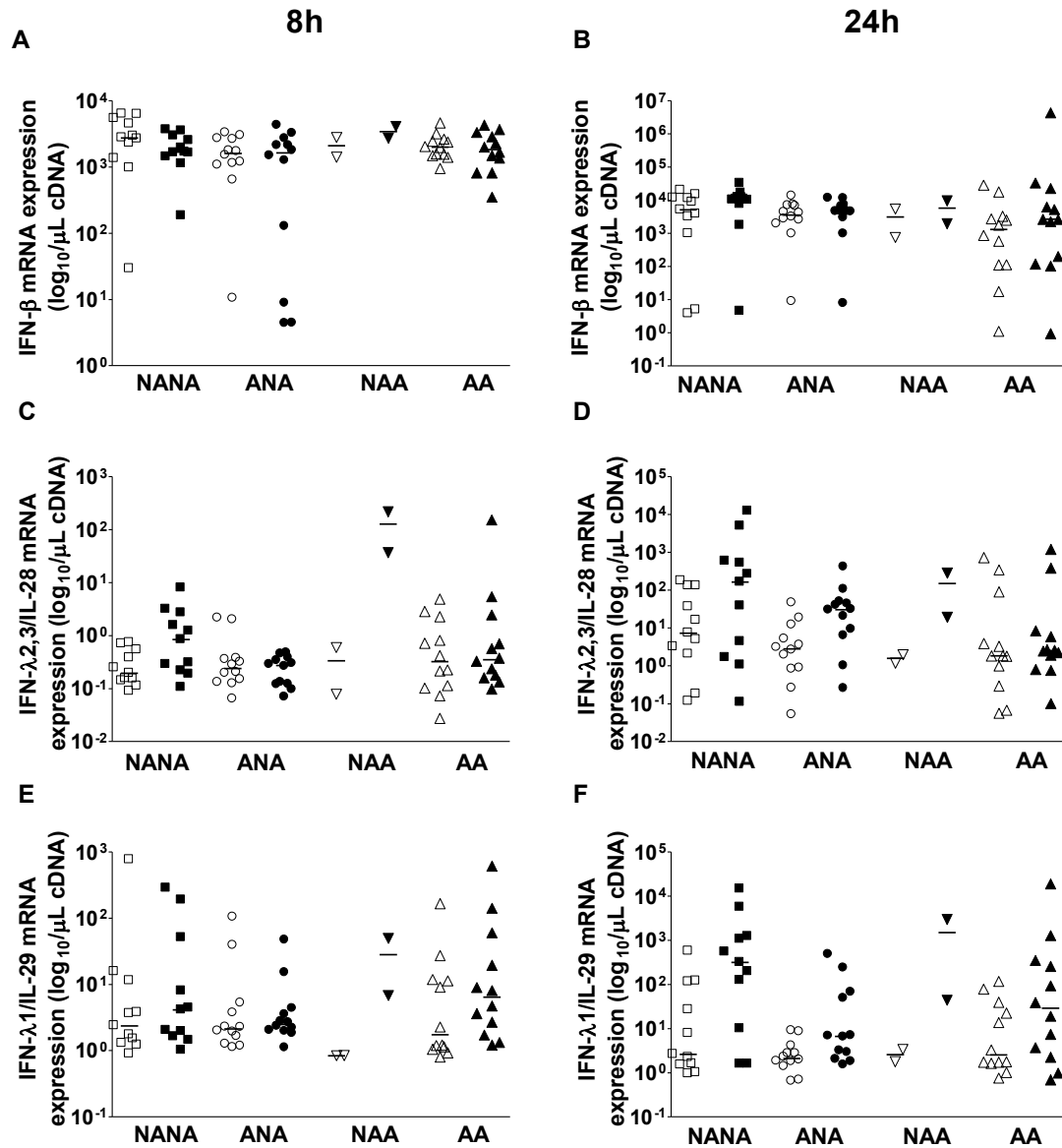


Figure 5.10. Interferon mRNA induction in HBECs post stimulation with LTA at 8h (A, C and E) and 24h (B, D and F). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, black shapes denote LTA. IFN- β (A and B), IFN- λ 2,3/IL-28 (C and D), IFN- λ 1/IL-29 (E and F). Induction of IFN- β mRNA was not observed. Non-significant weak induction of IFN- λ 2,3/IL-28 mRNA was observed at 8h in NAA and in NANA, ANA and NAA at 24h. Non-significant induction of IFN- λ 1/IL-29 mRNA was observed in all groups at 24h. No differences were observed between groups at both time points and with all IFNs measured.

Proinflammatory cytokines were measured in HBECs stimulated with LTA. Induction of IL-6 mRNA was not observed at 8h or 24h in any group (figure 5.11 A and B). At 8h, non-significant induction of CXCL8/IL-8 mRNA was observed in all groups (figure 5.11 C). Induction was not observed at 24h (figure 5.11 D). With both IL-6 and CXCL8/IL-8 mRNA no significant differences were observed between groups.

At 24h following stimulation with LTA non-significant weak induction of IL-1 β , and CXCL8/IL-8 was observed in all groups and IL-6 and TNF- α protein in NANA and ANA (figure 5.12). No differences were observed between the four volunteer groups.

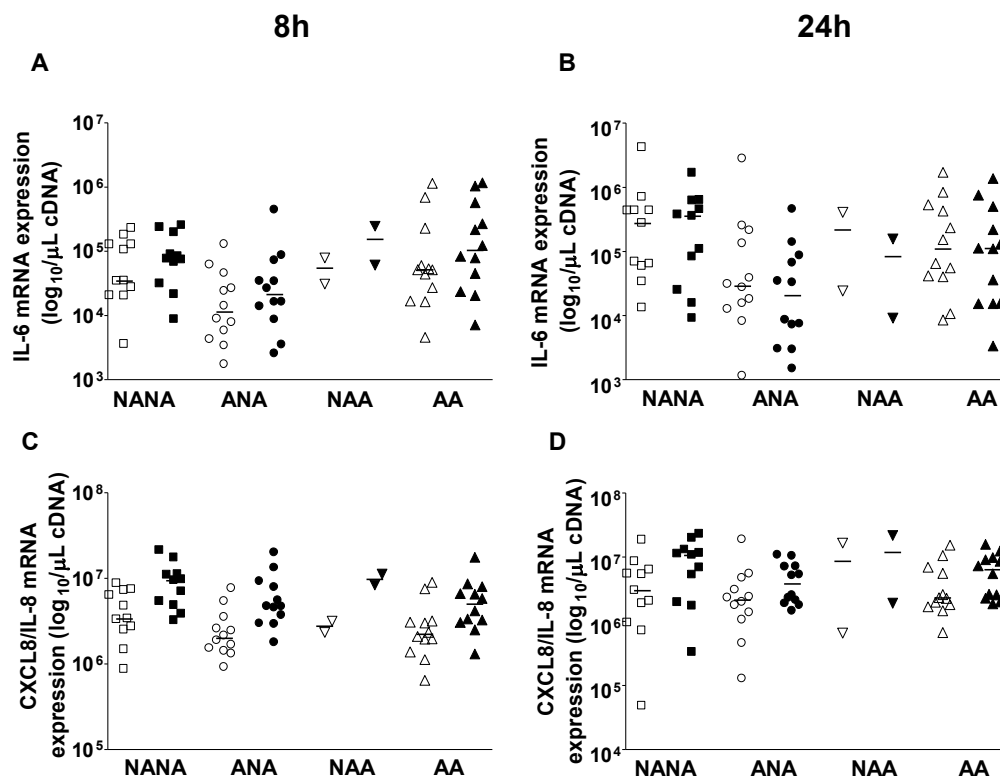


Figure 5.11. IL-6 and CXCL8/IL-8 mRNA induction in HBECs post stimulation with LTA at 8h (A and C) and 24h (B and D). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, black shapes denote LTA. IL-6 (A and B), CXCL8/IL-8 (C and D). Induction of IL-6 mRNA was not observed in any group. Non-significant induction of CXCL8/IL-8 mRNA was observed in all groups at 8h. No differences were observed between groups.

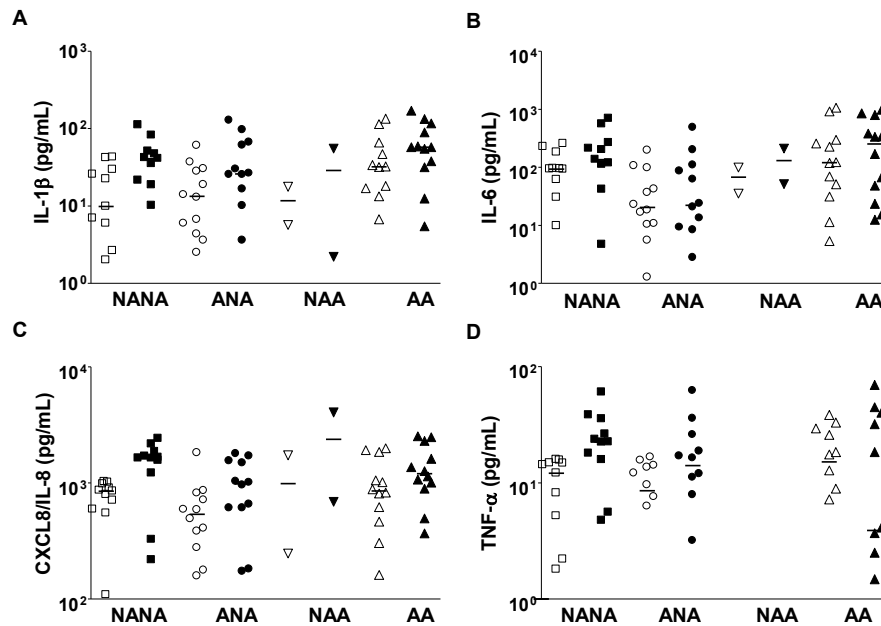


Figure 5.12. Proinflammatory cytokine protein induction in HBECs post stimulation with LTA at 24h. Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, black shapes denote LTA. IL-1 β (A), IL-6 (B), CXCL8/IL-8 (C), TNF- α (D). Non-significant increases of IL-1 β , IL-6, CXCL8/IL-8 and TNF- α protein were observed. Induction of TNF- α was not observed in NAA or AA. There were no differences between groups.

5.2.2.3 Peptidoglycan (PGN)

Type I and III IFN and proinflammatory cytokine response to PGN was assessed in HBECs. IFN- β and IFN- λ 2,3/IL-28 mRNA was not induced by PGN at 8h or 24h (figure 5.13 A-D). IFN- λ 1/IL-29 mRNA was non-significantly induced by PGN at 8h and 24h in NAA only (figure 5.13 E and F) but sample size was small. No significant differences were observed between groups.

No significant induction of IFN- β protein was observed at 24h post stimulation with PGN in any group (appendix A, figure A2, A). In NAA induction of IFN- λ 1/IL-29 protein was observed (appendix A, figure A2, B), but this failed to reach statistical significance with a small sample size. No statistically significant differences were observed between groups.

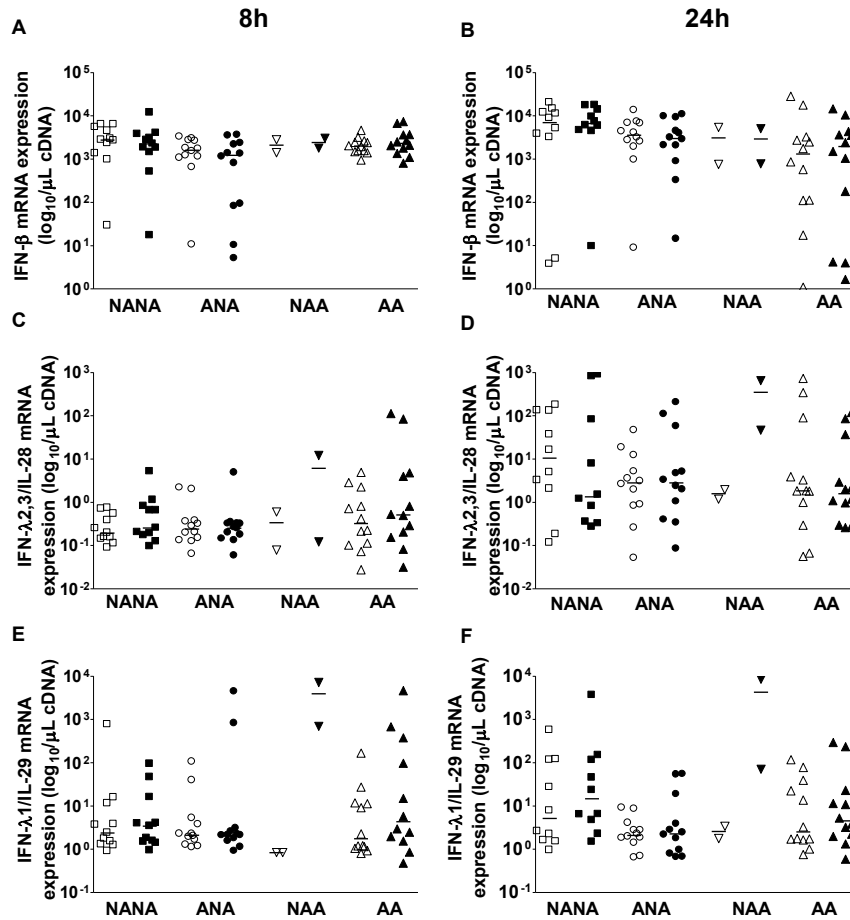


Figure 5.13. Interferon mRNA induction in HBECs post stimulation with PGN at 8h (A, C and E) and 24h (B, D and F). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, black shapes denote PGN. IFN- β (A and B), IFN- λ 2,3/IL-28 (C and D), IFN- λ 1/IL-29 (E and F). Induction of IFN- β and IFN- λ 2,3/IL-28 mRNA was not observed. Non-significant induction of IFN- λ 1/IL-29 mRNA was observed in NAA only. No significant differences were observed between groups.

Proinflammatory cytokines were measured in HBECs stimulated with PGN. Induction of IL-6 mRNA was not observed at 8h or 24h in any group (figure 5.14 A and B). At 8h, non-significant induction of CXCL8/IL-8 mRNA was observed in NAA (figure 5.14 C). Induction was not observed at 24h (figure 5.14 D). No significant differences were observed between groups.

At 24h following stimulation with PGN non-significant weak induction of IL-1 β , IL-6, CXCL8/IL-8 and TNF- α protein was observed in NAA only (figure 5.15). No differences were observed between the four volunteer groups.

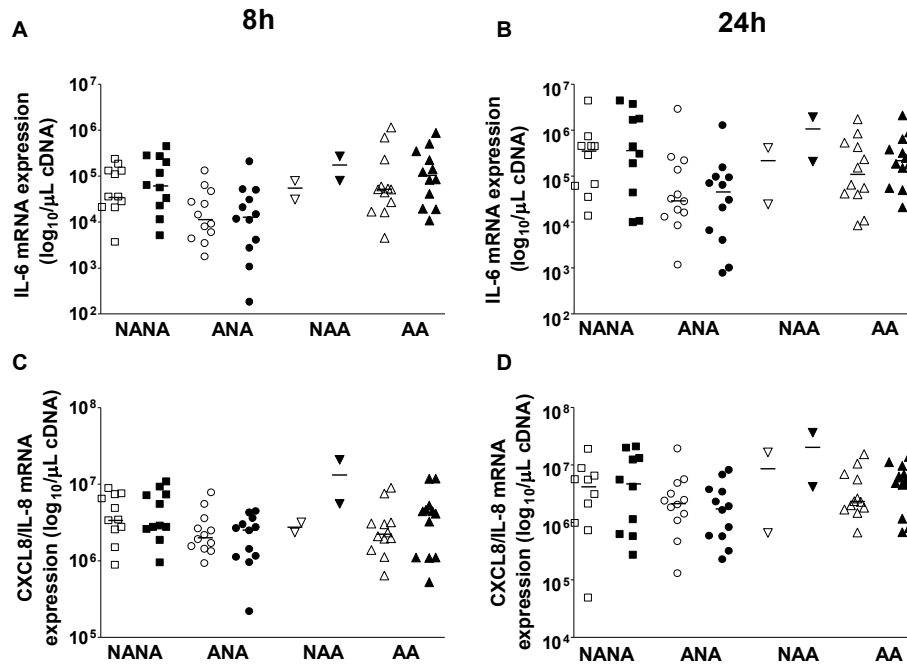


Figure 5.14. IL-6 and CXCL8/IL-8 mRNA induction in HBECs post stimulation with PGN at 8h (A and C) and 24h (B and D). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, black shapes denote PGN. IL-6 (A and B), CXCL8/IL-8 (C and D). Induction of IL-6 mRNA was not observed in any group. At 8h a non-significant induction of CXCL8/IL-8 mRNA was observed in the NAA group, with no induction in other groups or at 24h. No significant differences were observed between groups.

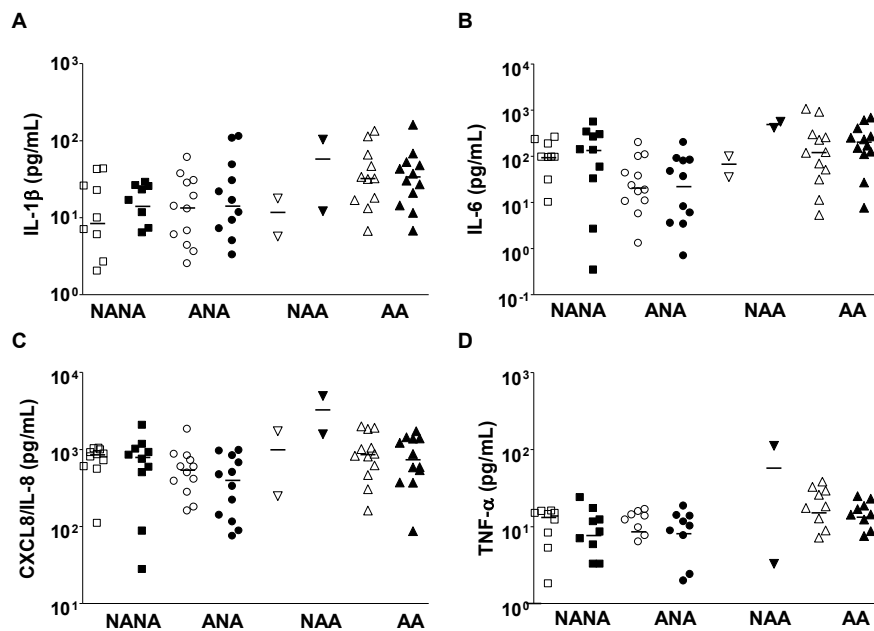


Figure 5.15. Proinflammatory cytokine protein induction in HBECs post stimulation with PGN at 24h. Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, black shapes denote PGN. IL-1β (A), IL-6 (B), CXCL8/IL-8 (C), TNF-α (D). Non-significant weak induction of IL-1β, IL-6, CXCL8/IL-8 and TNF-α protein was observed in NAA. Induction of proinflammatory cytokines was not observed in other groups. There were no differences between groups.

5.2.2.4 Synthetic lipoprotein (FSL)

Type I and III IFN and proinflammatory cytokine response to FSL was assessed in HBECs. IFN- β and IFN- λ 2,3/IL-28 mRNA was not induced by FSL at 8h or 24h (figure 5.16 A-D). IFN- λ 1/IL-29 mRNA was non-significantly induced by FSL at 8h in NAA only (figure 5.16 E). No significant differences were observed between groups.

No significant induction of IFN- β or IFN- λ 1/IL-29 protein was observed at 24h post stimulation with FSL in any group (appendix A, figure A3). No statistically significant differences were observed between groups.

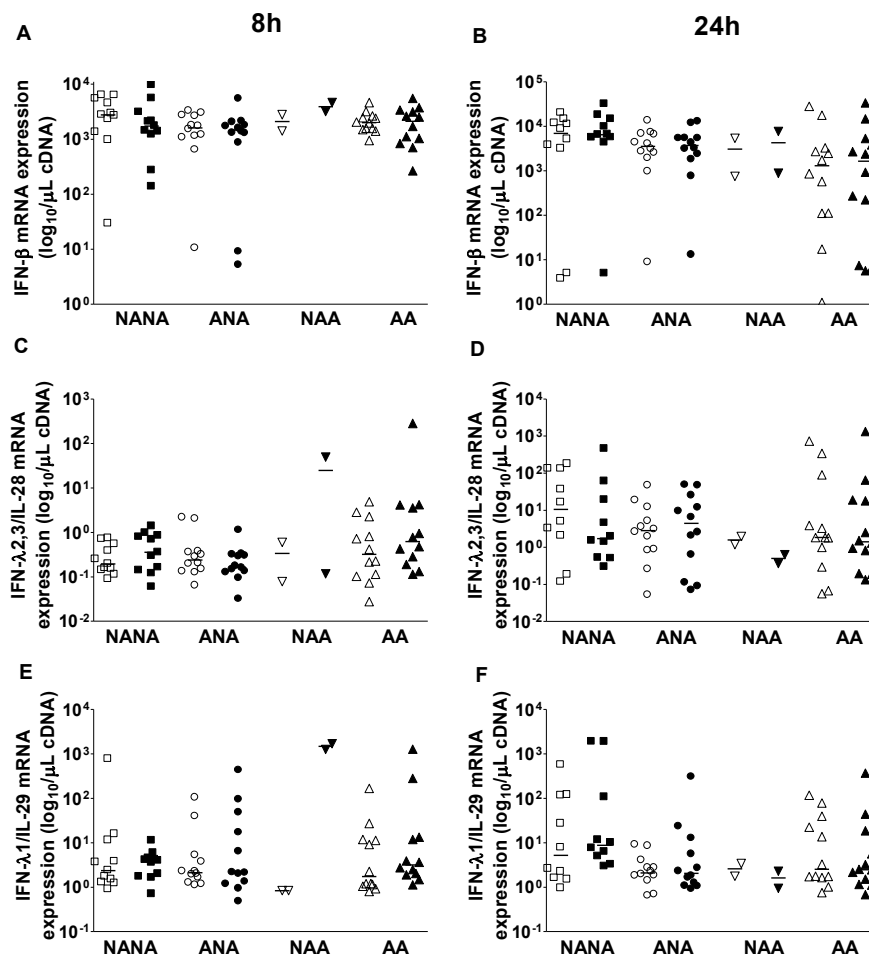


Figure 5.16. Interferon mRNA induction in HBECs post stimulation with FSL at 8h (A, C and E) and 24h (B, D and F). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, black shapes denote FSL. IFN- β (A and B), IFN- λ 2,3/IL-28 (C and D), IFN- λ 1/IL-29 (E and F). No induction of IFN- β or IFN- λ 2,3/IL-28 mRNA was observed. Non-significant induction of IFN- λ 1/IL-29 mRNA was observed in NAA at 8h. No significant differences were observed between groups.

Proinflammatory cytokines were measured in HBECs stimulated with FSL. Non-significant induction of IL-6 mRNA was observed at 8h in NANA, ANA and NAA and in ANA at 24h (figure 5.17 A and B). At 8h, significant induction of CXCL8/IL-8 mRNA was observed in NANA, ANA and AA (all $p < 0.05$), with a non-significant increase observed in NAA (figure 5.17 C). Induction was less marked at 24h (figure 5.17 D). No significant differences were observed between groups.

At 24h following stimulation with FSL non-significant weak induction of IL-1 β , IL-6 protein was observed in all groups (figure 5.18 A and B). Induction of CXCL8/IL-8 protein was observed in all groups, with statistical significance reached in ANA ($p < 0.01$) and AA ($p < 0.05$) (figure 5.18 C). TNF- α protein induction was observed in all groups with statistical significance reached in ANA only ($p < 0.05$) (figure 5.18 D). No differences were observed between the four volunteer groups.

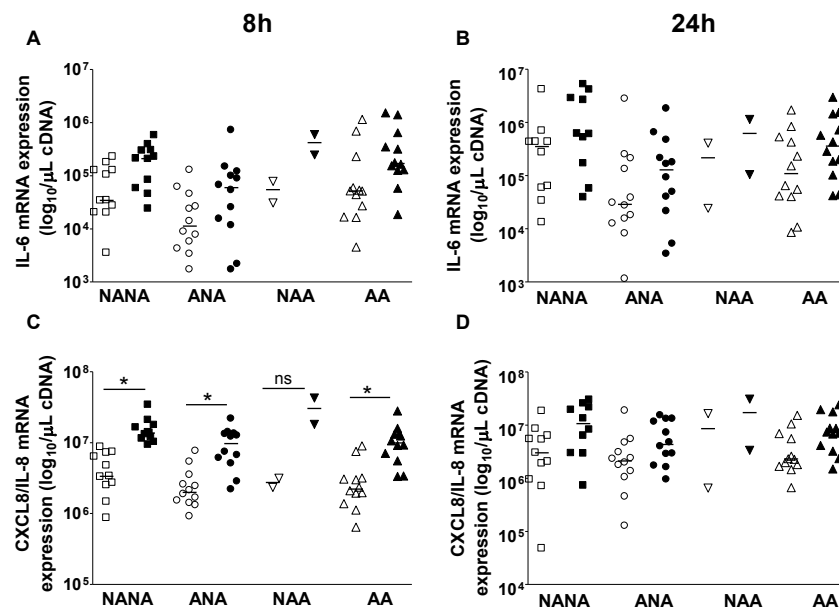


Figure 5.17. IL-6 and CXCL8/IL-8 mRNA induction in HBECs post stimulation with FSL at 8h (A and C) and 24h (B and D). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, black shapes denote FSL; * = $p < 0.05$, ns = non-significant. IL-6 (A and B), CXCL8/IL-8 (C and D). Non-significant induction of IL-6 mRNA was observed at 8h in NANA, ANA and NAA and in ANA at 24h. Significant induction of CXCL8/IL-8 mRNA was observed at 8h. No significant differences were observed between groups.

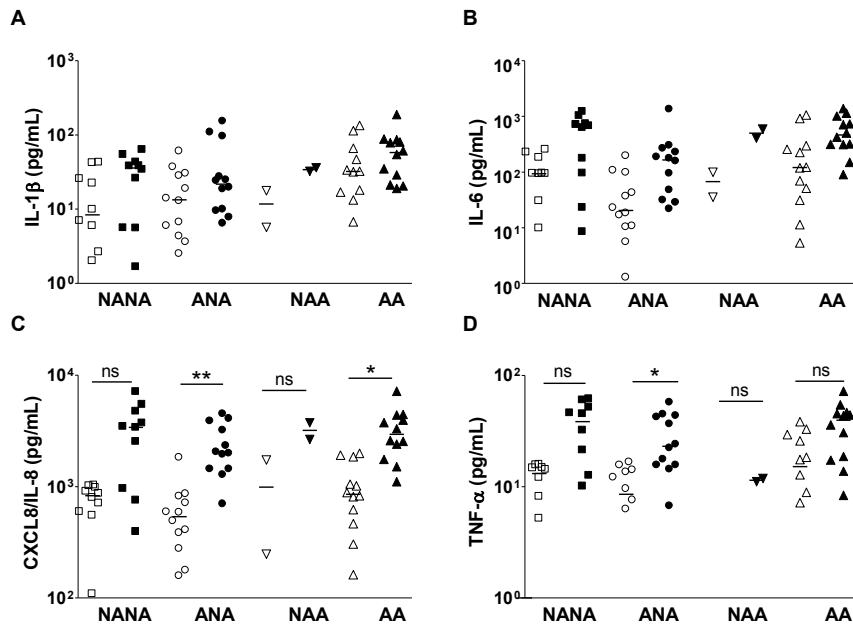


Figure 5.18. Proinflammatory cytokine protein induction in HBECs post stimulation with FSL at 24h. Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, black shapes denote FSL; * = $p < 0.05$, ** = $p < 0.01$, ns = non-significant. IL-1 β (A), IL-6 (B), CXCL8/IL-8 (C), TNF- α (D). Non-significant increases in induction of IL-1 β and IL-6 protein were observed in all groups. Significant induction of CXCL8/IL-8 protein was observed in ANA and AA, with non-significant increases observed in NANA and NAA. Significant induction of TNF- α protein was observed in ANA, with non-significant increases in induction in other groups. There were no differences between groups.

5.2.3 TLR4 stimulation of HBECs: type I and III IFN and proinflammatory cytokine mRNA and protein induction

Macrophages from asthmatics stimulated *ex vivo* with the TLR4 ligand lipopolysaccharide (LPS) have been shown to have a deficient IFN- λ response and IFN- λ responses correlated with exacerbation severity *in vivo*⁶¹. In order to further investigate this type I and III IFN and proinflammatory cytokine expression was examined in HBECs in order to assess whether other cell types were involved and whether the asthmatic or atopic status of the volunteer had an impact. Cells were stimulated with LPS and type I and III IFN and proinflammatory cytokines were measured; mRNA at 8h and 24h, and protein at 24h post stimulation.

IFN- β and IFN- λ 2,3/IL-28 mRNA was not induced by LPS at 8h or 24h (figure 5.19 A-D). IFN- λ 1/IL-29 mRNA was non-significantly induced by LPS at 8h in NAA

only but induction at 24h was not observed (figure 5.19 E and F). No significant differences were observed between groups.

No significant induction of IFN- β or IFN- λ 1/IL-29 protein was observed at 24h post stimulation with LPS in any group (appendix A, figure A4). No differences were observed between groups.

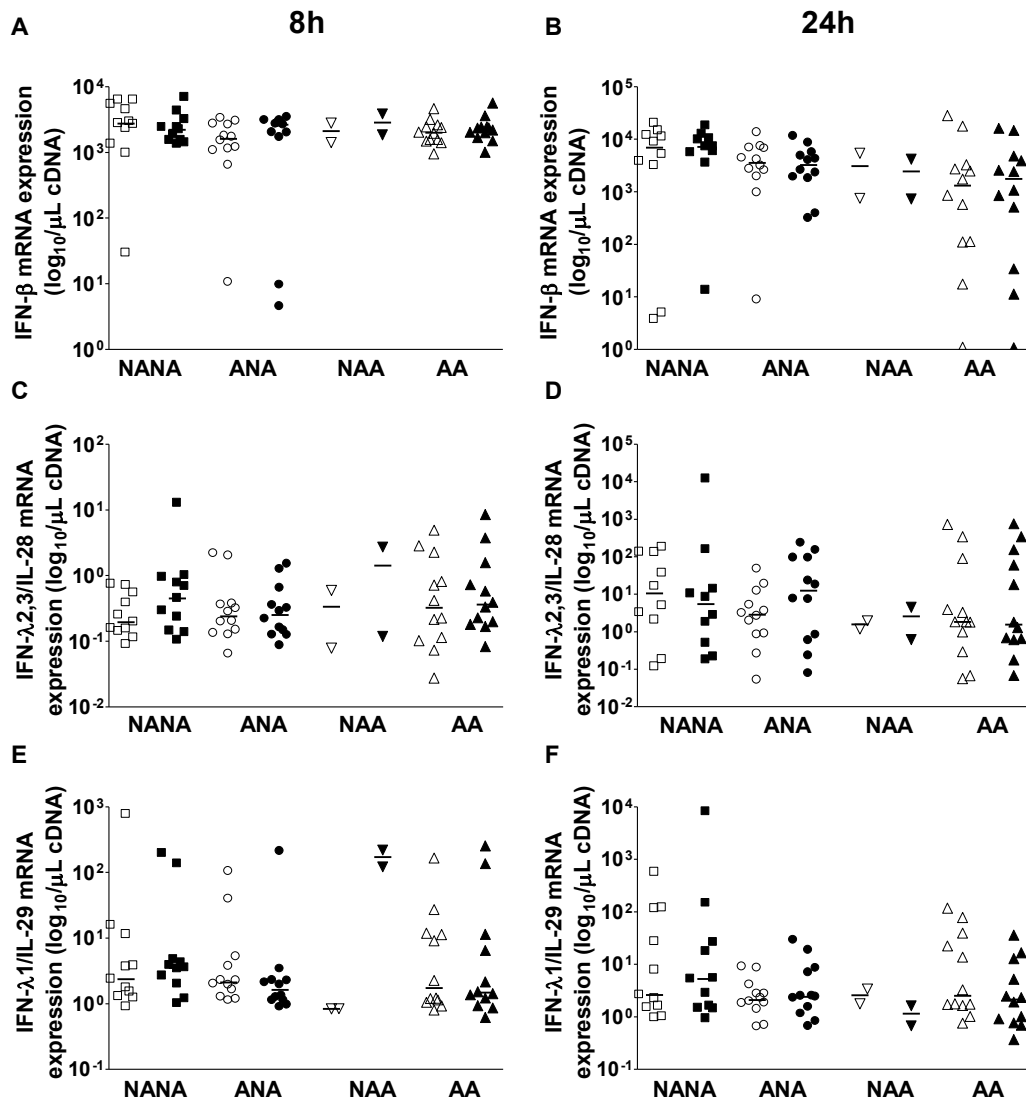


Figure 5.19. Interferon mRNA induction in HBECs post stimulation with LPS at 8h (A, C and E) and 24h (B, D and F). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, black shapes denote LPS. IFN- β (A and B), IFN- λ 2,3/IL-28 (C and D), IFN- λ 1/IL-29 (E and F). Induction of IFN- β and IFN- λ 2,3/IL-28 mRNA was not observed. Weak, non-significant induction of IFN- λ 1/IL-29 mRNA was observed at 8h in NAA only. No differences were observed between groups.

Proinflammatory cytokines were measured in HBECs stimulated with LPS. Non-significant weak induction of IL-6 mRNA was observed at 8h in all groups, but not at 24h (figure 5.20 A and B). At 8h, non-significant induction of CXCL8/IL-8 mRNA was observed in all groups other than ANA (figure 5.20 C). Induction was not observed at 24h (figure 5.20 D). No significant differences were observed between groups.

At 24h following stimulation with LPS non-significant weak induction of IL-1 β , IL-6 and CXCL8/IL-8 protein was observed in NANA, ANA and NAA (figure 5.21). No differences were observed between the groups.

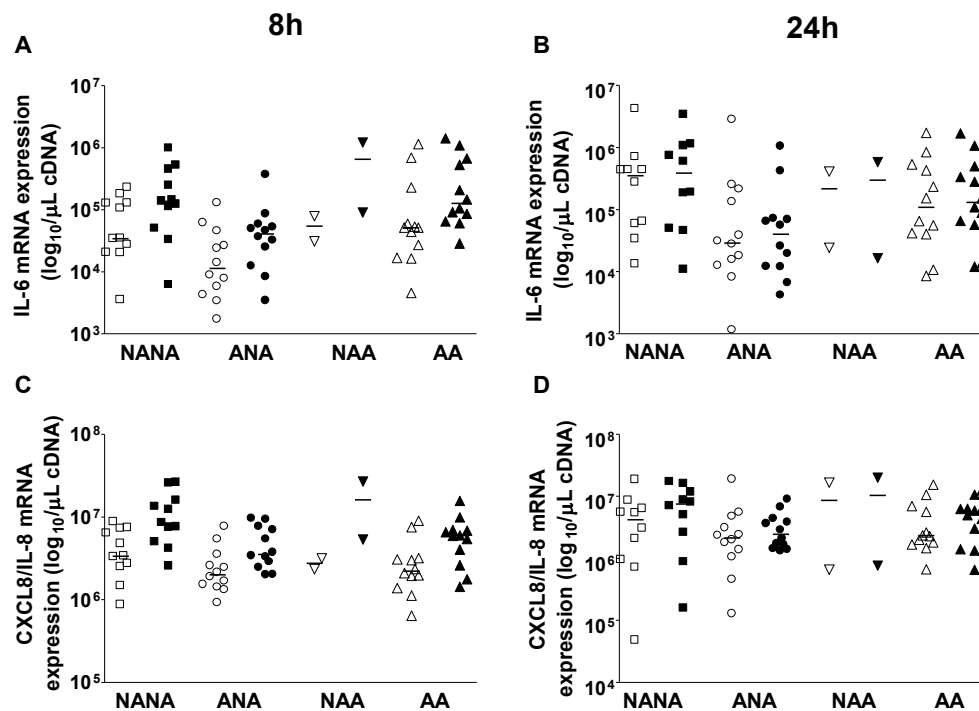


Figure 5.20. IL-6 and CXCL8/IL-8 mRNA induction in HBECs post stimulation with LPS at 8h (A and C) and 24h (B and D). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, black shapes denote LPS. IL-6 (A and B), CXCL8/IL-8 (C and D). Non-significant weak induction of IL-6 and CXCL8/IL-8 mRNA was observed at 8h in all groups. No induction was observed at 24h. No differences were observed between groups.

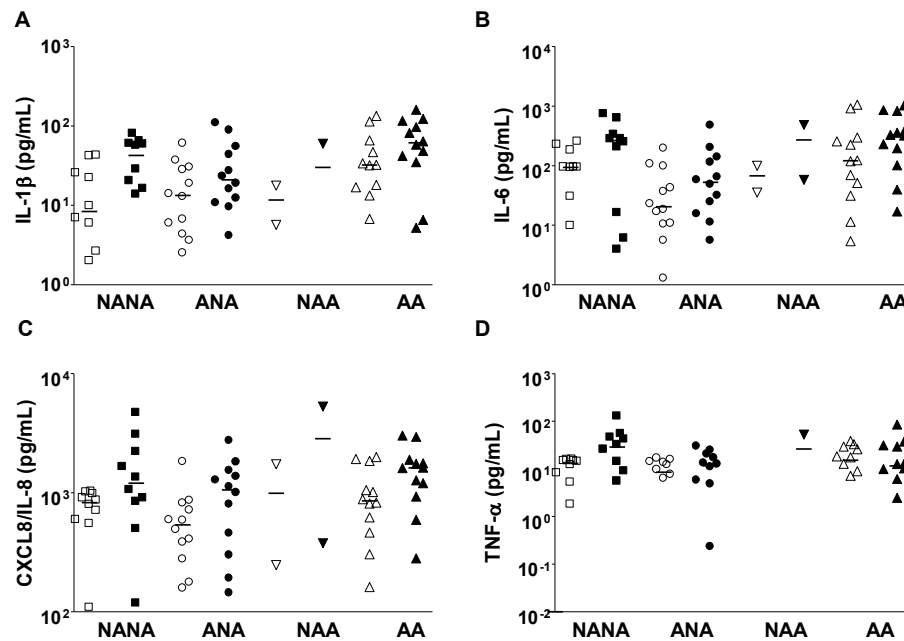


Figure 5.21. Proinflammatory cytokine protein induction in HBECs post stimulation with LPS at 24h. Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, black shapes denote LPS. IL-1 β (A), IL-6 (B), CXCL8/IL-8 (C), TNF- α (D). Non-significant weak induction of IL-1 β , IL-6 and CXCL8/IL-8 protein was observed NANA, ANA and NAA. There were no differences between groups.

5.2.4 TLR5 stimulation of HBECs: type I and III IFN and proinflammatory cytokine mRNA and protein induction

The type I and III IFN and proinflammatory cytokine response to the TLR5 ligand, flagellin (FLA), was assessed in HBECs. IFN- β mRNA was not induced by FLA at 8h or 24h (figure 5.22 A and B). IFN- λ 2,3/IL-28 and IFN- λ 1/IL-29 mRNA was non-significantly weakly induced by FLA at 8h in NAA only albeit with a small sample size (figure 5.22 C and E). Induction at 24h was not observed (figure 5.22 D and F). No significant differences were observed between groups.

No significant induction of IFN- β or IFN- λ 1/IL-29 protein was observed at 24h post stimulation with FLA in any group (appendix A, figure A5). No differences were observed between groups.

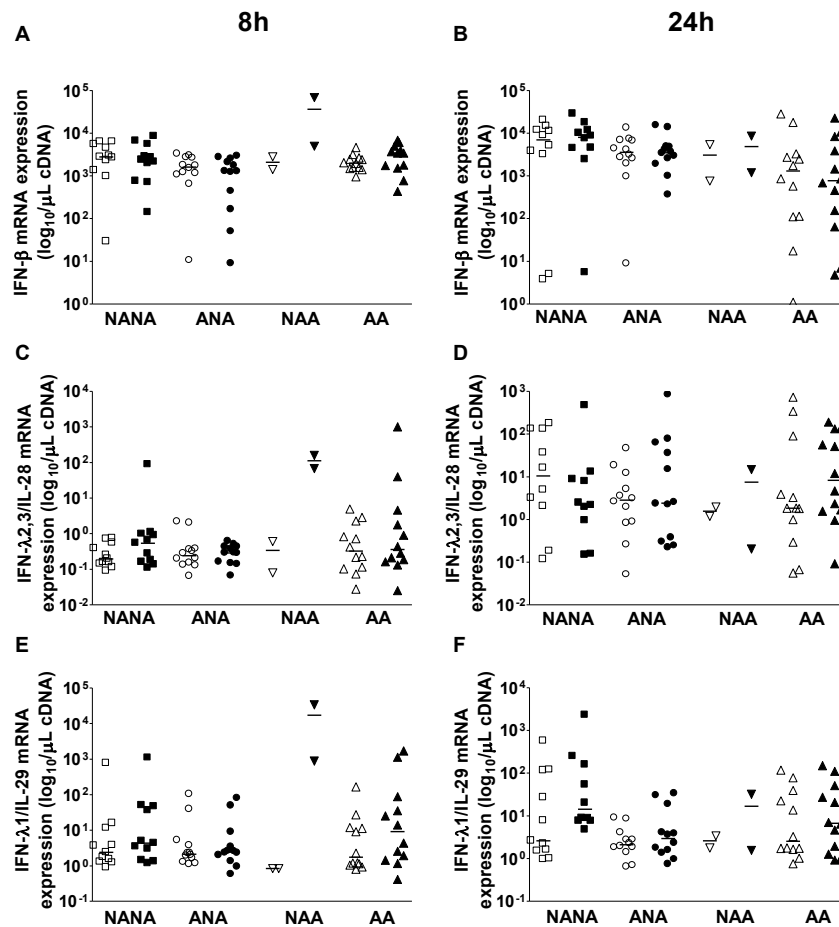


Figure 5.22. Interferon mRNA induction in HBECs post stimulation with FLA at 8h (A, C and E) and 24h (B, D and F). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, black shapes denote FLA. IFN-β (A and B), IFN-λ2,3/IL-28 (C and D), IFN-λ1/IL-29 (E and F). Induction of IFN-β mRNA was not observed. A weak non-significant induction of IFN-λ2,3/IL-28 and IFN-λ1/IL-29 mRNA was observed at 8h in NAA only with no induction in other groups or at 24h. No differences were observed between groups.

As with the other TLR ligands proinflammatory cytokine mRNA and protein was measured in HBECs stimulated with FLA. No significant induction of IL-6 mRNA was observed at 8h or 24h, though there was a suggestion of induction in NAA at 8h (figure 5.23 A and B). More convincing induction of CXCL8/IL-8 mRNA was observed at 8h, though this failed to reach statistical significance (figure 5.23 C). Induction of CXCL8/IL-8 mRNA was not observed at 24h (figure 5.23 D). No differences were observed between groups.

Proinflammatory cytokine protein was measured at 24h post stimulation with FLA. Non-significant induction of IL-6 and CXCL8/IL-8 protein was observed in all groups (figure 5.24 B and C). Induction of IL-1β and TNF-α was less convincing. No differences were observed between groups.

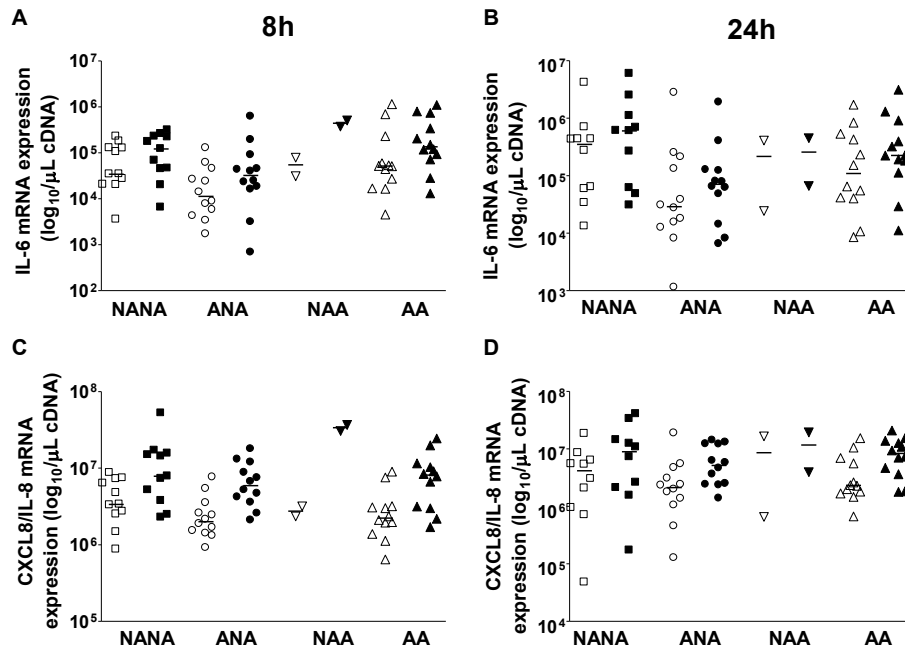


Figure 5.23. IL-6 and CXCL8/IL-8 mRNA induction in HBECs post stimulation with FLA at 8h (A and C) and 24h (B and D). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, black shapes denote FLA. IL-6 (A and B), CXCL8/IL-8 (C and D). No significant induction of IL-6 mRNA was observed. Non-significant induction of CXCL8/IL-8 mRNA was observed at 8h and not 24h. No differences were observed between groups.

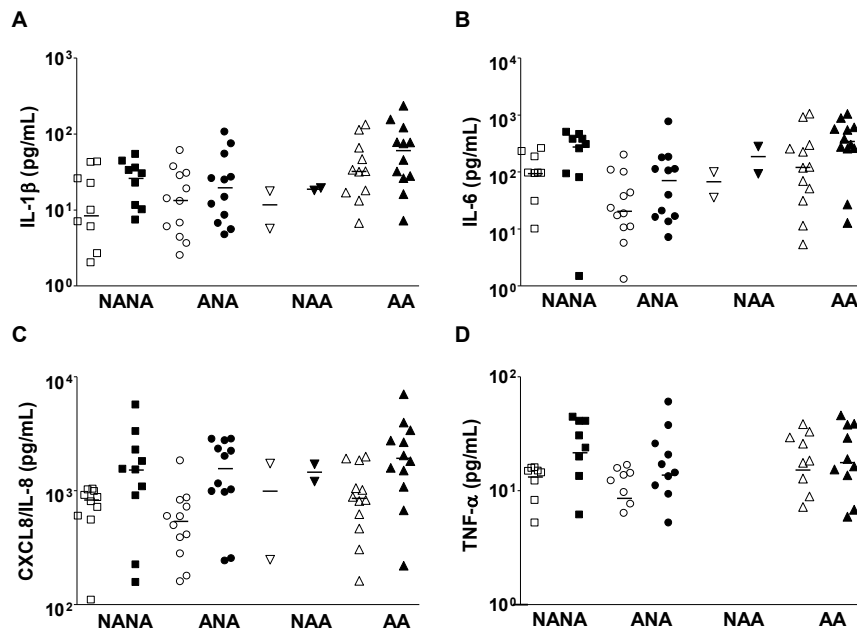


Figure 5.24. Proinflammatory cytokine protein induction in HBECs post stimulation with FLA at 24h. Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, black shapes denote FLA. IL-1β (A), IL-6 (B), CXCL8/IL-8 (C), TNF-α (D). Non-significant induction of IL-6 and CXCL8/IL-8 protein was observed in all groups. There were no differences between groups.

5.2.5 TLR9 stimulation of HBECs: type I and III IFN and proinflammatory cytokine mRNA and protein induction

TLR9 has been shown to induce IFN via the MyD88 pathway³¹⁷. This IFN signalling pathway may be of importance in IFN deficiency in asthma. HBECs were stimulated with the TLR9 ligand CpG-C to assess whether type I and III IFNs and proinflammatory cytokines were expressed and whether the asthmatic and/or atopic status of the cells had any impact on expression.

No significant induction of type I or III IFN mRNA was observed in HBECs stimulated with CpG-C at 8h or 24h (appendix A, figure A6). Similarly, no induction of type I or III protein was observed at 24h post stimulation (appendix A, figure A7).

Proinflammatory cytokines were measured in HBECs stimulated with CpG-C. No significant induction of IL-6 or CXCL8/IL-8 mRNA was observed in HBECs at 8h or 24h (appendix A, figure A8). No increases in IL-1 β , IL-6, CXCL8/IL-8 and TNF- α protein were observed (figure 5.25). There was a non-significant reduction in CXCL8/IL-8 protein levels in NANA, ANA and AA (figure 5.25 C). No significant differences were observed between the 4 volunteer groups.

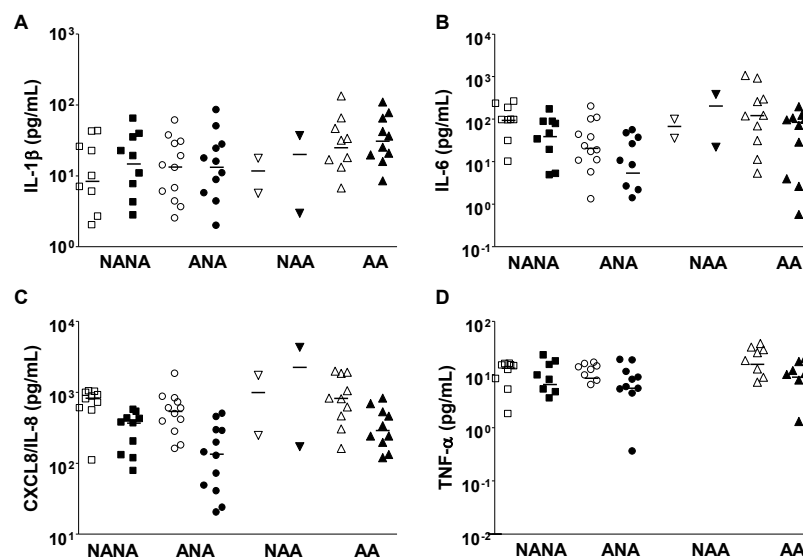


Figure 5.25. Proinflammatory cytokine protein induction in HBECs post stimulation with CpG-C at 24h. Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, black shapes denote CpG-C. IL-1 β (A), IL-6 (B), CXCL8/IL-8 (C), TNF- α (D). Induction of IL-1 β , IL-6, CXCL8/IL-8 and TNF- α protein was not observed in all groups. Indeed a suggestion of lower levels of CXCL8/IL-8 in CpG-C treated cells compared to medium treated cells was observed in all groups other than NAA. There were no differences between groups.

5.2.6 Summary of type I and III IFN and proinflammatory cytokine induction in HBECs stimulated with TLR1/2/4/5/6/9 ligands

Tables 5.1 to 5.7 summarise the induction of type I and III IFNs and proinflammatory cytokines in HBECs stimulated with “bacterial” TLR ligands.

Table 5.1 Summary of IFN and proinflammatory cytokine mRNA and protein induction post PAM stimulation in HBECs ↑ = significant induction, ns = non-significant increase observed, - = no induction, black squares = not performed

	8h				24h			
	NANA	ANA	NAA	AA	NANA	ANA	NAA	AA
IFN-β mRNA	-	-	-	-	-	-	-	-
IFN-β protein					-	-	-	-
IFNλ2,3/IL-28 mRNA	-	-	-	-	ns	ns	-	-
IFNλ1/IL-29 mRNA	-	-	ns	-	ns	-	-	-
IFNλ1/IL-29 protein					ns	-	-	-
IL-6 mRNA	-	-	ns	-	-	-	-	-
CXCL8/IL-8 mRNA	↑	ns	ns	ns	-	-	-	-
IL-1β protein					-	-	-	-
IL-6 protein					-	-	-	-
CXCL8/IL-8 protein					ns	ns	ns	ns
TNF-α protein					-	-	-	-

Table 5.2 Summary of IFN and proinflammatory cytokine mRNA and protein induction post LTA stimulation in HBECs ns = non-significant increase observed, - = no induction, black squares = not performed

	8h				24h			
	NANA	ANA	NAA	AA	NANA	ANA	NAA	AA
IFN- β mRNA	-	-	-	-	-	-	-	-
IFN- β protein					-	-	-	-
IFN λ 2,3/IL-28 mRNA	-	-	ns	-	ns	ns	ns	-
IFN λ 1/IL-29 mRNA	-	-	ns	-	ns	ns	ns	ns
IFN λ 1/IL-29 protein					-	-	-	-
IL-6 mRNA	-	-	-	-	-	-	-	-
CXCL8/IL-8 mRNA	ns	ns	ns	ns	-	-	-	-
IL-1 β protein					ns	ns	ns	ns
IL-6 protein					ns	ns	ns	-
CXCL8/IL-8 protein					ns	ns	ns	ns
TNF- α protein					ns	ns	-	-

Table 5.3 Summary of IFN and proinflammatory cytokine mRNA and protein induction post PGN stimulation in HBECs ns = non-significant increase observed, - = no induction, black squares = not performed

	8h				24h			
	NANA	ANA	NAA	AA	NANA	ANA	NAA	AA
IFN- β mRNA	-	-	-	-	-	-	-	-
IFN- β protein					-	-	-	-
IFN λ 2,3/IL-28 mRNA	-	-	-	-	-	-	-	-
IFN λ 1/IL-29 mRNA	-	-	ns	-	-	-	ns	-
IFN λ 1/IL-29 protein					-	-	ns	-
IL-6 mRNA	-	-	-	-	-	-	-	-
CXCL8/IL-8 mRNA	-	-	ns	-	-	-	-	-
IL-1 β protein					-	-	ns	-
IL-6 protein					-	-	ns	-
CXCL8/IL-8 protein					-	-	ns	-
TNF- α protein					-	-	ns	-

Table 5.4 Summary of IFN and proinflammatory cytokine mRNA and protein induction post FSL stimulation in HBECs ↑ = significant induction, ns = non-significant increase observed, - = no induction, black squares = not performed

	8h				24h			
	NANA	ANA	NAA	AA	NANA	ANA	NAA	AA
IFN-β mRNA	-	-	-	-	-	-	-	-
IFN-β protein					-	-	-	-
IFNλ2,3/IL-28 mRNA	-	-	-	-	-	-	-	-
IFNλ1/IL-29 mRNA	-	-	ns	-	-	-	-	-
IFNλ1/IL-29 protein					-	-	-	-
IL-6 mRNA	ns	ns	ns	-	ns	-	-	-
CXCL8/IL-8 mRNA	↑	↑	ns	↑	-	-	-	-
IL-1β protein					ns	ns	ns	ns
IL-6 protein					ns	ns	ns	ns
CXCL8/IL-8 protein					ns	↑	ns	↑
TNF-α protein					ns	↑	ns	ns

Table 5.5 Summary of IFN and proinflammatory cytokine mRNA and protein induction post LPS stimulation in HBECs ns = non-significant increase observed, - = no induction, black squares = not performed

	8h				24h			
	NANA	ANA	NAA	AA	NANA	ANA	NAA	AA
IFN-β mRNA	-	-	-	-	-	-	-	-
IFN-β protein					-	-	-	-
IFNλ2,3/IL-28 mRNA	-	-	-	-	-	-	-	-
IFNλ1/IL-29 mRNA	-	-	ns	-	-	-	-	-
IFNλ1/IL-29 protein					-	-	-	-
IL-6 mRNA	ns	ns	ns	ns	-	-	-	-
CXCL8/IL-8 mRNA	ns	ns	ns	ns	-	-	-	-
IL-1β protein					ns	ns	ns	-
IL-6 protein					ns	ns	ns	-
CXCL8/IL-8 protein					ns	ns	ns	-
TNF-α protein					-	-	-	-

Table 5.6 Summary of IFN and proinflammatory cytokine mRNA and protein induction post FLA stimulation in HBECS ns = non-significant increase observed, - = no induction, black squares = not performed

	8h				24h			
	NANA	ANA	NAA	AA	NANA	ANA	NAA	AA
IFN- β mRNA	-	-	-	-	-	-	-	-
IFN- β protein					-	-	-	-
IFN λ 2,3/IL-28 mRNA	-	-	ns	-	-	-	-	-
IFN λ 1/IL-29 mRNA	-	-	ns	-	-	-	-	-
IFN λ 1/IL-29 protein					-	-	-	-
IL-6 mRNA	-	-	-	-	-	-	-	-
CXCL8/IL-8 mRNA	ns	ns	ns	ns	-	-	-	-
IL-1 β protein					-	-	-	-
IL-6 protein					ns	ns	ns	ns
CXCL8/IL-8 protein					ns	ns	ns	ns
TNF- α protein					-	-	-	-

Table 5.7 Summary of IFN and proinflammatory cytokine mRNA and protein induction post CpG stimulation in HBECS - = no induction, black squares = not performed

	8h				24h			
	NANA	ANA	NAA	AA	NANA	ANA	NAA	AA
IFN- β mRNA	-	-	-	-	-	-	-	-
IFN- β protein					-	-	-	-
IFN λ 2,3/IL-28 mRNA	-	-	-	-	-	-	-	-
IFN λ 1/IL-29 mRNA	-	-	-	-	-	-	-	-
IFN λ 1/IL-29 protein					-	-	-	-
IL-6 mRNA	-	-	-	-	-	-	-	-
CXCL8/IL-8 mRNA	-	-	-	-	-	-	-	-
IL-1 β protein					-	-	-	-
IL-6 protein					-	-	-	-
CXCL8/IL-8 protein					-	-	-	-
TNF- α protein					-	-	-	-

5.2.7 TLR1, 2 and 6 stimulation of PBMCs: type I and III IFN and proinflammatory cytokine protein induction

As with other viral and bacterial stimuli, PBMCs were stimulated with “bacterial” TLR ligands to investigate whether type I and III IFNs were induced by these ligands, and to observe any differences between the atopic and asthmatic phenotype of volunteers’ samples. As in the HBEC section above, results were grouped into the 4 groups of bacterial TLR. Cells were stimulated with different TLR ligands and type I and III IFN and proinflammatory cytokine protein induction was measured at 24h and 48h post stimulation. mRNA was not analysed due to time constraints of the project.

5.2.7.1 Pam₃CSK₄

Type I and III IFNs were not significantly induced following stimulation with Pam₃CSK₄ (figure 5.26). IFN- α 2a was not induced (figure 5.26 A and B). IFN- β protein induction was only observed in a small number of ANA, though this failed to reach statistical significance (figure 5.26 C and D). IFN- λ 1/IL-29 protein was induced in a small number of AA and NAA, but this was not significant (figure 5.26 E and F). No statistically significant differences were observed between the 4 volunteer groups.

Marginal increases in proinflammatory cytokine protein induction were observed at 24h post stimulation of PBMCs with Pam₃CSK₄ (figure 5.27 A, C, E and G). At 48h non-significant increases in IL-1 β protein were observed in NANA, ANA and NAA (figure 5.27 B), in IL-6 protein in NANA, NAA and AA (figure 5.27 D), in CXCL8/IL-8 protein in NANA, ANA and NAA (figure 5.27 F) and in TNF- α protein in NANA and NAA (figure 5.27 H). No significant differences were observed between the volunteer groups. As non-significant trends for induction were observed at both 24h and 48h all groups were combined in order to establish whether PBMCs stimulated with Pam₃CSK₄ had significant proinflammatory cytokine induction. At 24h significant increases in all proinflammatory cytokines was observed (figure 5.28 A, C, E and G; $p < 0.001$ for all). No increase in IL-1 β protein was observed at 48h

(figure 5.28 B). Significant increases in IL-6 ($p<0.01$), CXCL8/IL-8 ($p<0.01$) and TNF- α ($p<0.05$) protein was observed in PBMCs at 48h (figure 5.28 D, F and H).

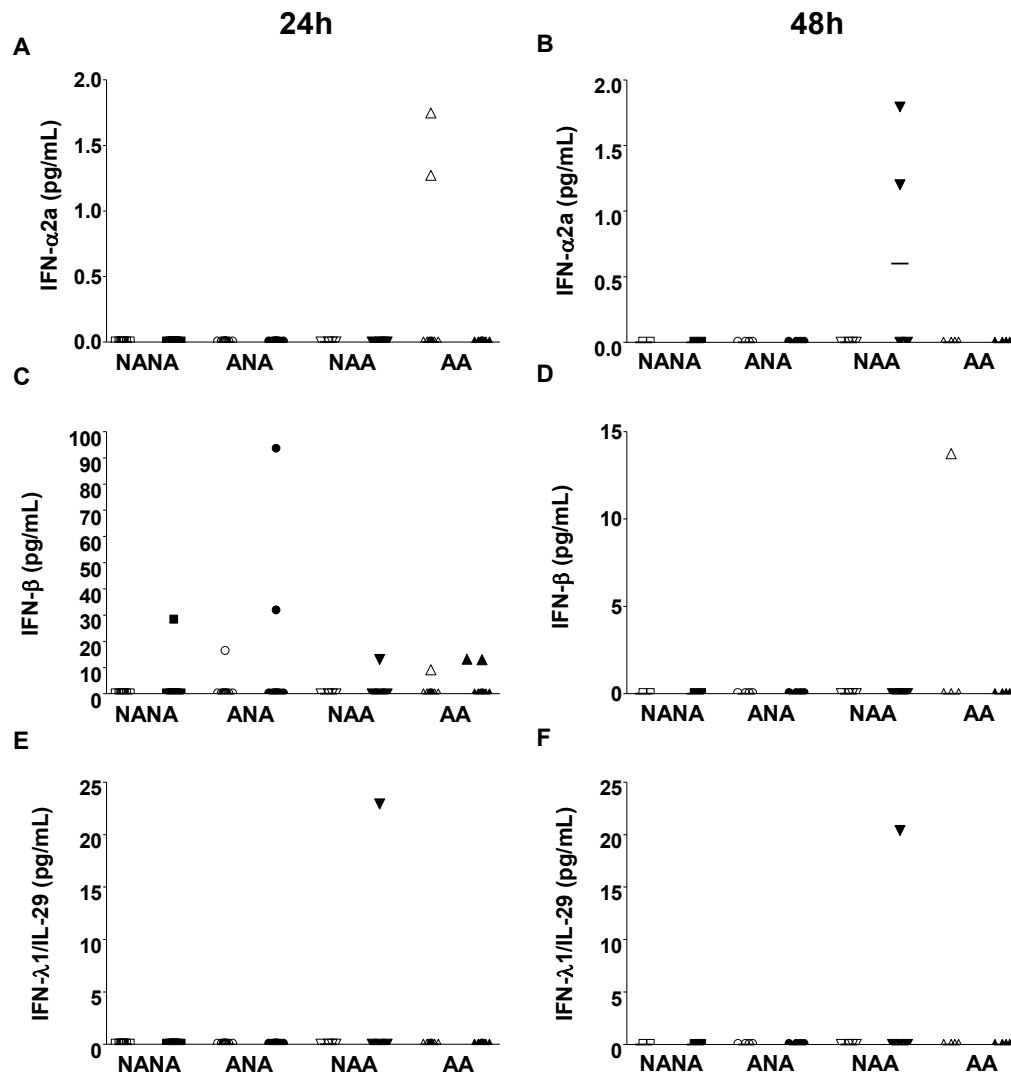


Figure 5.26. Interferon protein induction in PBMCs post stimulation with Pam₃CSK₄ at 24h (A, C and E) and 48h (B, D and F). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, black shapes denote Pam₃CSK₄. IFN- α 2a (A and B), IFN- β (C and D), IFN- λ 1/IL-29 (E and F). No significant IFN- α 2a induction was observed. No IFN- β protein induction was observed other than in a small number of ANA participants' samples. In a small number of NAA and AA participants' samples IFN- λ 1/IL-29 protein was observed, but this was non-significant.

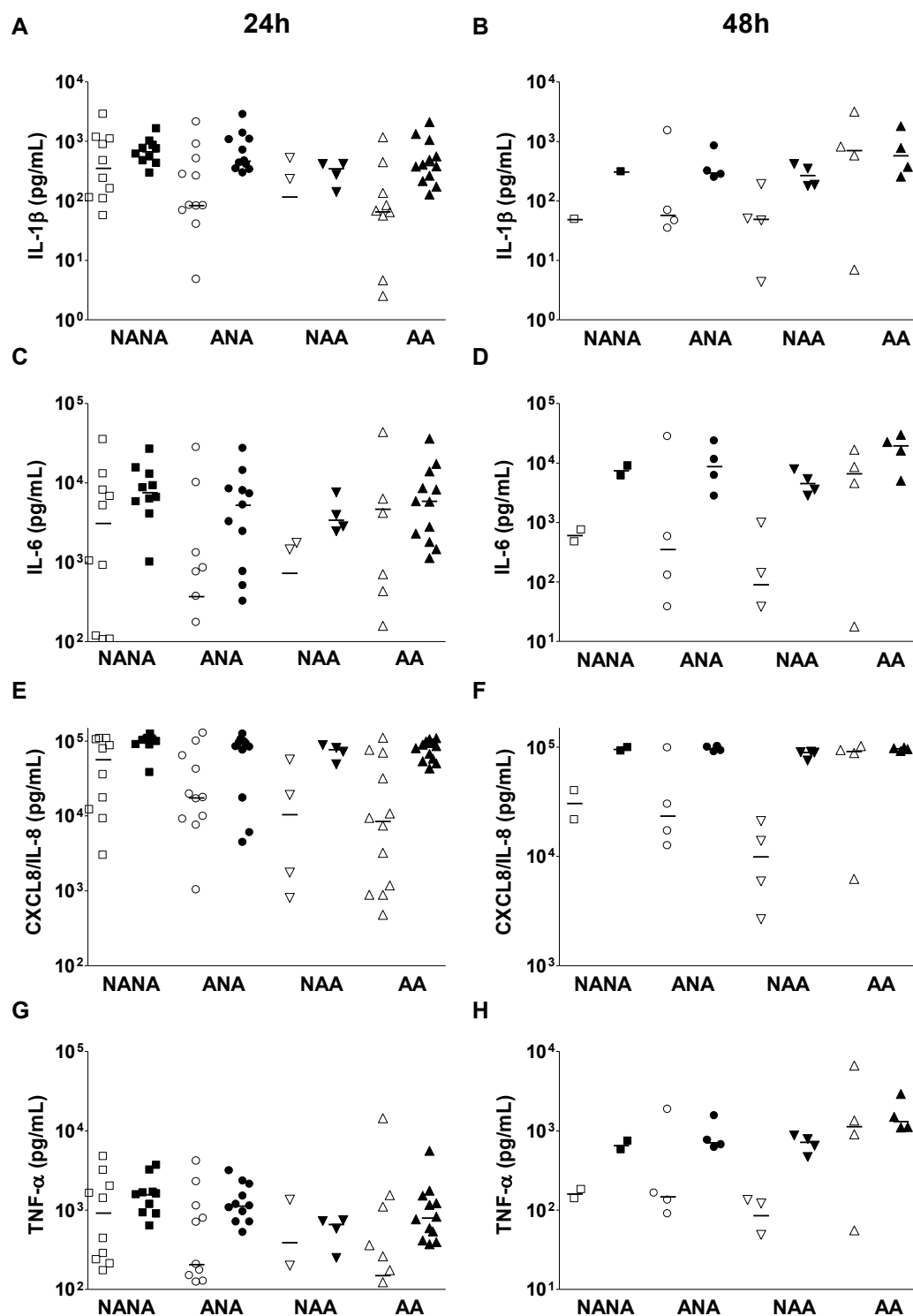


Figure 5.27. Proinflammatory cytokine protein induction in PBMCs post stimulation with Pam₃CSK₄ at 24h (A, C, E and G) and 48h (B, D, F and H). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, black shapes denote Pam₃CSK₄. IL-1 β (A and B), IL-6 (C and D), CXCL8/IL-8 (E and F), TNF- α (G and H). Non-significant marginal increases were observed in proinflammatory cytokine protein induction at 24h. At 48h non-significant increases in all proinflammatory cytokines measured in varying groups was observed (see text). No significant differences were observed between groups.

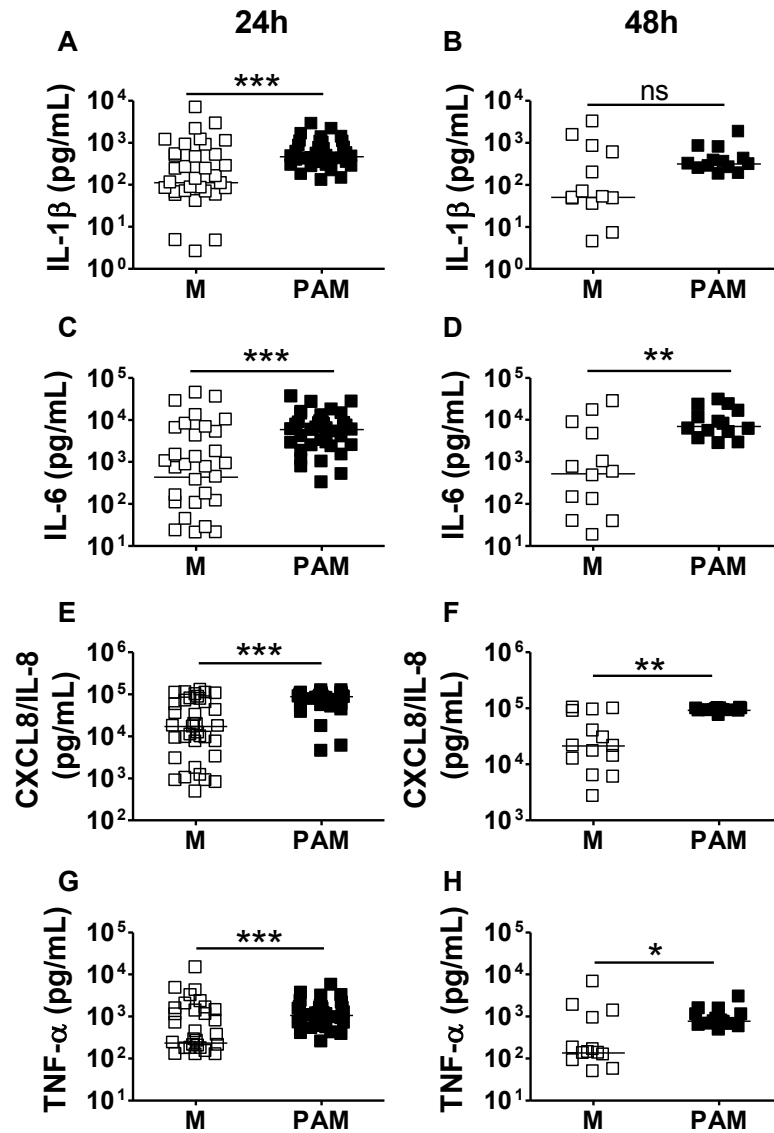


Figure 5.28. Proinflammatory cytokine protein induction at 24h (A, C, E and G) and 48h (B, D, F and H) in PBMCs from all groups combined stimulated with Pam₃CSK₄. IL-1 β (A and B), IL-6 (C and D), CXCL8/IL-8 (E and F) and TNF- α (G and H). Open squares denote medium (M); black squares denote Pam₃CSK₄; * = p < 0.05, ** = p < 0.01, *** = p < 0.001, ns = non-significant. Highly significant induction of all proinflammatory cytokines was observed at 24h in all groups combined. No significant induction of IL-1 β was observed at 48h. Significant induction of IL-6, CXCL8/IL-8 and TNF- α protein induction was observed at 48h.

5.2.7.2 LTA

Type I and III IFNs were not significantly induced following stimulation with LTA (figure 5.29). IFN- α 2a was not induced (figure 5.29 A and B). IFN- β protein induction was only observed at 24h in a small number of NAA and AA, though this failed to reach statistical significance (figure 5.29 C). No induction was observed at 48h (figure 5.29 D). IFN- λ 1/IL-29 protein was induced in a single AA volunteer at 24h, but not in other groups or at 48h (figure 5.29 E and F). No statistically significant differences were observed between groups.

Induction of all proinflammatory cytokine protein was observed in all groups at 24h and 48h (figure 5.30). At 24h significant induction of IL-1 β and IL-6 was observed in ANA ($p < 0.05$) and AA ($p < 0.001$), with non-significant induction observed in NANA and NAA (figure 5.30 A and C). Significant induction of CXCL8/IL-8 protein was observed in ANA ($p < 0.05$) and AA ($p < 0.01$), with non-significant induction observed in NANA and NAA (figure 5.30 E). Significant induction of TNF- α protein was observed in AA only ($p < 0.001$), with non-significant induction observed in other groups (figure 5.30 G). At 48h trends towards induction of IL-1 β , IL-6, CXCL8/IL-8 and TNF- α protein were observed in all groups but these failed to reach statistical significance (figure 5.30 B, D, F and H). Both at 24h and 48h no significant difference in proinflammatory cytokine induction was observed between groups. As a result, in order to determine whether cytokine induction at 24h and 48h was significant, all groups were combined. Highly significant induction of IL-1 β , IL-6, CXCL8/IL-8 and TNF- α protein as observed in all groups combined at both 24h and 48h ($p < 0.001$ for all; figure 5.31).

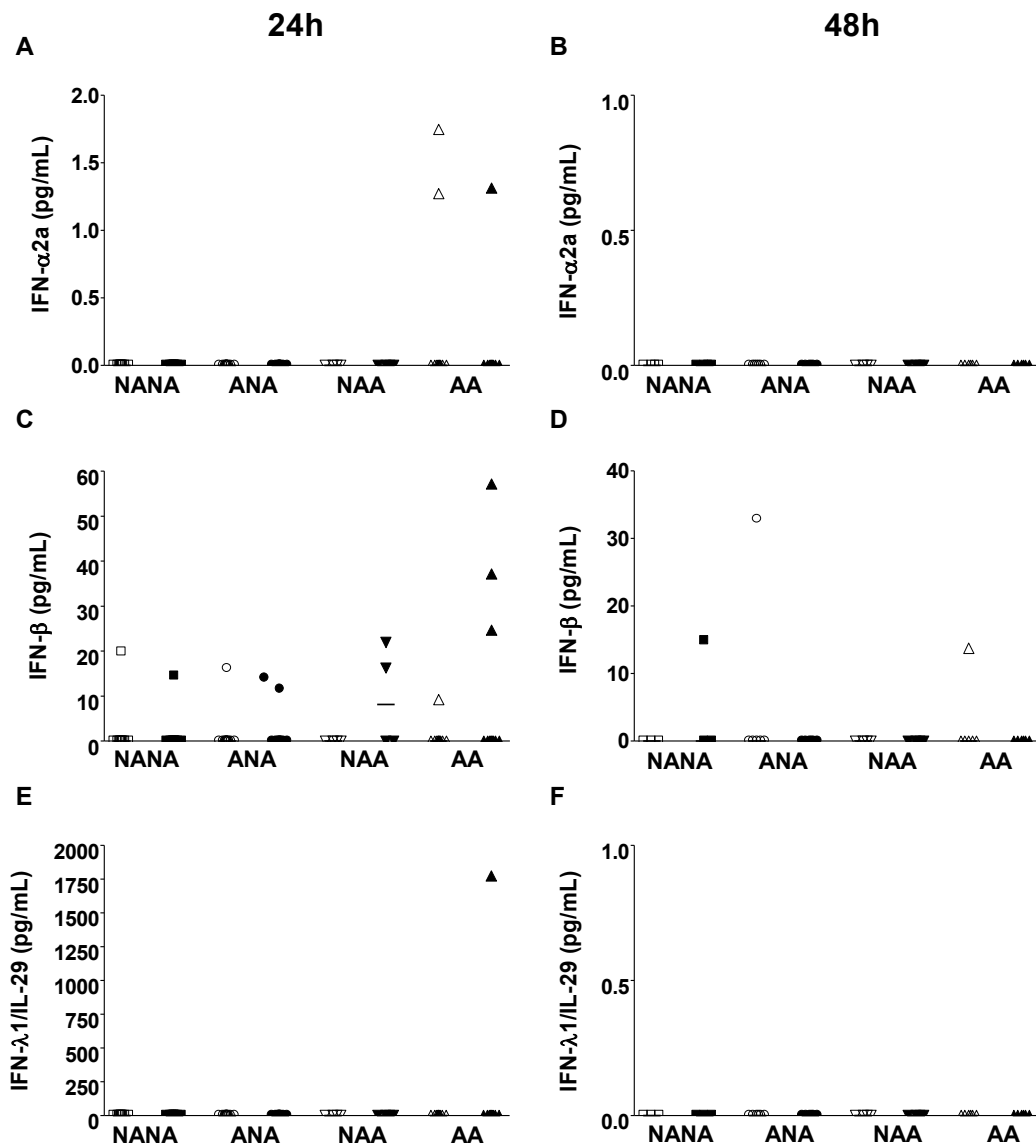


Figure 5.29. Interferon protein induction in PBMCs post stimulation with LTA at 24h (A, C and E) and 48h (B, D and F). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, black shapes denote LTA. IFN-α2a (A and B), IFN-β (C and D), IFN-λ1/IL-29 (E and F). No significant IFN-α2a induction was observed. A non-significant weak increase in IFN-β protein induction was observed in NAA and AA at 24h but not at 48h. In a single AA participant's sample IFN-λ1/IL-29 protein at 24h was observed, but this was non-significant.

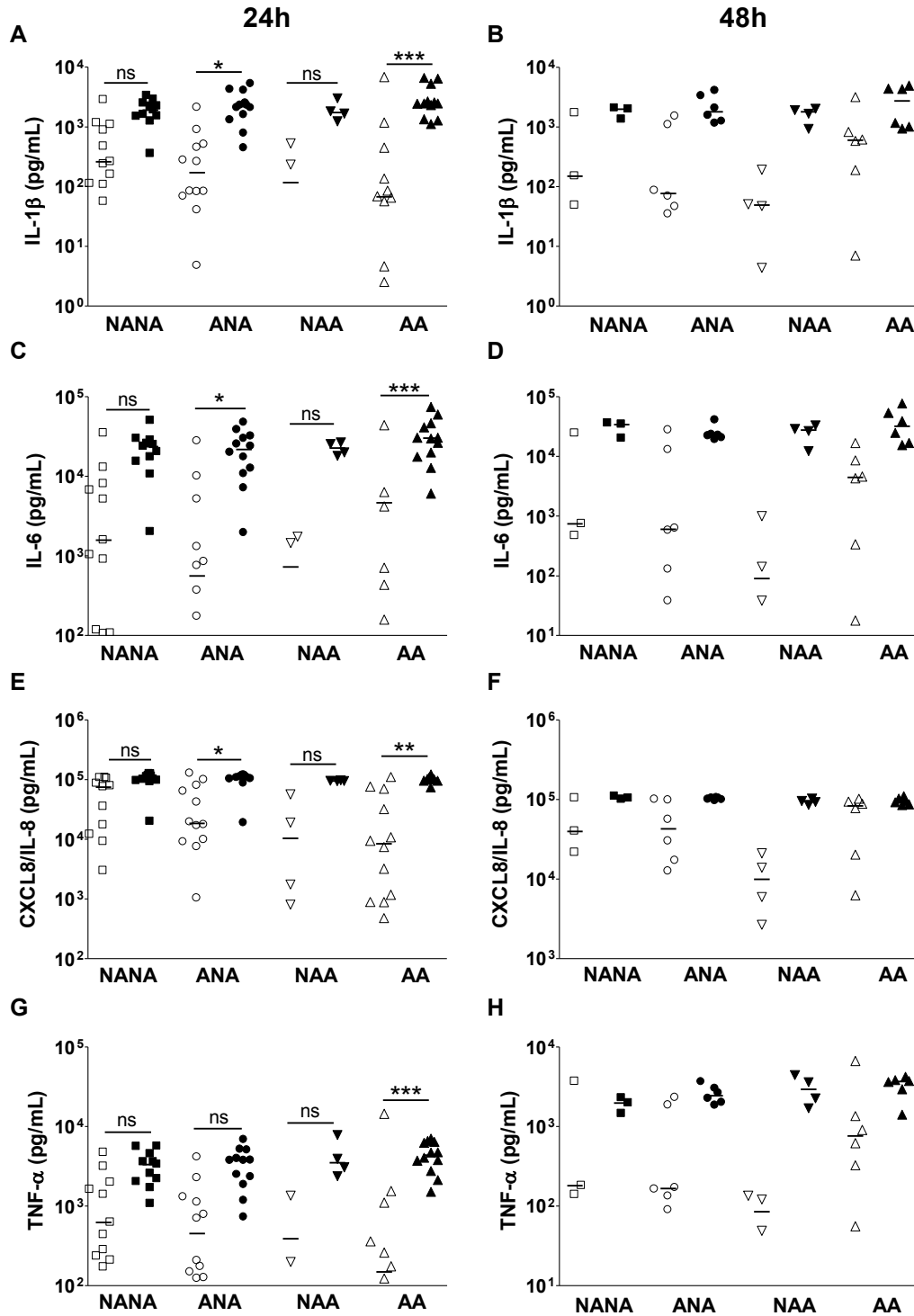


Figure 5.30. Proinflammatory cytokine protein induction in PBMCs post stimulation with LTA at 24h (A, C, E and G) and 48h (B, D, F and H). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, black shapes denote LTA; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, ns = non-significant. IL-1 β (A and B), IL-6 (C and D), CXCL8/IL-8 (E and F), TNF- α (G and H). Induction of all proinflammatory cytokines measured was observed in all groups at 24h, reaching statistical significance in ANA and AA for IL-1 β , IL-6 and CXCL8/IL-8 and in AA for TNF- α , with non-significant increases in other groups. At 48h increases were observed, but these failed to reach statistical significance. No differences were observed between groups.

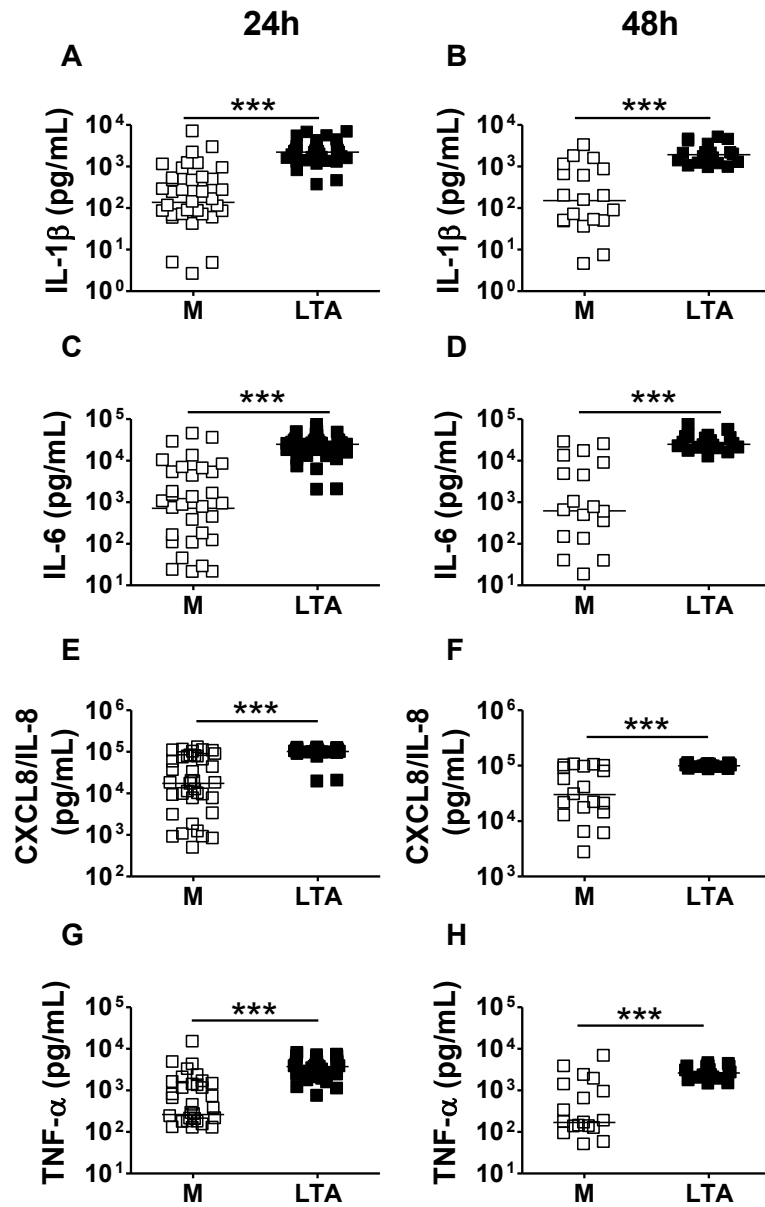


Figure 5.31. Proinflammatory cytokine protein induction at 24h (A, C, E and G) and 48h (B, D, F and H) in PBMCs from all groups combined stimulated with LTA. IL-1 β (A and B), IL-6 (C and D), CXCL8/IL-8 (E and F) and TNF- α (G and H). Open squares denote medium (M); black squares denote LTA; *** = $p < 0.001$. Significant increases in all proinflammatory cytokines measured were observed in PBMCs at both 24h and 48h from all groups combined.

5.2.7.3 PGN

As with Pam₃CSK₄ and LTA, type I and III IFN and proinflammatory cytokine protein was measured in PBMCs stimulated with PGN. No induction of IFN- α 2a was observed at 24h or 48h post stimulation (figure 5.32 A and B). A non-significant increase in IFN- β protein induction was observed in ANA and AA at 24h, but not at 48h other than in a single ANA participant's sample (figure 5.32 C and D). A non-significant trend for induction of IFN- λ /IL-29 was observed in NAA at 48h (figure 5.32 E and F). No significant difference was observed between groups.

Induction of proinflammatory cytokine protein was observed in all groups, reaching statistical significance at 24h in AA with IL-1 β ($p < 0.01$), IL-6 ($p < 0.01$), CXCL8/IL-8 ($p < 0.05$) and TNF- α ($p < 0.01$) and non-significant trends in all other groups (figure 5.33 A, C, E and G). No statistically significant differences were observed between groups. At 48h trends for an increase in induction were observed in all groups but these failed to reach statistical significance (figure 5.33 B, D, F and H). As no differences were observed between groups and as there was a small sample size of samples obtained at 48h, in order to observe whether PGN stimulated PBMCs induced significant amounts of proinflammatory cytokines at 48h, all groups were combined. Likewise all groups were combined at 24h. Significant amounts of proinflammatory cytokines was observed at 24h and 48h with all groups combined ($p < 0.001$ for all at 24h; $p < 0.01$ for all at 48h, figure 5.34).

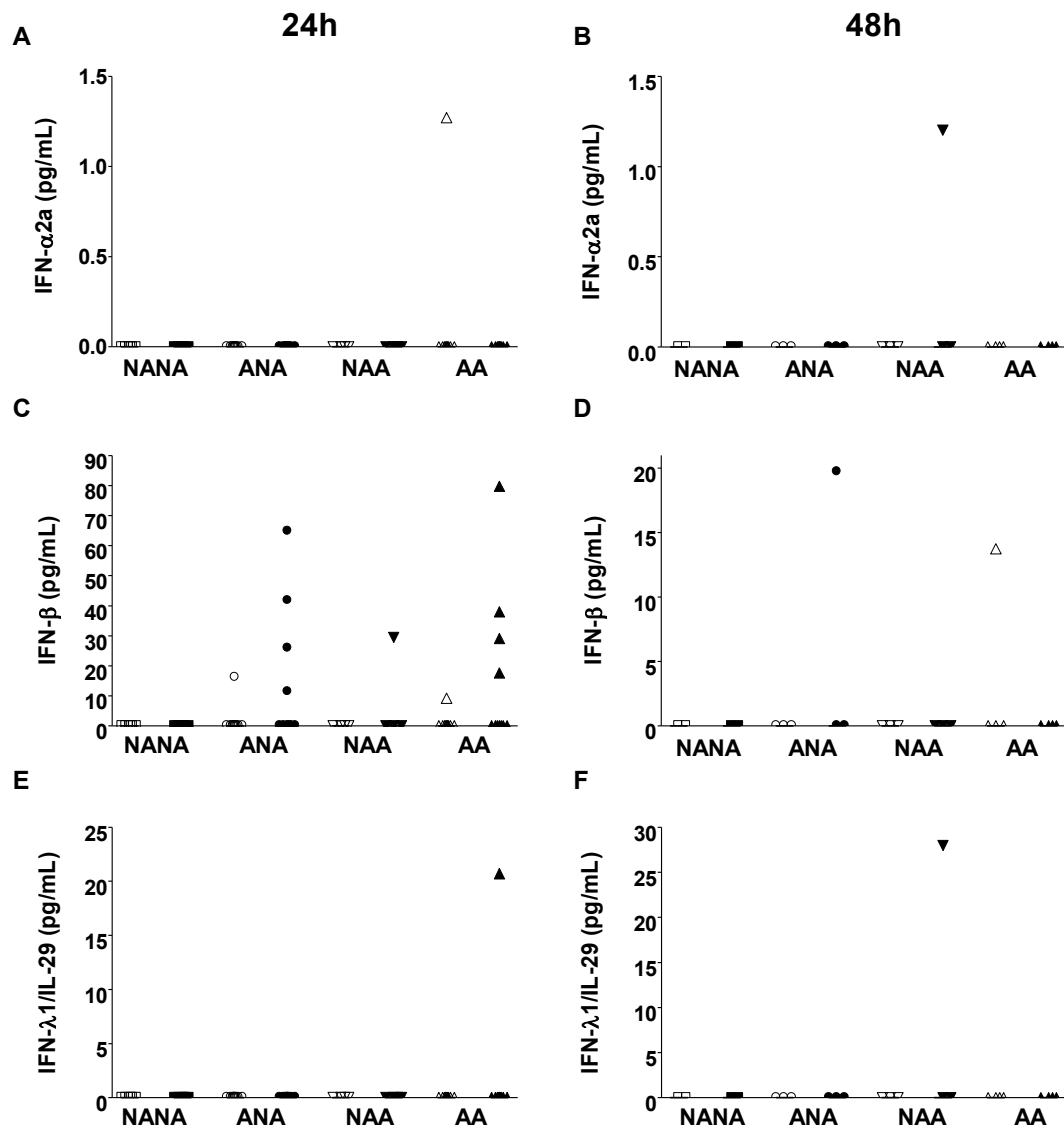


Figure 5.32. Interferon protein induction in PBMCs post stimulation with PGN at 24h (A, C and E) and 48h (B, D and F). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, black shapes denote PGN. IFN-α2a (A and B), IFN-β (C and D), IFN-λ1/IL-29 (E and F). No significant IFN-α2a induction was observed. A non-significant increase in IFN-β protein induction was observed in ANA and AA at 24h, but not at 48h other than in a single ANA participant's sample. A non-significant increase in IFN-λ1/IL-29 protein induction was observed in NAA at 48h

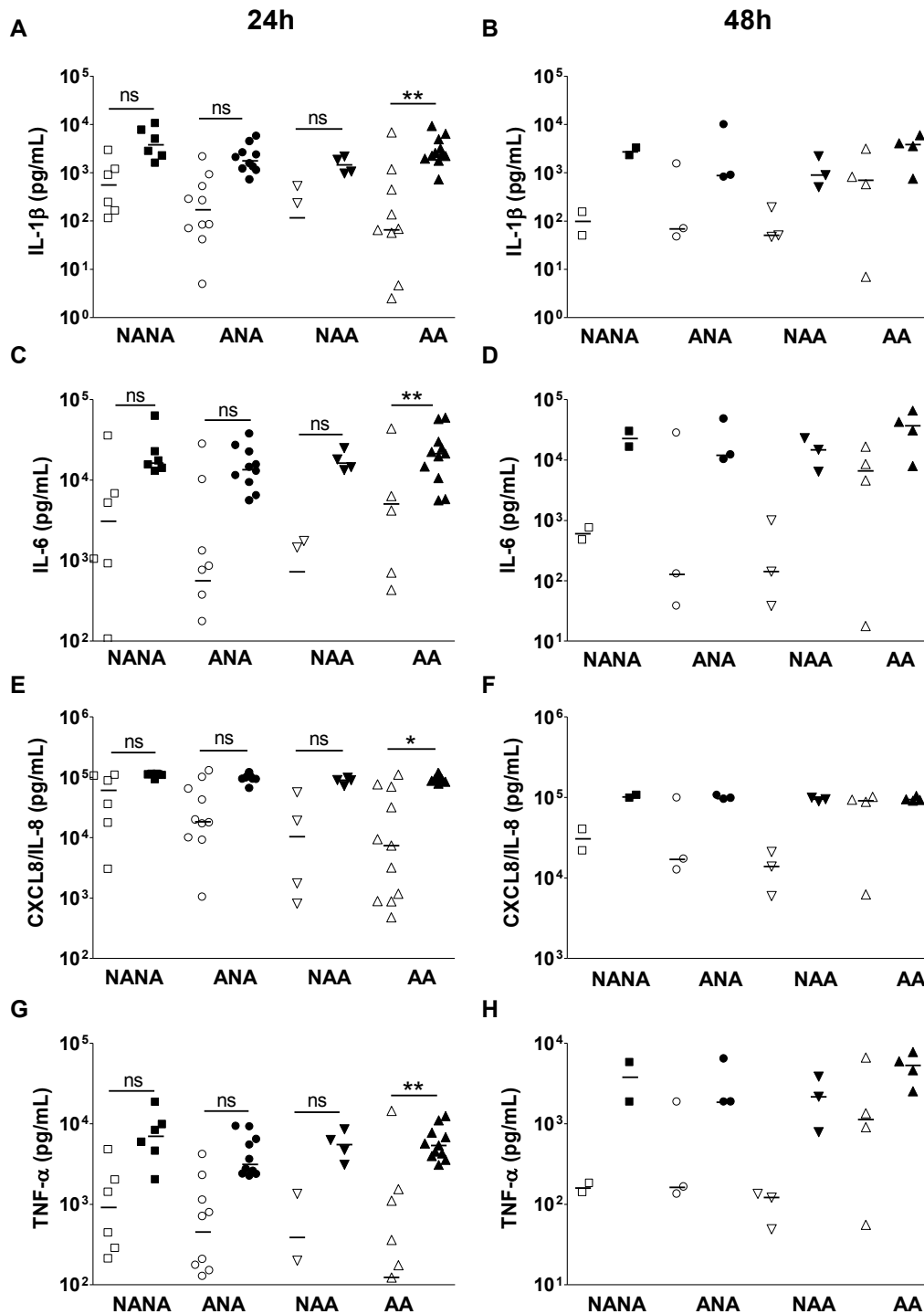


Figure 5.33. Proinflammatory cytokine protein induction in PBMCs post stimulation with PGN at 24h (A, C, E and G) and 48h (B, D, F and H). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, black shapes denote PGN; * = $p < 0.05$, ** = $p < 0.01$, ns = non-significant. IL-1 β (A and B), IL-6 (C and D), CXCL8/IL-8 (E and F), TNF- α (G and H). Increases in proinflammatory cytokine protein were observed in all groups at 24h, reaching statistical significance in AA. Increases were also observed at 48h, but this failed to reach statistical significance. No differences were observed between volunteer groups at both time points.

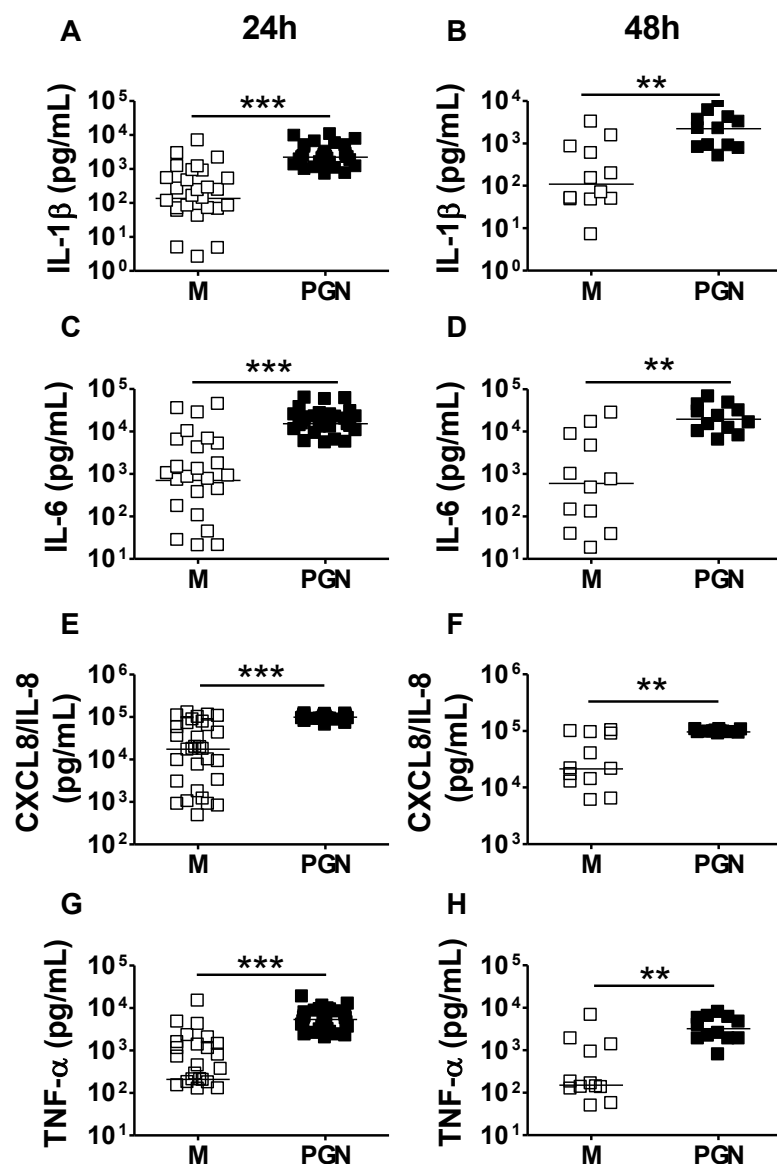


Figure 5.34. Proinflammatory cytokine protein induction at 24h (A, C, E and G) and 48h (B, D, F and H) in PBMCs from all groups combined stimulated with PGN. IL-1 β (A and B), IL-6 (C and D), CXCL8/IL-8 (E and F) and TNF- α (G and H). Open squares denote medium (M); black squares denote PGN; ** = $p < 0.01$, *** = $p < 0.001$. Significant induction of all proinflammatory cytokines was observed in all groups combined.

5.2.7.4 FSL

The final TLR 2/6 ligand to be examined in PBMCs was FSL. As with the other ligands, type I and III IFN and proinflammatory cytokine protein was measured at 24h and 48h. Induction of IFN- α 2a was not observed (figure 5.35 A and B). A non-significant trend for induction of IFN- β protein was observed in ANA and AA at 24h only (figure 5.35 C). An increased induction of IFN- λ 1/IL-29 protein was observed in single volunteers in the NAA and AA groups at 48h (figure 5.35 F). No significant difference was observed between groups.

No significant induction of proinflammatory cytokine protein was observed in PBMCs at 24h post stimulation with FSL (figure 5.36 A, C, E and G). A trend for induction of TNF- α was observed in ANA only (figure 5.36 G). At 48h trends for induction of proinflammatory cytokines was observed in NANA, ANA and NAA, with less obvious induction in AA (figure 5.36 B, D, F and H). These failed to reach statistical significance. No differences were observed between groups. As no differences were observed between groups, as with the other ligands, all groups were combined at 24h and 48h, in order to observe whether FSL stimulated PBMCs induced significant amounts of proinflammatory cytokines. Significant amounts of IL-1 β ($p < 0.01$ at 24h; non-significant at 48h), IL-6 ($p < 0.001$ for 24h; $p < 0.05$ for 48h), CXCL8/IL-8 ($p < 0.01$ at both 24h and 48h) and TNF- α ($p < 0.01$ at 24h; $p < 0.05$ at 48h) was observed with all groups combined (figure 5.37).

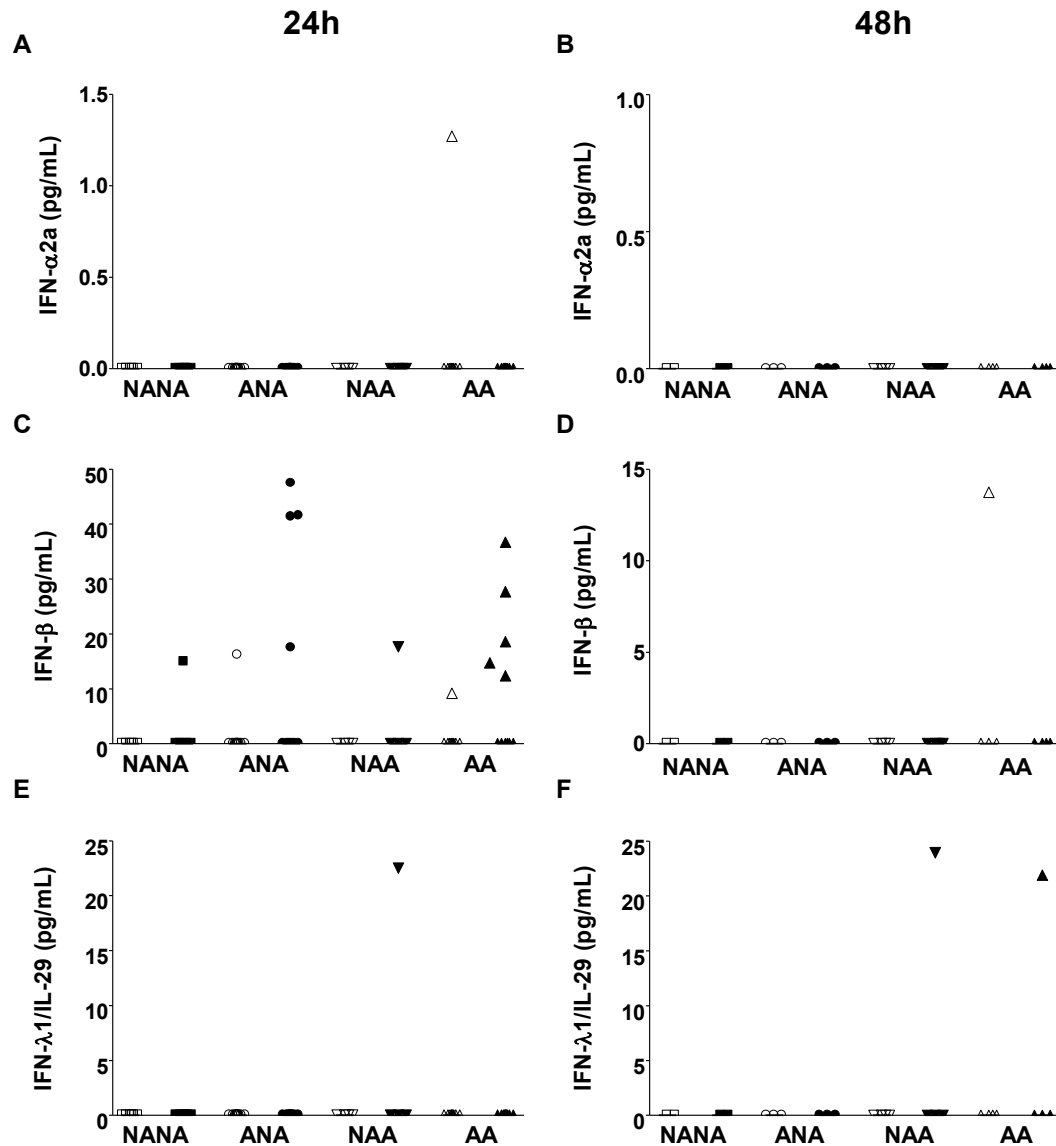


Figure 5.35. Interferon protein induction in PBMCs post stimulation with FSL at 24h (A, C and E) and 48h (B, D and F). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, black shapes denote FSL. IFN-α2a (A and B), IFN-β (C and D), IFN-λ1/IL-29 (E and F). No significant IFN-α2a induction was observed. A non-significant trend in IFN-β protein induction was observed in ANA and AA at 24h, but not at 48h. An increase in IFN-λ1/IL-29 protein induction was observed in a single volunteer in the NAA and AA groups at 48h.

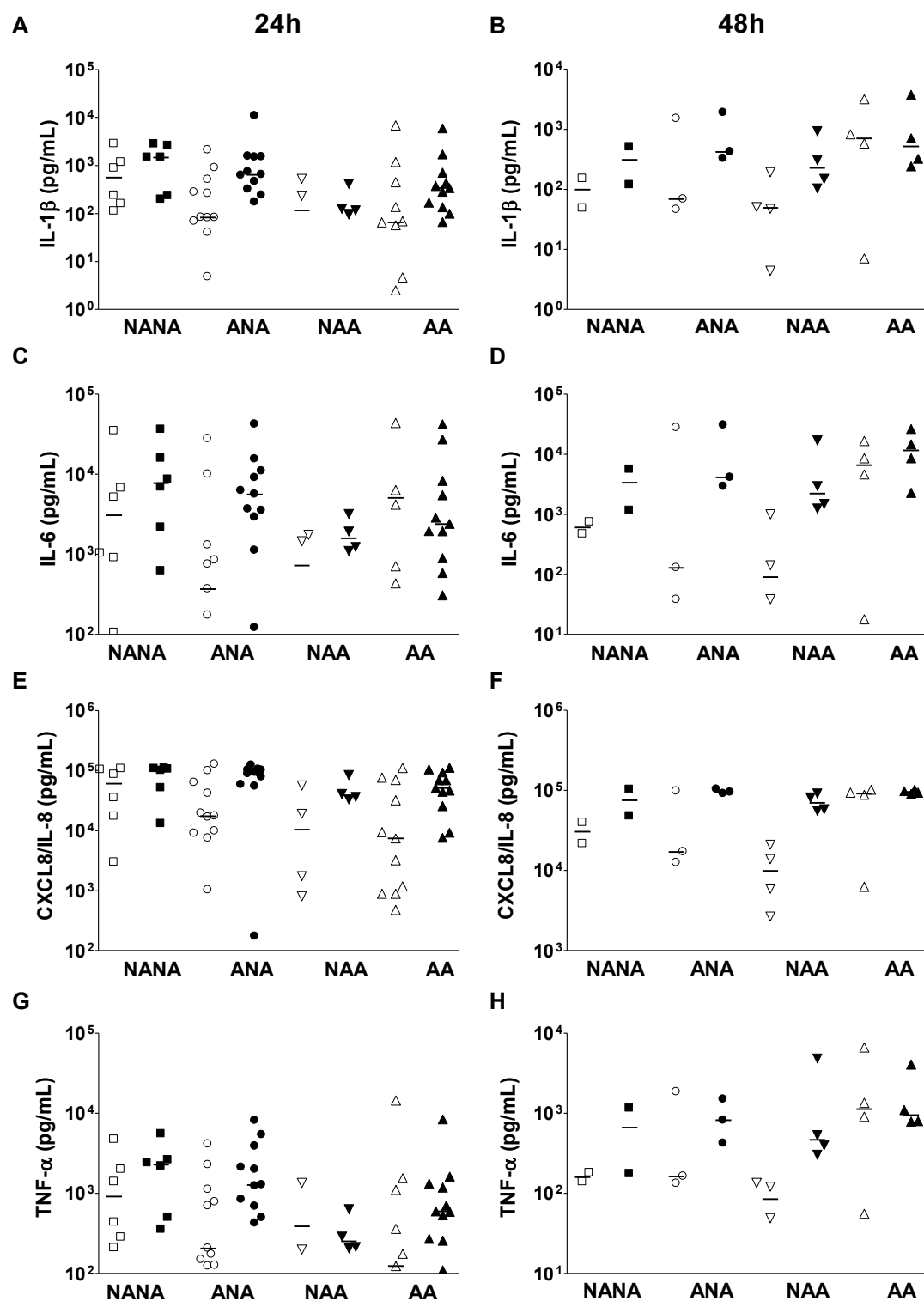


Figure 5.36. Proinflammatory cytokine protein induction in PBMCs post stimulation with FSL at 24h (A, C, E and G) and 48h (B, D, F and H). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, black shapes denote FSL. IL-1 β (A and B), IL-6 (C and D), CXCL8/IL-8 (E and F), TNF- α (G and H). No induction of proinflammatory cytokines was observed at 24h. A trend for induction of TNF- α in ANA was observed. At 48h, trends for induction were observed in NANA, ANA and NAA.

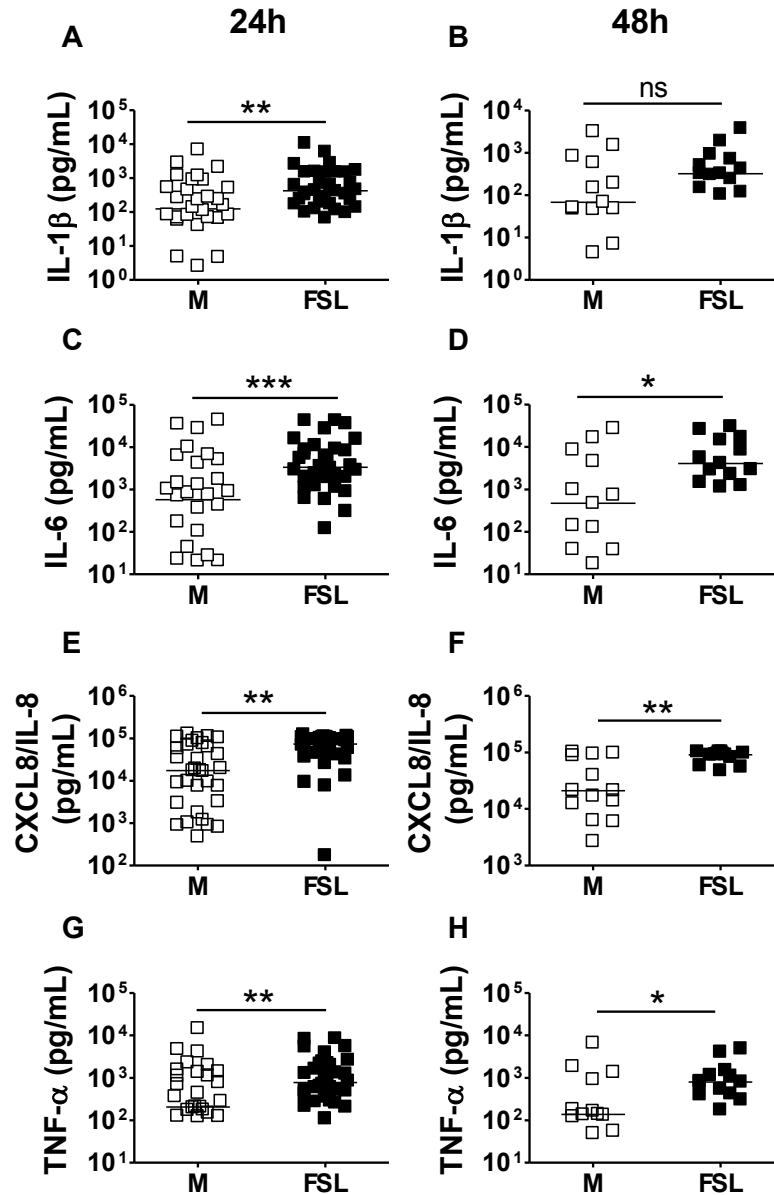


Figure 5.37. Proinflammatory cytokine protein induction at 24h (A, C, E and G) and 48h (B, D, F and H) in PBMCs from all groups combined stimulated with FSL. IL-1 β (A and B), IL-6 (C and D), CXCL8/IL-8 (E and F) and TNF- α (G and H). Open squares denote medium (M); black squares denote FSL; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, ns = non-significant. Significant amounts of IL-6, CXCL8/IL-8 and TNF- α were observed. Induction of IL-1 β was observed at 24h and not 48h.

5.2.8 TLR 4 stimulation of PBMCs: type I and III IFN and proinflammatory cytokine protein induction

PBMCs were stimulated with the TLR 4 ligand LPS and induction of type I and III IFN and proinflammatory cytokine protein was assessed at 24h and 48h post stimulation. Induction of IFN- α 2a was not observed (figure 5.38 A and B). A trend for induction of IFN- β was observed at 24h in NAA and AA, but this was not statistically significant (figure 5.38 C). No induction was observed at 48h (figure 5.38 D). Induction of IFN- λ 1/IL-29 was not observed other than in single NAA and AA participants' samples at 48h (figure 5.38 E and F). No significant differences were observed between the 4 volunteer groups.

In order to establish whether stimulation of PBMCs with LPS resulted in induction of proinflammatory cytokines, cell supernatants were assessed for cytokine protein and 24h and 48h. At 24h induction of IL-1 β , IL-6, CXCL8/IL-8 and TNF- α was observed, reaching statistical significance in ANA and AA and with trends for induction observed in NANA and NAA (figure 5.39 A, C, E and G). Non-significant trends for induction of proinflammatory cytokine protein were observed at 48h (figure 5.39 B, D, F and H). No significant differences were observed between groups at 24h or 48h. As with the other TLR ligands examined, as no significant difference had been identified between volunteer groups, all the groups were combined. Highly significant induction of IL-1 β , IL-6, CXCL8/IL-8 and TNF- α protein were observed at both 24h and 48h post LPS-stimulation of PBMCs in all groups combined (all $p < 0.001$, figure 5.40).

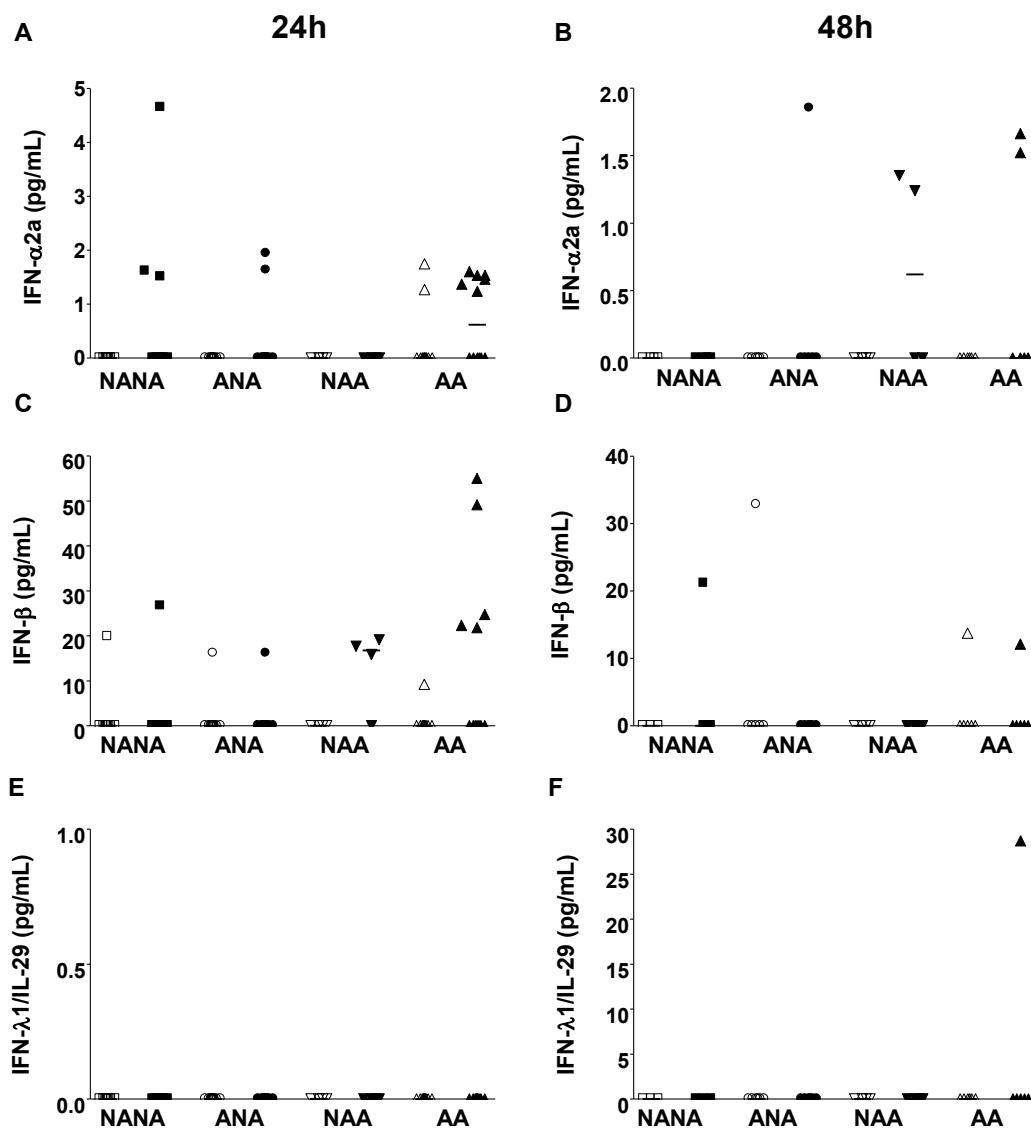


Figure 5.38. Interferon protein induction in PBMCs post stimulation with LPS at 24h (A, C and E) and 48h (B, D and F). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, black shapes denote LPS. IFN-α2a (A and B), IFN-β (C and D), IFN-λ1/IL-29 (E and F). No significant IFN-α2a induction was observed. A non-significant trend in IFN-β protein induction was observed in NAA and AA at 24h, but not at 48h. In a single NAA and AA participants' samples IFN-λ1/IL-29 protein was observed at 48h, but this was non-significant. No significant differences were observed between groups.

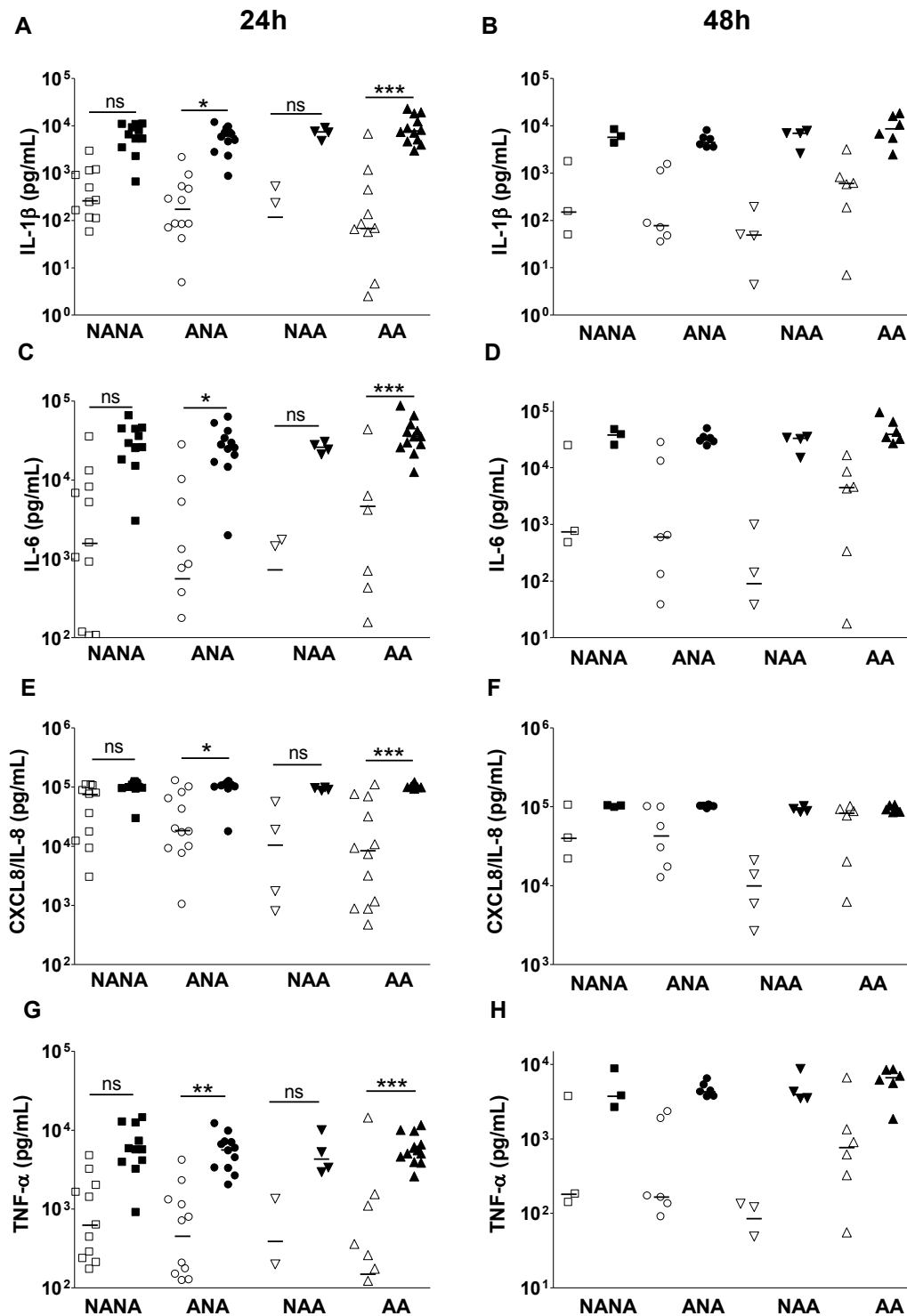


Figure 5.39. Proinflammatory cytokine protein induction in PBMCs post stimulation with LPS at 24h (A, C, E and G) and 48h (B, D, F and H). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, black shapes denote LPS; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, ns = non-significant. IL-1 β (A and B), IL-6 (C and D), CXCL8/IL-8 (E and F), TNF- α (G and H). At 24h induction of all proinflammatory cytokines was observed, reaching statistical significance in ANA and AA. At 48h, non-significant trends for induction were observed. No significant differences were observed between groups.

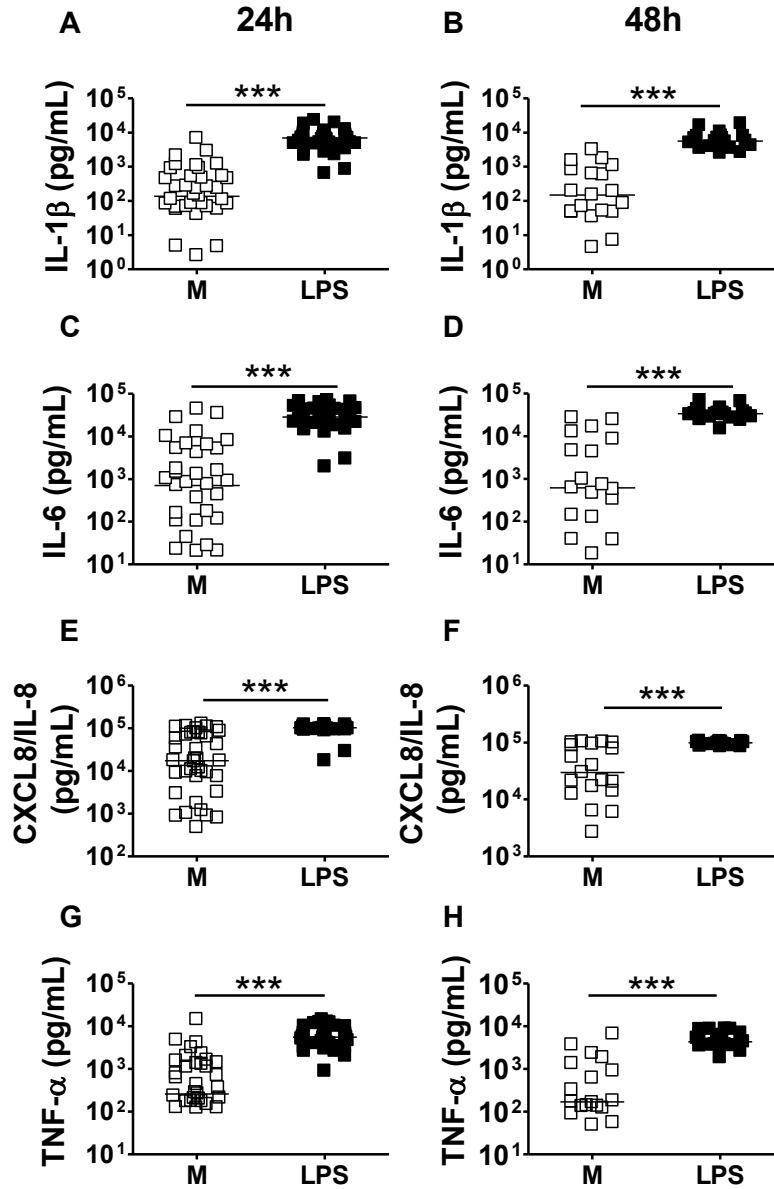


Figure 5.40. Proinflammatory cytokine protein induction at 24h (A, C, E and G) and 48h (B, D, F and H) in PBMCs from all groups combined stimulated with LPS. IL-1 β (A and B), IL-6 (C and D), CXCL8/IL-8 (E and F) and TNF- α (G and H). Open squares denote medium (M); black squares denote LPS; *** = $p < 0.001$. Highly significant induction of all proinflammatory cytokine protein was observed in all groups combined at both 24h and 48h.

5.2.9 TLR 5 stimulation of PBMCs: type I and III IFN and proinflammatory cytokine protein induction

Induction of type I and III IFN and proinflammatory cytokine protein was examined in PBMC supernatants stimulated with FLA. No significant induction of type I or III IFNs was observed at 24h or 48h (appendix A, figure A9). In 2 separate AA volunteers induction of IFN- β protein at 24h and IFN- λ 1/IL-29 protein at 48h was observed (appendix A, figure A9, C and F).

Proinflammatory cytokine induction was observed at 24h and 48h, reaching statistical significance in AA at 24h, with non-significant trends in other groups. At 48h, trends for induction were observed but, due to the small sample size, statistical significance was not reached (figure 5.41). No significant differences were observed between groups. Groups were combined to assess whether statistically significant induction at 24h and 48h was observed. At 24h significant induction of all proinflammatory cytokines was observed ($p < 0.001$ for all). At 48h induction of IL-1 β ($p < 0.05$), IL-6 ($p < 0.001$), CXCL8/IL-8 ($p < 0.01$) and TNF- α ($p < 0.01$) protein was observed in PBMCs stimulated with FLA (figure 5.42).

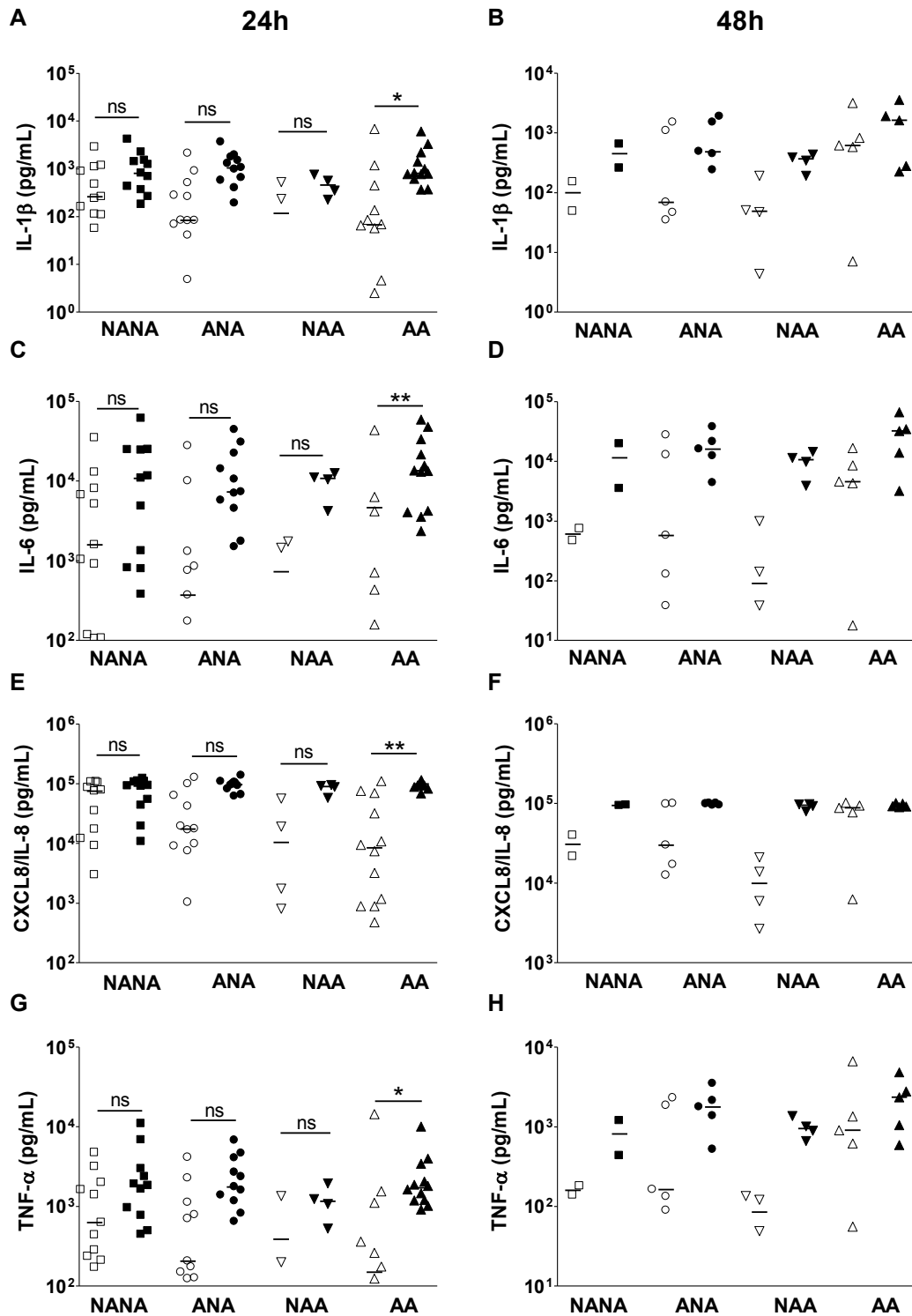


Figure 5.41. Proinflammatory cytokine protein induction in PBMCs post stimulation with FLA at 24h (A, C, E and G) and 48h (B, D, F and H). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, black shapes denote FLA; * = $p < 0.05$, ** = $p < 0.01$, ns = non-significant. IL-1 β (A and B), IL-6 (C and D), CXCL8/IL-8 (E and F), TNF- α (G and H). In AA significant induction of all proinflammatory cytokines was observed at 24h, with non-significant increases observed in other groups. At 48h trends for increased induction was observed, but these failed to reach statistical significance. No differences were observed between groups.

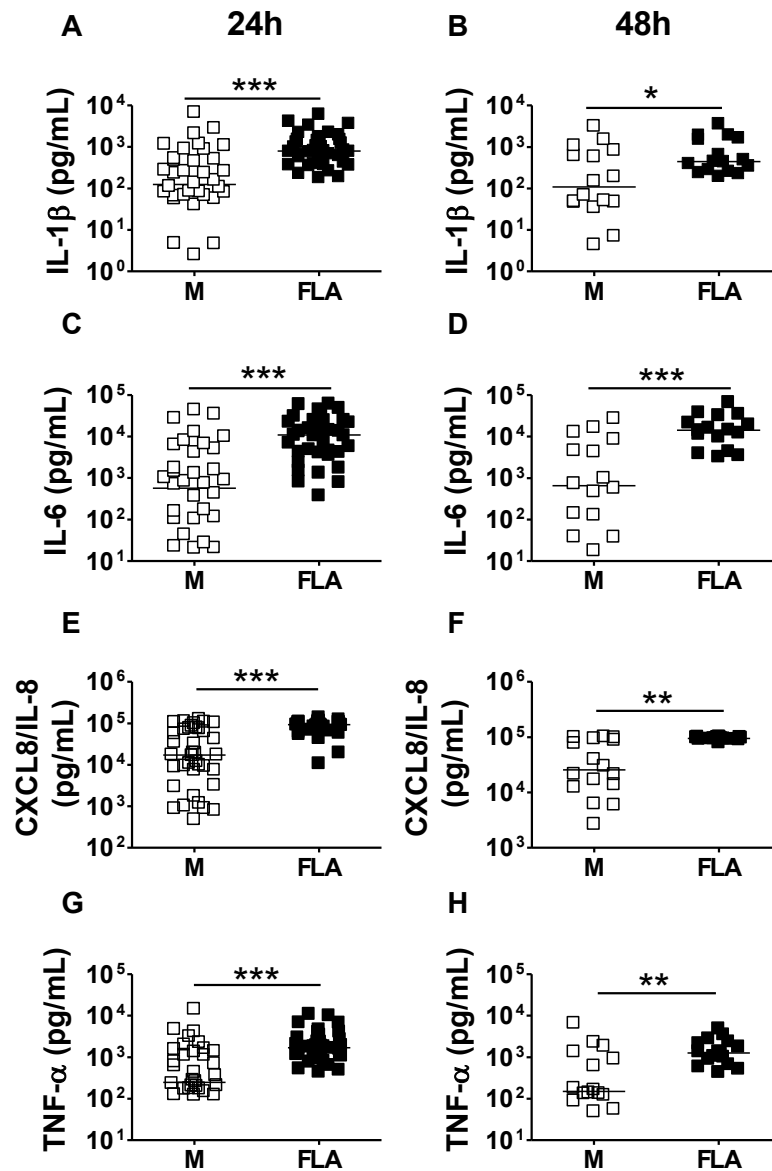


Figure 5.42. Proinflammatory cytokine protein induction at 24h (A, C, E and G) and 48h (B, D, F and H) in PBMCs from all groups combined stimulated with FLA. IL-1 β (A and B), IL-6 (C and D), CXCL8/IL-8 (E and F) and TNF- α (G and H). Open squares denote medium (M); black squares denote FLA; * = p<0.05, ** = p<0.01, *** = p<0.001. Significant induction of all proinflammatory cytokine protein was observed in all groups combined at 24h and 48h.

5.2.10 TLR 9 stimulation of PBMCs: type I and III IFN and proinflammatory cytokine protein induction

PBMCs were stimulated with the TLR 9 ligand CpG-C and induction of type I and III IFN and proinflammatory cytokine protein was assessed at 24h and 48h post stimulation. Type I and III IFNs were not significantly induced following stimulation with CpG-C (appendix A, figure A10). No statistically significant differences were observed between the 4 volunteer groups.

Proinflammatory cytokine induction from PBMCs stimulated with CpG-C was assessed at 24h and 48h. In AA, at 24h, significant induction of IL-1 β ($p<0.05$), IL-6 ($p<0.05$), CXCL8/IL-8 ($p<0.01$) and TNF- α ($p<0.05$) protein was observed. Non-significant trends in induction were observed in other groups (figure 5.43 A, C, E and G). No significant differences were observed between groups. At 48h, induction of IL-1 β protein was not observed, and weak non-significant trends in induction of IL-6, CXCL8/IL-8 and TNF- α protein were observed (figure 5.43 B, D, F and H). No significant difference was observed between groups. All samples were combined to assess whether significant induction of proinflammatory cytokine protein occurred at 24h and 48h. At 24h significant induction of all proinflammatory cytokines was observed in PBMCs stimulated with CpG-C ($p<0.001$, figure 5.44 A, C, E and G). At 48h only induction of CXCL8/IL-8 protein reached statistical significance ($p<0.05$, figure 5.44 F).

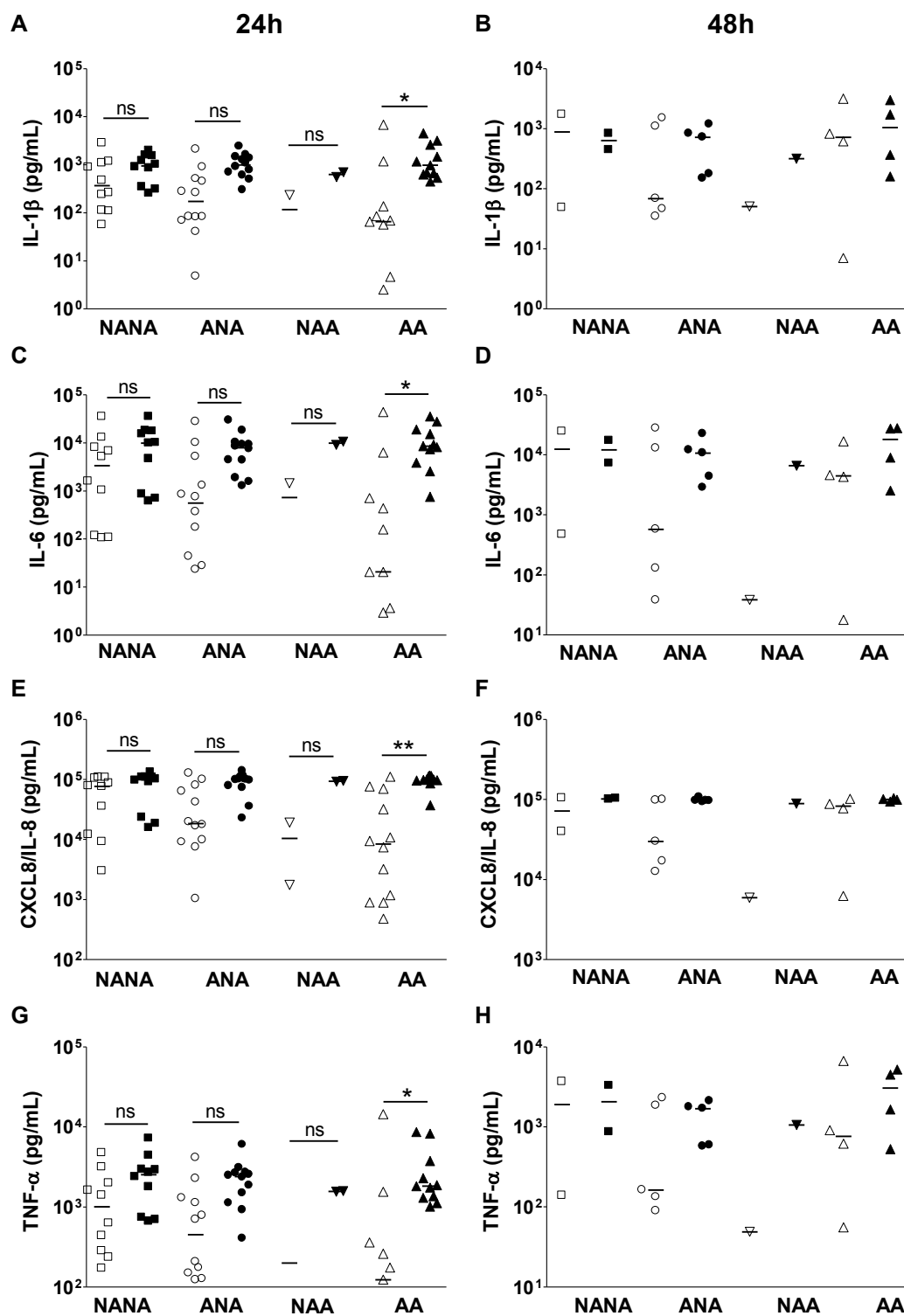


Figure 5.43. Proinflammatory cytokine protein induction in PBMCs post stimulation with CpG-C at 24h (A, C, E and G) and 48h (B, D, F and H). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, black shapes denote CpG-C; * = $p < 0.05$, ** = $p < 0.01$, ns = non-significant. IL-1 β (A and B), IL-6 (C and D), CXCL8/IL-8 (E and F), TNF- α (G and H). At 24h, significant induction of proinflammatory cytokine protein was observed in AA, with non-significant trends observed in other groups. At 48h, induction of IL-1 β was not observed. Only non-significant weak trends in the other cytokines were observed at 48h. No significant differences were observed between groups.

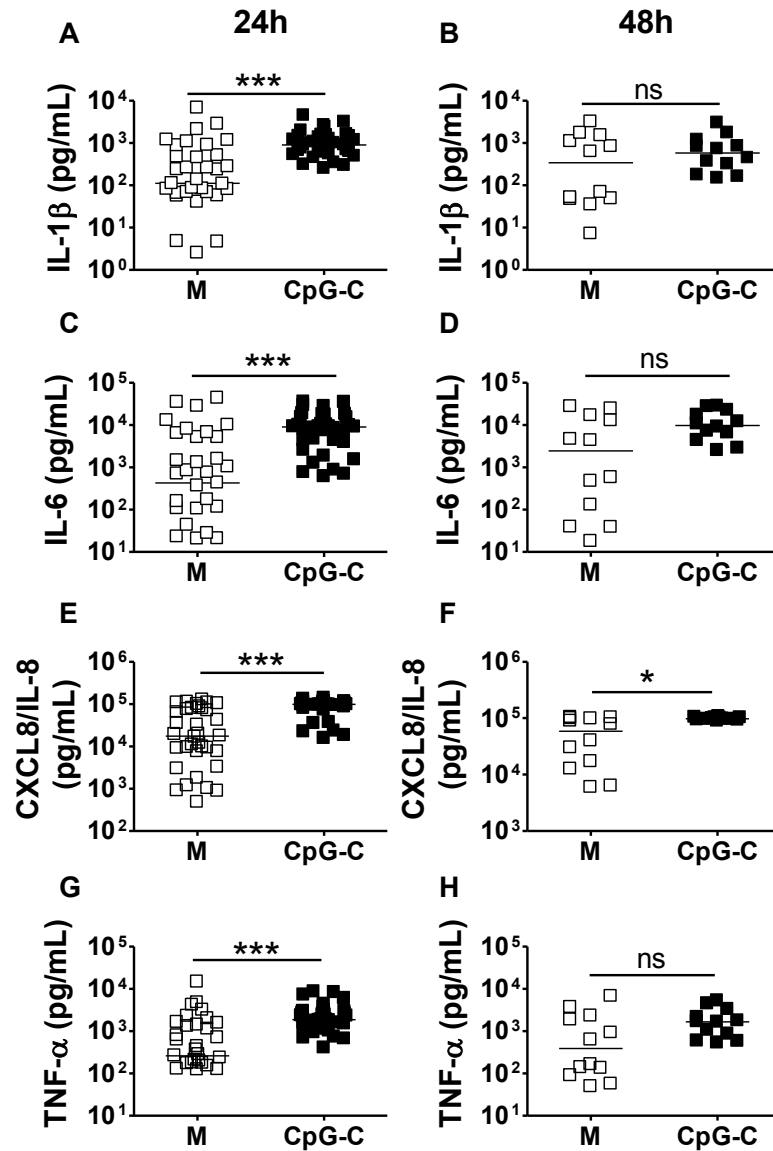


Figure 5.44. Proinflammatory cytokine protein induction at 24h (A, C, E and G) and 48h (B, D, F and H) in PBMCs from all groups combined stimulated with CpG-C. IL-1 β (A and B), IL-6 (C and D), CXCL8/IL-8 (E and F) and TNF- α (G and H). Open squares denote medium (M); black squares denote CpG-C; * = $p < 0.05$, *** = $p < 0.001$, ns = non-significant. In all groups combined, significant induction of all cytokines was observed at 24h. At 48h significant induction of CXCL8/IL-8 protein was observed. Other proinflammatory cytokine protein induction was not observed.

5.2.11 Summary of type I and III IFN and proinflammatory cytokine induction in PBMCs stimulated with TLR1/2/4/5/6/9 ligands

Tables 5.8 to 5.14 summarise the induction of type I and III IFNs and proinflammatory cytokines in PBMCs stimulated with “bacterial” TLR ligands.

Table 5.8 Summary of IFN and proinflammatory cytokine protein induction post PAM stimulation in PBMCs ns = non-significant increase observed, - = no induction

	24h				48h			
	NANA	ANA	NAA	AA	NANA	ANA	NAA	AA
IFN- α 2a	-	-	-	-	-	-	-	-
IFN- β	-	-	-	-	-	-	-	-
IFN λ 1/IL-29	-	-	-	-	-	-	-	-
IL-1 β	ns	ns	ns	ns	ns	ns	ns	-
IL-6	ns	ns	ns	-	ns	ns	ns	ns
CXCL8/IL-8	ns	ns	ns	ns	ns	ns	ns	-
TNF- α	-	ns	-	ns	ns	ns	ns	-

Table 5.9 Summary of IFN and proinflammatory cytokine protein induction post LTA stimulation in PBMCs $\uparrow$ = significant induction, ns = non-significant increase observed, - = no induction

	24h				48h			
	NANA	ANA	NAA	AA	NANA	ANA	NAA	AA
IFN- α 2a	-	-	-	-	-	-	-	-
IFN- β	-	-	-	-	-	-	-	-
IFN λ 1/IL-29	-	-	-	-	-	-	-	-
IL-1 β	ns	$\uparrow$	ns	$\uparrow$	ns	ns	ns	ns
IL-6	ns	$\uparrow$	ns	$\uparrow$	ns	ns	ns	ns
CXCL8/IL-8	ns	$\uparrow$	ns	$\uparrow$	ns	ns	ns	-
TNF- α	ns	ns	ns	$\uparrow$	ns	ns	ns	ns

Table 5.10 Summary of IFN and proinflammatory cytokine protein induction post PGN stimulation in PBMCs ↑ = significant induction, ns = non-significant increase observed, - = no induction

	24h				48h			
	NANA	ANA	NAA	AA	NANA	ANA	NAA	AA
IFN-α2a	-	-	-	-	-	-	-	-
IFN-β	-	ns	-	ns	-	-	-	-
IFNλ1/IL-29	-	-	-	-	-	-	ns	-
IL-1β	ns	ns	ns	↑	ns	ns	ns	ns
IL-6	ns	ns	ns	↑	ns	ns	ns	ns
CXCL8/IL-8	ns	ns	ns	↑	ns	ns	ns	ns
TNF-α	ns	ns	ns	↑	ns	ns	ns	ns

Table 5.11 Summary of IFN and proinflammatory cytokine protein induction post FSL stimulation in PBMCs ns = non-significant increase observed, - = no induction

	24h				48h			
	NANA	ANA	NAA	AA	NANA	ANA	NAA	AA
IFN-α2a	-	-	-	-	-	-	-	-
IFN-β	-	ns	-	ns	-	-	-	-
IFNλ1/IL-29	-	-	-	-	-	-	ns	-
IL-1β	-	ns	-	-	-	ns	ns	-
IL-6	-	ns	-	-	ns	ns	ns	-
CXCL8/IL-8	-	ns	-	ns	ns	ns	ns	-
TNF-α	-	ns	-	ns	ns	ns	ns	-

Table 5.12 Summary of IFN and proinflammatory cytokine protein induction post LPS stimulation in PBMCs ↑ = significant induction, ns = non-significant increase observed, - = no induction

	24h				48h			
	NANA	ANA	NAA	AA	NANA	ANA	NAA	AA
IFN- α 2a	-	-	-	-	-	-	-	-
IFN- β	-	-	ns	ns	-	-	-	-
IFN λ 1/IL-29	-	-	-	-	-	-	-	-
IL-1 β	ns	↑	ns	↑	ns	ns	ns	ns
IL-6	ns	↑	ns	↑	ns	ns	ns	ns
CXCL8/IL-8	ns	↑	ns	↑	ns	ns	ns	ns
TNF- α	ns	↑	ns	↑	ns	ns	ns	ns

Table 5.13 Summary of IFN and proinflammatory cytokine protein induction post FLA stimulation in PBMCs ↑ = significant induction, ns = non-significant increase observed, - = no induction

	24h				48h			
	NANA	ANA	NAA	AA	NANA	ANA	NAA	AA
IFN- α 2a	-	-	-	-	-	-	-	-
IFN- β	-	-	-	-	-	-	-	-
IFN λ 1/IL-29	-	-	-	-	-	-	-	-
IL-1 β	ns	ns	ns	↑	ns	ns	ns	ns
IL-6	ns	ns	ns	↑	ns	ns	ns	ns
CXCL8/IL-8	ns	ns	ns	↑	ns	ns	ns	ns
TNF- α	ns	ns	ns	↑	ns	ns	ns	ns

Table 5.14 Summary of IFN and proinflammatory cytokine protein induction post CpG stimulation in PBMCs ↑ = significant induction, ns = non-significant increase observed, - = no induction

	24h				48h			
	NANA	ANA	NAA	AA	NANA	ANA	NAA	AA
IFN-α2a	-	-	-	-	-	-	-	-
IFN-β	-	-	-	-	-	-	-	-
IFNλ1/IL-29	-	-	-	-	-	-	-	-
IL-1β	ns	ns	ns	↑	-	ns	ns	-
IL-6	ns	ns	ns	↑	-	ns	ns	-
CXCL8/IL-8	ns	ns	ns	↑	-	ns	ns	-
TNF-α	ns	ns	ns	↑	-	ns	ns	ns

5.3 Discussion

In order to investigate IFN signalling in response to bacterial infection, responses of TLRs which recognise bacterial products were examined in HBECs and PBMCs. These “bacterial” ligands to TLR1, 2, 4, 5, 6 and 9 were used to stimulate cells from asthmatics and non-asthmatics, atopics and non-atopics and responses of type I and III IFN and proinflammatory cytokines important in host defence against bacterial infections were assessed.

5.3.1 TLR1, 2 and 6 stimulation

In HBECs no induction of type I IFN mRNA or protein was observed in any group. For each of the ligands stimulating TLR1, 2 and 6 a trend for induction of type III IFN was observed (see tables 5.1 to 5.4), but no particular pattern in the different groups was observed, suggesting the asthmatic or atopic nature of the cells made little difference to the induction pattern. This is the first study to suggest type III IFN induction in HBECs in response to these TLR ligands, though induction failed to reach statistical significance. Proinflammatory cytokine mRNA and protein was

observed with Pam, LTA and FSL, reaching significance in certain groups (see tables 5.1 to 5.4). No significant difference was observed between asthmatics or control groups. Previous studies have only observed weak proinflammatory responses to TLR2 stimulation in airway epithelial cells ³¹⁷.

Non-significant induction of IFN- β protein was observed in PBMCs from AA and ANA at 24h following stimulation with the TLR2/6 ligands peptidoglycan and lipoprotein. Type I and III IFNs were not induced at any other time point or by TLR1/2 ligand Pam3CSK4 or the TLR2/6 ligand LTA. Induction of proinflammatory cytokines was observed in PBMCs stimulated with LTA and PGN at 24h and 48h, and with Pam at 48h in NANA and NAA, and FSL at 24h in ANA and 48h in NANA and NAA (see tables 5.1 to 5.4). No significant difference was observed between asthmatics or control groups. Expression of TLR1, 2 and 6 on PBMCs has been identified previously as significantly reduced in asthmatics compared to non-asthmatics ³³⁵. Although TLR expression was not examined in this chapter, no difference in TLR function was identified between asthmatics and non-asthmatics. Previous studies have not reported TLR2 as an inducer of type I IFNs although in recognising viral TLR2 ligands (several DNA viruses, including vaccinia, cytomegalovirus and herpes simplex virus) and not bacterial TLR ligands, induction of type I IFN has been reported ²⁵⁹. In contrast to this, TLR2 signalling has been identified as inhibitory to type I IFN induction by TLR9 ³³⁶. LTA has previously been reported not to be critical in TNF- α release from PBMCs in that no difference was observed in TNF- α induction from PBMCs incubated with LTA-rich or LTA-free wall fragments from *Spn* ³³⁷. The increases in proinflammatory cytokines following stimulation with these TLR ligands and only weak data to suggest type III IFN induction in HBECs would suggest that TLR1, 2 and 6 signalling in HBECs and PBMCs is more important for the induction of proinflammatory cytokines than IFNs. Several other anti-bacterial cytokines could also be involved in host defence against bacteria, particularly IFN- γ . This has a role in anti-bacterial immunity, though it appears to be microbe-specific ³²². Due to time and financial constraints of the project and the multiplex used not having IFN- γ as a cytokine to measure, the production of IFN- γ was not analysed. IFN- γ responses to rhinovirus infection are deficient in atopic asthmatics ⁷⁸. As part of future work, induction of IFN- γ could be

analysed from cells stimulated with bacterial ligands to determine whether IFN- γ responses to bacterial infection are deficient in asthma.

5.3.2 TLR4 stimulation

Induction of type I and III IFN mRNA and protein was not observed in HBECs stimulated with LPS. Non-significant increases in proinflammatory cytokines was observed in all groups other than AA, which suggests confirmation of previous reports of cytokine induction from HBECs in response to LPS³³⁸. No significant type I or III IFN protein was induced from PBMCs stimulated with LPS. A trend for induction of IFN- β was observed in asthmatics, but this was not significant. This would suggest that PBMCs are not a major source of IFNs and the previously observed IFN deficiency in HBECs^{60, 61} does not extend to PBMCs stimulated with LPS. Other investigators have found airway epithelial cells unresponsive to LPS³³⁹ unless infected with RSV which leads to TLR4 upregulation³⁴⁰. Induction of proinflammatory cytokines in PBMCs was observed in all groups, reaching significance at 24h in ANA and AA. No difference was observed between groups. Previous studies have found that LPS stimulated TNF- α induction from PBMCs is higher in mild-moderate than in severe asthmatics³⁴¹, which differs from other studies suggesting reduced TLR4 responses in asthma³³⁰ and decreased induction of TNF- α and IL-1 β from PBMCs stimulated with LPS in asthma³³⁵.

To link the earlier investigation of *Spn* and IFN induction, pneumolysin, which is recognised by TLR4 and is a virulence factor for *Spn*, could be examined in a further study of HBECs and PBMCs in asthmatics.

5.3.3 TLR5 stimulation

No significant induction of type I or III mRNA or protein was observed in HBECs stimulated with the TLR ligand flagellin. A non-significant increase in type III mRNA was observed in NAA only, but sample size was small. No induction of type I or III IFN protein was observed in PBMCs, suggesting TLR5 is not important in IFN induction in these cell types. This differs from a previous study suggesting TLR5

stimulation of mouse bone marrow derived macrophages results in IFN- β induction
268 .

A non-significant increase in CXCL8/IL-8 mRNA at 8h and IL-6 and CXCL8/IL-8 protein at 24h was observed in HBECs, with no differences between groups. Increases in induction of proinflammatory cytokines was observed in PBMCs stimulated with flagellin, with significance obtained in AA. No differences however were observed between groups. This differs from the study which showed decreased *ex-vivo* induction of IL-1 β , IL-10 and TNF- α from asthmatic PBMCs stimulated with flagellin ³³⁵. The finding that TLR5 appears to lead to the induction of proinflammatory cytokines suggests the role of TLR5 lies more in anti-bacterial immunity than in IFN induction.

5.3.4 TLR9 stimulation

The TLR9 ligand CpG-C did not induce type I or III IFN mRNA or protein in HBECs or PBMCs. This would suggest TLR9 is not important in IFN induction, however studies have identified TLR9 as an important receptor mediating the induction of type I IFNs in mouse dendritic cells in response to bacterial infection ³⁴². Recently, IFN- α induction in human PBMCs stimulated with TLR9 ligands has been observed ³⁴³ and TLR9 has been shown to be a strong inducer of IFN- β in mice *in vivo* ³⁴⁴, findings not supported by this study.

No increases in proinflammatory cytokine mRNA or protein were observed in HBECs. Increases in proinflammatory cytokine protein were observed in PBMCs from all groups at 24h, with significance in AA, and a non-significant increase observed at 48h in NAA only. This increase in proinflammatory cytokines and not type I and III IFNs suggests that, in PBMCs, TLR9 has more of a role in anti-bacterial immunity than in IFN induction. No differences were observed between groups, suggesting the asthmatic or atopic status of the subjects' cell type is of no importance.

5.4 Conclusions

This is the first report of type I and III IFN and proinflammatory cytokine responses to TLR1, 2, 6, 4, 5 and 9 stimulation in HBECs and PBMCs from AA, NAA, ANA and NANA and aimed to determine whether differences were observed between asthmatics and/or atopics. Although induction of type I IFN was not observed in any cell type, non-significant induction of type III IFN was observed in HBECs, but not PBMCs. Proinflammatory cytokines were induced, particularly in PBMCs, suggesting these TLRs have a role in anti-bacterial immunity and less of a role in IFN induction. No differences were observed between AA, NAA, ANA or NANA. The study hypothesis must therefore be rejected.

5.5 Summary

This chapter investigated the type I and III IFN and proinflammatory cytokine responses to TLR1, 2, 6, 4, 5 and 9 stimulation in HBECs and PBMCs in AA, NAA, ANA and NANA. HBECs responded to TLR1, 2, 6, 4 and 5 stimulation with largely non-significant increases in proinflammatory cytokines, but only limited data to support type III IFN induction. PBMCs responded to TLR2, 6, 4, 5 and 9 stimulation with no induction of type I or III IFNs, but both significant and non-significant increases in proinflammatory cytokine protein. No differences were observed between asthmatics or non-asthmatics, atopics or non-atopics.

Chapter 6: Discussion

6.1 Key study findings

6.1.1 RV16 induced type I and III IFN in BAL cells, whereas RV1B only induced type III IFN, but no deficiency was identified in asthmatics

The lack of deficiency identified in this study differs from that of another study where type I IFN induction in BAL cells was reduced in AA compared to NANA ²⁰⁸. The differences between the two studies may be a result of this study having a group of less severe asthmatics. Whilst it would have been preferable to assess this earlier in the study, the nature of the project was such that data was only analysed at the end of the study period; an inherent flaw in the study design. The observed lack of type III IFN deficiency as seen in another study at 48h post infection ⁶¹ may also have been due to only an 8h time point being used in the present study, due to the limited number of cells obtained at bronchoscopy. The aim had been to perform both an 8h and 24h time point as these time points had revealed significant data in other work within the department and subsequent published work ²⁰⁸, though as there were a limited number of cells obtained, only an 8h time point was analysed. It could be argued that a 24h time point would have revealed more pertinent results, in that the viral replication studies in HBECs demonstrated peak RV release at 24h with RV16 and 48h with RV1B.

6.1.2 IFN induction in HBECs differed for major and minor group RVs, but no difference was observed between asthmatics and non-asthmatics

RV16 infection resulted in both type I and III IFN protein being induced at 48h, but RV1B infection only induced type I IFN protein. The lack of IFN deficiency in HBECs identified in this study may not be surprising given that no deficiency was observed in BAL cells and the conclusion that asthmatics had a milder form of the disease. It may be that the previously observed IFN deficiency ^{60, 61} is due to that being found in a subgroup of asthmatics and possibly related to disease severity. It should be noted that absolute levels of IFN- β differed in this study compared to

original reports of defective RV-induced IFN (up to 500pg/mL compared to levels up to 1900pg/mL⁶⁰). This is consistent with other studies that have shown lower levels of RV16-induced IFN- β protein than the original Wark et al. report. A number of studies, published recently and long after this study was designed, have identified relationships between IFN deficiency and markers of severity. Sykes et al. identified deficient type I IFN production from BAL cells and this was inversely proportional to airway hyperresponsiveness and skin prick test positivity²⁰⁸. Gill et al. identified deficient IFN- α from plasmacytoid dendritic cells in atopic asthmatics and that secretion was inversely correlated with serum IgE levels, suggesting the severity of atopy may be important²⁰⁵. Baraldo et al. identified deficient RV-induced IFN responses in asthmatic children irrespective of their atopic status and in atopic non-asthmatic children, suggesting these deficiencies are not limited to asthma alone²⁰⁹. These findings would certainly support the hypothesis that IFN deficiencies are identified in a subgroup of more severe asthmatics. Due to problems of volunteer recruitment (over 20 suspected NAA volunteers were screened) and cells being lost to contamination, sample size was insufficient to determine whether a deficiency was observed in NAA. Other investigators have also failed to report defective RV induced IFN production in asthma^{203, 204}. Differences between the two RVs was also observed in that viral replication peaked at 24h with RV16 and 48h with RV1B, but no differences in viral replication were observed between asthmatics and non-asthmatics. The different responses between the strains of RV may be due to cells having differing levels RV receptor expression, different efficiencies of viral replication or different intracellular responses. These differences have implications for the further investigation of RVs in asthma. It would have been interesting to observe whether ICAM-1 and LDL-receptor expression differed between groups and whether a difference was related to asthma disease severity.

6.1.3 RV16 and not RV1B infection induced type I and III IFN in PBMCs

RV1B induced type III IFNs in BAL cells, but not in PBMCs. RV16 induced type I and III IFN in PBMCs, which is similar to that seen in BAL cells, but no differences were observed between asthmatics and controls. The data presented in this study does not support RV infection of unsorted PBMCs as a useful clinical test

in asthma, which differs from previous studies^{82, 206}. As in BAL cells, RV failed to induce proinflammatory cytokine production in PBMCs. This is surprising. High baseline levels of cytokines were observed in both BAL and PBMCs, suggesting the acquisition of BAL cells at bronchoscopy and the processing of PBMCs itself results in production of these cytokines. The lack of induction differs from previous studies where proinflammatory cytokines have been shown to be deficient in adolescent asthmatics⁸¹. The differences observed between BAL cells and PBMCs may mean that further studies would have difficulties in extrapolating the findings of PBMCs as a surrogate to BAL cells in an attempt to search for a less invasive way to identify IFN deficiencies in asthma.

6.1.4 TLR3 stimulation of HBECs resulted in induction of type I and III IFN, whereas TLR7/8 stimulation resulted in induction of type III IFN only

Induction of type I and III IFNs and proinflammatory cytokines was observed in HBECs stimulated with poly(IC). No difference was observed between asthmatics and controls. Induction differed in PBMCs with proinflammatory cytokines and not IFNs being induced suggesting that the expression/function of TLR3 may differ in different cell types. Due to an insufficient number of cells being acquired at bronchoscopy the response to TLR stimulation in BAL cells was not investigated. It would be of interest to observe whether similar findings to PBMCs were observed in BAL cells in order to further assess whether PBMCs are reliable cell surrogates for airway cells in the investigation of IFN production in asthmatics.

HBECs stimulated with the TLR7/8 ligand R848 produced type III and not type I IFN. Significant induction of CXCL8/IL-8 and non-significant trends for induction of IL-1 β and TNF- α were observed. These findings support the expression of TLR7/8 on HBECs, where previous reports have had conflicting data^{317, 318}. No differences were observed between asthmatics and controls. By contrast, significant induction of type I IFN was observed in PBMCs, again indicating that the expression/function of TLRs may differ in different cell types.

6.1.5 *Spn* failed to induce type I or III IFN mRNA or protein in BAL cells, HBECs or PBMCs

The *in vitro* model of pneumococcal infection of all cell types presented in this report failed to demonstrate the induction of type I and III IFN in response to *Spn*. Proinflammatory/anti-bacterial cytokines were not induced in BAL cells or HBECs, but induction was well demonstrated in PBMCs. No differences were observed between asthmatics and controls. The study failed to identify type I and III IFN induction in the innate immune response to *Spn*. The lack of induction may be due to a number of factors. The 8h time point chosen, to be in line with experiments performed with RV, may have been too early; the concentration of bacteria and/or the strain used may have been insufficient to elicit a response. Other innate immune pathways may be involved, such as the anti-microbial peptides.

This study also failed to find differences in proinflammatory/anti-bacterial cytokine induction in asthmatic subjects, in response to *Spn* infection of these cell types. Thus a mechanistic explanation for increased susceptibility to invasive pneumococcal disease in asthma ^{104, 319} remains elusive. This again may be because the asthmatics studied were too mild for deficiencies to be observed. Klemets et al. reported susceptibility to invasive pneumococcal disease to be much greater in more severe asthma (odds ratio ~12), than in milder asthma (odds ratio ~3), supporting this hypothesis ¹⁰⁴.

6.1.6 HBEC responses to stimulation of TLRs which recognise bacterial products

Induction of type I IFN was not observed in HBECs from asthmatics or controls stimulated with TLR1/2/6/4/5/9 ligands. In HBECs stimulated with TLR1/2/6 ligands a trend for induction of type III IFN was observed, but no difference was observed between asthmatics and controls. Although only a trend, this is the first report to make this observation. Induction of anti-bacterial cytokines was observed, particularly with TLR1/2/6 ligands, which confirms previous reports that these TLRs are expressed on HBECs ³¹⁷. No deficiencies in induction of anti-bacterial cytokines were observed in asthmatics.

6.1.7 PBMC responses to stimulation of TLRs which recognise bacterial products

No robust induction of type I or III IFN was observed in PBMCs stimulated with TLR1/2/6/4/5/9 ligands. Induction of anti-bacterial cytokines was observed, but no difference was observed between asthmatics and controls. This would suggest that, in PBMCs, signalling of TLRs which recognise bacterial products results in the induction of anti-bacterial cytokines with subsequent anti-bacterial immunity and not in IFN induction.

6.2 Future Work

The most important question to answer from future work is whether IFN deficiency is related to asthma severity and whether differences between more severe atopic and/or non-atopic asthmatics and atopic non-asthmatics, or non-atopic healthy controls would be observed. More recent studies, published after this study was designed and underway suggest this to be so^{205, 208, 209}. As part of future work, volunteers with more severe asthma, and in particular those with a high exacerbation frequency, should be recruited to determine whether the deficiency is identified in different cell types and, in particular, in an adult population. Due to the difficulties with recruitment, sample size for non-atopic asthmatics was small, so a larger sample size may provide more robust data. Other work related to this study which could be performed includes:

- To investigate a reliable surrogate to airway cells use of separated blood monocytes rather than unsorted PBMCs may identify a deficiency in asthma
- To investigate whether IFN deficiency is acquired or present from birth, similar experiments could be performed in cells from asthmatic and non-asthmatic children
- In order to investigate *Spn* further, the TLR4 ligand, pneumolysin, could be studied

- Although attempted in this study and abandoned due to time constraints, use of atypical bacteria such as *Chlamydophila pneumoniae* may be of more relevance to asthma exacerbations
- Investigation of other proinflammatory/anti-bacterial cytokines such as IFN- γ may identify deficiencies in asthma
- Investigation of other innate immune systems, such as anti-microbial peptides, may be of more relevance for the investigation of innate immunity against bacteria in asthma

6.3 Conclusions

This is the first report of type I and III IFN and proinflammatory/anti-bacterial cytokine responses to stimulation with RV, *Spn* and TLR1-9 ligands in cells from adult asthmatics and non-asthmatics, atopics and non-atopics. The hypothesised deficiencies in IFN production in asthmatic subjects was not observed in BAL cells, HBECs or PBMCs. The most likely cause for this deficiency not being identified is the relatively mild nature of the disease in the asthmatics recruited. *Spn* did not induce type I and III IFN production in any cell type. A weak trend for induction of type III IFN was observed in HBECs stimulated with TLR1/2/6 ligands.

Although the question of whether asthma or atopy is important for IFN deficiency in asthma has not yet been answered, the finding that atopic non-asthmatics had no deficiency and that the group of asthmatics recruited had mild disease has advanced knowledge of the field. It has also suggested that IFN pathways appear not to be involved in the response to bacterial infection and that deficiencies in production of anti-bacterial cytokines are not observed in any cell type in the asthmatics included in this study. Thus a mechanism to explain increased susceptibility to invasive pneumococcal infection in asthma remains unknown. This again may be because the asthmatic subjects were at the milder end of the asthma spectrum of disease and studies in more severe asthma are warranted.

References

1. Masoli M, Fabian D, Holt S, Beasley R. The global burden of asthma: executive summary of the GINA Dissemination Committee report. *Allergy* 2004;59(5):469-478.
2. Eder W, Ege MJ, von ME. The asthma epidemic. *N Engl J Med* 2006;355(21):2226-2235.
3. Beasley R, Crane J, Lai CK, Pearce N. Prevalence and etiology of asthma. *J Allergy Clin Immunol* 2000;105(2 Pt 2):S466-S472.
4. de Marco R., Cappa V, Accordini S et al. Trends in the prevalence of asthma and allergic rhinitis in Italy between 1991 and 2010. *Eur Respir J* 2012;39(4):883-892.
5. GINA. Global strategy for asthma management and prevention. www.ginasthma.org 2010.
6. Bateman ED, Hurd SS, Barnes PJ et al. Global strategy for asthma management and prevention: GINA executive summary. *Eur Respir J* 2008;31(1):143-178.
7. Pearce N, Pekkanen J, Beasley R. How much asthma is really attributable to atopy? *Thorax* 1999;54(3):268-272.
8. Martin PE, Matheson MC, Gurrin L et al. Childhood eczema and rhinitis predict atopic but not nonatopic adult asthma: a prospective cohort study over 4 decades. *J Allergy Clin Immunol* 2011;127(6):1473-1479.
9. Rackemann FM. A working classification of asthma. *Am J Med* 1947;3(5):601-606.
10. Davies RJ, Holford-Strevens VC, Wells ID, Pepys J. Bacterial precipitins and their immunoglobulin class in atopic asthma, non-atopic asthma, and chronic bronchitis. *Thorax* 1976;31(4):419-424.
11. Leynaert B, Sunyer J, Garcia-Esteban R et al. Gender differences in prevalence, diagnosis and incidence of allergic and non-allergic asthma: a population-based cohort. *Thorax* 2012;67(7):625-631.
12. Upton MN, McConnachie A, McSharry C et al. Intergenerational 20 year trends in the prevalence of asthma and hay fever in adults: the Midspan family study surveys of parents and offspring. *BMJ* 2000;321(7253):88-92.
13. Basagana X, Sunyer J, Kogevinas M et al. Socioeconomic status and asthma prevalence in young adults: the European Community Respiratory Health Survey. *Am J Epidemiol* 2004;160(2):178-188.
14. Court CS, Cook DG, Strachan DP. Comparative epidemiology of atopic and non-atopic wheeze and diagnosed asthma in a national sample of English adults. *Thorax* 2002;57(11):951-957.
15. Loymans RJ, Ter RG, Sterk PJ. Definitions of asthma exacerbations. *Curr Opin Allergy Clin Immunol* 2011;11(3):181-186.

16. Reddel H, Ware S, Marks G, Salome C, Jenkins C, Woolcock A. Differences between asthma exacerbations and poor asthma control. *Lancet* 1999;353(9150):364-369.
17. Moorman JE, Rudd RA, Johnson CA et al. National surveillance for asthma--United States, 1980-2004. *MMWR Surveill Summ* 2007;56(8):1-54.
18. Greenstone IR, Ni Chroinin MN, Masse V et al. Combination of inhaled long-acting beta2-agonists and inhaled steroids versus higher dose of inhaled steroids in children and adults with persistent asthma. *Cochrane Database Syst Rev* 2005;(4):CD005533.
19. Lasserson TJ, Cates CJ, Ferrara G, Casali L. Combination fluticasone and salmeterol versus fixed dose combination budesonide and formoterol for chronic asthma in adults and children. *Cochrane Database Syst Rev* 2008;(3):CD004106.
20. Joos S, Miksch A, Szecsenyi J et al. Montelukast as add-on therapy to inhaled corticosteroids in the treatment of mild to moderate asthma: a systematic review. *Thorax* 2008;63(5):453-462.
21. Barnes NC, Miller CJ. Effect of leukotriene receptor antagonist therapy on the risk of asthma exacerbations in patients with mild to moderate asthma: an integrated analysis of zafirlukast trials. *Thorax* 2000;55(6):478-483.
22. Johnston NW, Mandhane PJ, Dai J et al. Attenuation of the September epidemic of asthma exacerbations in children: a randomized, controlled trial of montelukast added to usual therapy. *Pediatrics* 2007;120(3):e702-e712.
23. Adcock IM, Barnes PJ. Molecular mechanisms of corticosteroid resistance. *Chest* 2008;134(2):394-401.
24. Umland SP, Schleimer RP, Johnston SL. Review of the molecular and cellular mechanisms of action of glucocorticoids for use in asthma. *Pulm Pharmacol Ther* 2002;15(1):35-50.
25. Barnes PJ. Molecular mechanisms and cellular effects of glucocorticosteroids. *Immunol Allergy Clin North Am* 2005;25(3):451-468.
26. Atmar RL, Guy E, Guntupalli KK et al. Respiratory tract viral infections in inner-city asthmatic adults. *Arch Intern Med* 1998;158(22):2453-2459.
27. Green RM, Custovic A, Sanderson G, Hunter J, Johnston SL, Woodcock A. Synergism between allergens and viruses and risk of hospital admission with asthma: case-control study. *BMJ* 2002;324(7340):763.
28. Johnston NW, Johnston SL, Duncan JM et al. The September epidemic of asthma exacerbations in children: a search for etiology. *J Allergy Clin Immunol* 2005;115(1):132-138.
29. Johnston SL, Pattemore PK, Sanderson G et al. Community study of role of viral infections in exacerbations of asthma in 9-11 year old children. *BMJ* 1995;310(6989):1225-1229.
30. Khetsuriani N, Kazerouni NN, Erdman DD et al. Prevalence of viral respiratory tract infections in children with asthma. *J Allergy Clin Immunol* 2007;119(2):314-321.

31. Kistler A, Avila PC, Rouskin S et al. Pan-viral screening of respiratory tract infections in adults with and without asthma reveals unexpected human coronavirus and human rhinovirus diversity. *J Infect Dis* 2007;196(6):817-825.
32. Nicholson KG, Kent J, Ireland DC. Respiratory viruses and exacerbations of asthma in adults. *BMJ* 1993;307(6910):982-986.
33. Tan WC, Xiang X, Qiu D, Ng TP, Lam SF, Hegele RG. Epidemiology of respiratory viruses in patients hospitalized with near-fatal asthma, acute exacerbations of asthma, or chronic obstructive pulmonary disease. *Am J Med* 2003;115(4):272-277.
34. Vallet C, Pons-Catalano C, Mandelcwaig A et al. Human bocavirus: a cause of severe asthma exacerbation in children. *J Pediatr* 2009;155(2):286-288.
35. Johnston SL, Pattemore PK, Sanderson G et al. The relationship between upper respiratory infections and hospital admissions for asthma: a time-trend analysis. *Am J Respir Crit Care Med* 1996;154(3 Pt 1):654-660.
36. Minor TE, Baker JW, Dick EC et al. Greater frequency of viral respiratory infections in asthmatic children as compared with their nonasthmatic siblings. *J Pediatr* 1974;85(4):472-477.
37. Tarlo S, Broder I, Spence L. A prospective study of respiratory infection in adult asthmatics and their normal spouses. *Clin Allergy* 1979;9(3):293-301.
38. Corne JM, Marshall C, Smith S et al. Frequency, severity, and duration of rhinovirus infections in asthmatic and non-asthmatic individuals: a longitudinal cohort study. *Lancet* 2002;359(9309):831-834.
39. Kling S, Donninger H, Williams Z et al. Persistence of rhinovirus RNA after asthma exacerbation in children. *Clin Exp Allergy* 2005;35(5):672-678.
40. Rossmann MG, Arnold E, Erickson JW et al. Structure of a human common cold virus and functional relationship to other picornaviruses. *Nature* 1985;317(6033):145-153.
41. Bella J, Rossmann MG. Review: rhinoviruses and their ICAM receptors. *J Struct Biol* 1999;128(1):69-74.
42. Olson NH, Kolatkar PR, Oliveira MA et al. Structure of a human rhinovirus complexed with its receptor molecule. *Proc Natl Acad Sci U S A* 1993;90(2):507-511.
43. Greve JM, Davis G, Meyer AM et al. The major human rhinovirus receptor is ICAM-1. *Cell* 1989;56(5):839-847.
44. Hofer F, Gruenberger M, Kowalski H et al. Members of the low density lipoprotein receptor family mediate cell entry of a minor-group common cold virus. *Proc Natl Acad Sci U S A* 1994;91(5):1839-1842.
45. Gern JE. The ABCs of rhinoviruses, wheezing, and asthma. *J Virol* 2010;84(15):7418-7426.
46. Bochkov YA, Gern JE. Clinical and molecular features of human rhinovirus C. *Microbes Infect* 2012;14(6):485-494.

47. Miller EK, Edwards KM, Weinberg GA et al. A novel group of rhinoviruses is associated with asthma hospitalizations. *J Allergy Clin Immunol* 2009;123(1):98-104.
48. Cordey S, Gerlach D, Junier T, Zdobnov EM, Kaiser L, Tapparel C. The cis-acting replication elements define human enterovirus and rhinovirus species. *RNA* 2008;14(8):1568-1578.
49. Pathak HB, Arnold JJ, Wiegand PN, Hargittai MR, Cameron CE. Picornavirus genome replication: assembly and organization of the VPg uridylylation ribonucleoprotein (initiation) complex. *J Biol Chem* 2007;282(22):16202-16213.
50. Belsham GJ, Sonenberg N. Picornavirus RNA translation: roles for cellular proteins. *Trends Microbiol* 2000;8(7):330-335.
51. Belsham GJ, Sonenberg N. RNA-protein interactions in regulation of picornavirus RNA translation. *Microbiol Rev* 1996;60(3):499-511.
52. Cookson W. The immunogenetics of asthma and eczema: a new focus on the epithelium. *Nat Rev Immunol* 2004;4(12):978-988.
53. Bossios A, Psarras S, Gourgiotis D et al. Rhinovirus infection induces cytotoxicity and delays wound healing in bronchial epithelial cells. *Respir Res* 2005;6:114.
54. Oddera S, Silvestri M, Lantero S, Sacco O, Rossi GA. Downregulation of the expression of intercellular adhesion molecule (ICAM)-1 on bronchial epithelial cells by fenoterol, a beta2-adrenoceptor agonist. *J Asthma* 1998;35(5):401-408.
55. Wegner CD, Gundel RH, Reilly P, Haynes N, Letts LG, Rothlein R. Intercellular adhesion molecule-1 (ICAM-1) in the pathogenesis of asthma. *Science* 1990;247(4941):456-459.
56. Papi A, Papadopoulos NG, Stanciu LA et al. Reducing agents inhibit rhinovirus-induced up-regulation of the rhinovirus receptor intercellular adhesion molecule-1 (ICAM-1) in respiratory epithelial cells. *FASEB J* 2002;16(14):1934-1936.
57. Gern JE, Brockman-Schneider R, Bhattacharya S, Malter JS, Busse WW. Serum and low-density lipoprotein enhance interleukin-8 secretion by airway epithelial cells. *Am J Respir Cell Mol Biol* 2003;29(4):483-489.
58. Papadopoulos NG, Bates PJ, Bardin PG et al. Rhinoviruses infect the lower airways. *J Infect Dis* 2000;181(6):1875-1884.
59. Bossios A, Gourgiotis D, Skevaki CL et al. Rhinovirus infection and house dust mite exposure synergize in inducing bronchial epithelial cell interleukin-8 release. *Clin Exp Allergy* 2008;38(10):1615-1626.
60. Wark PA, Johnston SL, Bucchieri F et al. Asthmatic bronchial epithelial cells have a deficient innate immune response to infection with rhinovirus. *J Exp Med* 2005;201(6):937-947.
61. Contoli M, Message SD, Laza-Stanca V et al. Role of deficient type III interferon-lambda production in asthma exacerbations. *Nat Med* 2006;12(9):1023-1026.
62. Broug-Holub E, Toews GB, van Iwaarden JF et al. Alveolar macrophages are required for protective pulmonary defenses in murine *Klebsiella pneumoniae*: elimination of alveolar

- macrophages increases neutrophil recruitment but decreases bacterial clearance and survival. *Infect Immun* 1997;65(4):1139-1146.
63. Holt PG, Oliver J, Bilyk N et al. Downregulation of the antigen presenting cell function(s) of pulmonary dendritic cells in vivo by resident alveolar macrophages. *J Exp Med* 1993;177(2):397-407.
 64. Thepen T, McMenamin C, Oliver J, Kraal G, Holt PG. Regulation of immune response to inhaled antigen by alveolar macrophages: differential effects of in vivo alveolar macrophage elimination on the induction of tolerance vs. immunity. *Eur J Immunol* 1991;21(11):2845-2850.
 65. Tang C, Inman MD, van RN et al. Th type 1-stimulating activity of lung macrophages inhibits Th2-mediated allergic airway inflammation by an IFN-gamma-dependent mechanism. *J Immunol* 2001;166(3):1471-1481.
 66. Schmal H, Czermak BJ, Lentsch AB et al. Soluble ICAM-1 activates lung macrophages and enhances lung injury. *J Immunol* 1998;161(7):3685-3693.
 67. Gliemann J. Receptors of the low density lipoprotein (LDL) receptor family in man. Multiple functions of the large family members via interaction with complex ligands. *Biol Chem* 1998;379(8-9):951-964.
 68. Gern JE, Dick EC, Lee WM et al. Rhinovirus enters but does not replicate inside monocytes and airway macrophages. *J Immunol* 1996;156(2):621-627.
 69. Laza-Stanca V, Stanciu LA, Message SD, Edwards MR, Gern JE, Johnston SL. Rhinovirus replication in human macrophages induces NF-kappaB-dependent tumor necrosis factor alpha production. *J Virol* 2006;80(16):8248-8258.
 70. Hall DJ, Bates ME, Guar L, Cronan M, Korpi N, Bertics PJ. The role of p38 MAPK in rhinovirus-induced monocyte chemoattractant protein-1 production by monocytic-lineage cells. *J Immunol* 2005;174(12):8056-8063.
 71. Message SD, Laza-Stanca V, Mallia P et al. Rhinovirus-induced lower respiratory illness is increased in asthma and related to virus load and Th1/2 cytokine and IL-10 production. *Proc Natl Acad Sci U S A* 2008;105(36):13562-13567.
 72. Nagarkar DR, Bowman ER, Schneider D et al. Rhinovirus infection of allergen-sensitized and -challenged mice induces eotaxin release from functionally polarized macrophages. *J Immunol* 2010;185(4):2525-2535.
 73. Oliver BG, Lim S, Wark P et al. Rhinovirus exposure impairs immune responses to bacterial products in human alveolar macrophages. *Thorax* 2008;63(6):519-525.
 74. Plummeridge MJ, Armstrong L, Birchall MA, Millar AB. Reduced production of interleukin 12 by interferon gamma primed alveolar macrophages from atopic asthmatic subjects. *Thorax* 2000;55(10):842-847.
 75. Ho LP, Davis M, Denison A, Wood FT, Greening AP. Reduced interleukin-18 levels in BAL specimens from patients with asthma compared to patients with sarcoidosis and healthy control subjects. *Chest* 2002;121(5):1421-1426.

76. Laza-Stanca V, Message SD, Edwards MR et al. The role of IL-15 deficiency in the pathogenesis of virus-induced asthma exacerbations. *PLoS Pathog* 2011;7(7):e1002114.
77. Levandowski RA, Ou DW, Jackson GG. Acute-phase decrease of T lymphocyte subsets in rhinovirus infection. *J Infect Dis* 1986;153(4):743-748.
78. Papadopoulos NG, Stanciu LA, Papi A, Holgate ST, Johnston SL. A defective type 1 response to rhinovirus in atopic asthma. *Thorax* 2002;57(4):328-332.
79. Parry DE, Busse WW, Sukow KA, Dick CR, Swenson C, Gern JE. Rhinovirus-induced PBMC responses and outcome of experimental infection in allergic subjects. *J Allergy Clin Immunol* 2000;105(4):692-698.
80. Khaitov MR, Laza-Stanca V, Edwards MR et al. Respiratory virus induction of alpha-, beta- and lambda-interferons in bronchial epithelial cells and peripheral blood mononuclear cells. *Allergy* 2009;64(3):375-386.
81. Iikura K, Katsunuma T, Saika S et al. Peripheral blood mononuclear cells from patients with bronchial asthma show impaired innate immune responses to rhinovirus in vitro. *Int Arch Allergy Immunol* 2011;155 Suppl 1:27-33.
82. Gehlhar K, Bilitewski C, Reinitz-Rademacher K, Rohde G, Bufe A. Impaired virus-induced interferon-alpha2 release in adult asthmatic patients. *Clin Exp Allergy* 2006;36(3):331-337.
83. Biscione GL, Corne J, Chauhan AJ, Johnston SL. Increased frequency of detection of *Chlamydia pneumoniae* in asthma. *Eur Respir J* 2004;24(5):745-749.
84. Black PN, Scicchitano R, Jenkins CR et al. Serological evidence of infection with *Chlamydia pneumoniae* is related to the severity of asthma. *Eur Respir J* 2000;15(2):254-259.
85. Kocabas A, Avsar M, Hanta I, Koksai F, Kuleci S. *Chlamydia pneumoniae* infection in adult asthmatic patients. *J Asthma* 2008;45(1):39-43.
86. Cunningham AF, Johnston SL, Julious SA, Lampe FC, Ward ME. Chronic *Chlamydia pneumoniae* infection and asthma exacerbations in children. *Eur Respir J* 1998;11(2):345-349.
87. Wark PA, Johnston SL, Simpson JL, Hensley MJ, Gibson PG. *Chlamydia pneumoniae* immunoglobulin A reactivation and airway inflammation in acute asthma. *Eur Respir J* 2002;20(4):834-840.
88. Bisgaard H, Hermansen MN, Buchvald F et al. Childhood asthma after bacterial colonization of the airway in neonates. *N Engl J Med* 2007;357(15):1487-1495.
89. Hales BJ, Chai LY, Elliot CE et al. Antibacterial antibody responses associated with the development of asthma in house dust mite-sensitised and non-sensitised children. *Thorax* 2012;67(4):321-327.
90. Celedon JC, Fuhlbrigge A, Rifas-Shiman S, Weiss ST, Finkelstein JA. Antibiotic use in the first year of life and asthma in early childhood. *Clin Exp Allergy* 2004;34(7):1011-1016.
91. Friedman R, Ackerman M, Wald E, Casselbrant M, Friday G, Fireman P. Asthma and bacterial sinusitis in children. *J Allergy Clin Immunol* 1984;74(2):185-189.

92. Chen J, Deng Y, Zhao J et al. The polymorphism of IL-17 G-152A was associated with childhood asthma and bacterial colonization of the hypopharynx in bronchiolitis. *J Clin Immunol* 2010;30(4):539-545.
93. Jounio U, Juvonen R, Bloigu A et al. Pneumococcal carriage is more common in asthmatic than in non-asthmatic young men. *Clin Respir J* 2010;4(4):222-229.
94. Cardozo DM, Nascimento-Carvalho CM, Andrade AL et al. Prevalence and risk factors for nasopharyngeal carriage of *Streptococcus pneumoniae* among adolescents. *J Med Microbiol* 2008;57(Pt 2):185-189.
95. Zhang L, Prietsch SO, Mendes AP et al. Inhaled corticosteroids increase the risk of oropharyngeal colonization by *Streptococcus pneumoniae* in children with asthma. *Respirology* 2013;18(2):272-277.
96. Chodosh S. Acute bacterial exacerbations in bronchitis and asthma. *Am J Med* 1987;82(4A):154-163.
97. Schuller DE. Prophylaxis of otitis media in asthmatic children. *Pediatr Infect Dis* 1983;2(4):280-283.
98. Ansaldi F, Turello V, Lai P et al. Effectiveness of a 23-valent polysaccharide vaccine in preventing pneumonia and non-invasive pneumococcal infection in elderly people: a large-scale retrospective cohort study. *J Int Med Res* 2005;33(5):490-500.
99. Hales BJ, Martin AC, Pearce LJ et al. IgE and IgG anti-house dust mite specificities in allergic disease. *J Allergy Clin Immunol* 2006;118(2):361-367.
100. Hales BJ, Martin AC, Pearce LJ et al. Anti-bacterial IgE in the antibody responses of house dust mite allergic children convalescent from asthma exacerbation. *Clin Exp Allergy* 2009;39(8):1170-1178.
101. Preston JA, Thorburn AN, Starkey MR et al. *Streptococcus pneumoniae* infection suppresses allergic airways disease by inducing regulatory T-cells. *Eur Respir J* 2011;37(1):53-64.
102. Thorburn AN, Hansbro PM. Harnessing regulatory T cells to suppress asthma: from potential to therapy. *Am J Respir Cell Mol Biol* 2010;43(5):511-519.
103. Talbot TR, Hartert TV, Mitchel E et al. Asthma as a risk factor for invasive pneumococcal disease. *N Engl J Med* 2005;352(20):2082-2090.
104. Klemets P, Lyytikainen O, Ruutu P et al. Risk of invasive pneumococcal infections among working age adults with asthma. *Thorax* 2010;65(8):698-702.
105. Jung JA, Kita H, Dhillon R et al. Influence of asthma status on serotype-specific pneumococcal antibody levels. *Postgrad Med* 2010;122(5):116-124.
106. Jung JA, Kita H, Yawn BP et al. Increased risk of serious pneumococcal disease in patients with atopic conditions other than asthma. *J Allergy Clin Immunol* 2010;125(1):217-221.
107. Wang JH, Kwon HJ, Jang YJ. Rhinovirus enhances various bacterial adhesions to nasal epithelial cells simultaneously. *Laryngoscope* 2009;119(7):1406-1411.

108. Ishizuka S, Yamaya M, Suzuki T et al. Effects of rhinovirus infection on the adherence of *Streptococcus pneumoniae* to cultured human airway epithelial cells. *J Infect Dis* 2003;188(12):1928-1939.
109. Kawabata M, Kurono Y. Polyinosine-polycytidylic acid enhances cellular adherence of *Streptococcus pneumoniae*. *Laryngoscope* 2011;121(11):2443-2448.
110. Rijneveld AW, Weijer S, Florquin S et al. Improved host defense against pneumococcal pneumonia in platelet-activating factor receptor-deficient mice. *J Infect Dis* 2004;189(4):711-716.
111. Avadhanula V, Rodriguez CA, Devincenzo JP et al. Respiratory viruses augment the adhesion of bacterial pathogens to respiratory epithelium in a viral species- and cell type-dependent manner. *J Virol* 2006;80(4):1629-1636.
112. Peltola V, Heikkinen T, Ruuskanen O et al. Temporal association between rhinovirus circulation in the community and invasive pneumococcal disease in children. *Pediatr Infect Dis J* 2011;30(6):456-461.
113. Oliver BG, Lim S, Wark P et al. Rhinovirus exposure impairs immune responses to bacterial products in human alveolar macrophages. *Thorax* 2008;63(6):519-525.
114. Abeyta M, Hardy GG, Yother J. Genetic alteration of capsule type but not PspA type affects accessibility of surface-bound complement and surface antigens of *Streptococcus pneumoniae*. *Infect Immun* 2003;71(1):218-225.
115. Hyams C, Camberlein E, Cohen JM, Bax K, Brown JS. The *Streptococcus pneumoniae* capsule inhibits complement activity and neutrophil phagocytosis by multiple mechanisms. *Infect Immun* 2010;78(2):704-715.
116. Wartha F, Beiter K, Albiger B et al. Capsule and D-alanylated lipoteichoic acids protect *Streptococcus pneumoniae* against neutrophil extracellular traps. *Cell Microbiol* 2007;9(5):1162-1171.
117. Mitchell AM, Mitchell TJ. *Streptococcus pneumoniae*: virulence factors and variation. *Clin Microbiol Infect* 2010;16(5):411-418.
118. Gillespie SH, Balakrishnan I. Pathogenesis of pneumococcal infection. *J Med Microbiol* 2000;49(12):1057-1067.
119. Littmann M, Albiger B, Frentzen A, Normark S, Henriques-Normark B, Plant L. *Streptococcus pneumoniae* evades human dendritic cell surveillance by pneumolysin expression. *EMBO Mol Med* 2009;1(4):211-222.
120. Kadioglu A, Weiser JN, Paton JC, Andrew PW. The role of *Streptococcus pneumoniae* virulence factors in host respiratory colonization and disease. *Nat Rev Microbiol* 2008;6(4):288-301.
121. Paton JC, Rowan-Kelly B, Ferrante A. Activation of human complement by the pneumococcal toxin pneumolysin. *Infect Immun* 1984;43(3):1085-1087.

122. Riise GC, Larsson S, Andersson BA. Bacterial adhesion to oropharyngeal and bronchial epithelial cells in smokers with chronic bronchitis and in healthy nonsmokers. *Eur Respir J* 1994;7(10):1759-1764.
123. Adamou JE, Wizemann TM, Barren P, Langermann S. Adherence of *Streptococcus pneumoniae* to human bronchial epithelial cells (BEAS-2B). *Infect Immun* 1998;66(2):820-822.
124. Barthelson R, Mobasser A, Zopf D, Simon P. Adherence of *Streptococcus pneumoniae* to respiratory epithelial cells is inhibited by sialylated oligosaccharides. *Infect Immun* 1998;66(4):1439-1444.
125. van Alphen L, Jansen HM, Dankert J. Virulence factors in the colonization and persistence of bacteria in the airways. *Am J Respir Crit Care Med* 1995;151(6):2094-2099.
126. Sorrentino R, de Souza PM, Sriskandan S, Duffin C, Paul-Clark MJ, Mitchell JA. Pattern recognition receptors and interleukin-8 mediate effects of Gram-positive and Gram-negative bacteria on lung epithelial cell function. *Br J Pharmacol* 2008;154(4):864-871.
127. Shen H, Yoshida H, Yan F et al. Synergistic induction of MUC5AC mucin by nontypeable *Haemophilus influenzae* and *Streptococcus pneumoniae*. *Biochem Biophys Res Commun* 2008;365(4):795-800.
128. Hof DG, Repine JE, Peterson PK, Hoidal JR. Phagocytosis by human alveolar macrophages and neutrophils: qualitative differences in the opsonic requirements for uptake of *Staphylococcus aureus* and *Streptococcus pneumoniae* in vitro. *Am Rev Respir Dis* 1980;121(1):65-71.
129. Johnson JD, Hand WL, King NL, Hughes CG. Activation of alveolar macrophages after lower respiratory tract infection. *J Immunol* 1975;115(1):80-84.
130. Jonsson S, Musher DM, Chapman A, Goree A, Lawrence EC. Phagocytosis and killing of common bacterial pathogens of the lung by human alveolar macrophages. *J Infect Dis* 1985;152(1):4-13.
131. Gordon SB, Irving GR, Lawson RA, Lee ME, Read RC. Intracellular trafficking and killing of *Streptococcus pneumoniae* by human alveolar macrophages are influenced by opsonins. *Infect Immun* 2000;68(4):2286-2293.
132. Xu F, Droemann D, Rupp J et al. Modulation of the inflammatory response to *Streptococcus pneumoniae* in a model of acute lung tissue infection. *Am J Respir Cell Mol Biol* 2008;39(5):522-529.
133. Pittet LA, Quinton LJ, Yamamoto K et al. Earliest innate immune responses require macrophage RelA during pneumococcal pneumonia. *Am J Respir Cell Mol Biol* 2011;45(3):573-581.
134. Keller R, Fischer W, Keist R, Bassetti S. Macrophage response to bacteria: induction of marked secretory and cellular activities by lipoteichoic acids. *Infect Immun* 1992;60(9):3664-3672.

135. Fang R, Tsuchiya K, Kawamura I et al. Critical roles of ASC inflammasomes in caspase-1 activation and host innate resistance to *Streptococcus pneumoniae* infection. *J Immunol* 2011;187(9):4890-4899.
136. Houldsworth S, Andrew PW, Mitchell TJ. Pneumolysin stimulates production of tumor necrosis factor alpha and interleukin-1 beta by human mononuclear phagocytes. *Infect Immun* 1994;62(4):1501-1503.
137. Hachicha M, Rathanaswami P, Naccache PH, McColl SR. Regulation of chemokine gene expression in human peripheral blood neutrophils phagocytosing microbial pathogens. *J Immunol* 1998;160(1):449-454.
138. Arva E, Andersson B. Kinetics of cytokine release and expression of lymphocyte cell-surface activation markers after in vitro stimulation of human peripheral blood mononuclear cells with *Streptococcus pneumoniae*. *Scand J Immunol* 1999;49(3):237-243.
139. Winther B, Gwaltney JM, Hendley JO. Respiratory virus infection of monolayer cultures of human nasal epithelial cells. *Am Rev Respir Dis* 1990;141(4 Pt 1):839-845.
140. Gern JE, Vrtis R, Grindle KA, Swenson C, Busse WW. Relationship of upper and lower airway cytokines to outcome of experimental rhinovirus infection. *Am J Respir Crit Care Med* 2000;162(6):2226-2231.
141. Papadopoulos NG, Papi A, Meyer J et al. Rhinovirus infection up-regulates eotaxin and eotaxin-2 expression in bronchial epithelial cells. *Clin Exp Allergy* 2001;31(7):1060-1066.
142. Schroth MK, Grimm E, Frindt P et al. Rhinovirus replication causes RANTES production in primary bronchial epithelial cells. *Am J Respir Cell Mol Biol* 1999;20(6):1220-1228.
143. Shaw DE, Berry MA, Hargadon B et al. Association between neutrophilic airway inflammation and airflow limitation in adults with asthma. *Chest* 2007;132(6):1871-1875.
144. Jayaram L, Pizzichini MM, Cook RJ et al. Determining asthma treatment by monitoring sputum cell counts: effect on exacerbations. *Eur Respir J* 2006;27(3):483-494.
145. Heinrich PC, Behrmann I, Haan S, Hermanns HM, Muller-Newen G, Schaper F. Principles of interleukin (IL)-6-type cytokine signalling and its regulation. *Biochem J* 2003;374(Pt 1):1-20.
146. Rose-John S, Waetzig GH, Scheller J, Grotzinger J, Seegert D. The IL-6/sIL-6R complex as a novel target for therapeutic approaches. *Expert Opin Ther Targets* 2007;11(5):613-624.
147. Gabay C, Kushner I. Acute-phase proteins and other systemic responses to inflammation. *N Engl J Med* 1999;340(6):448-454.
148. Zhu Z, Tang W, Ray A et al. Rhinovirus stimulation of interleukin-6 in vivo and in vitro. Evidence for nuclear factor kappa B-dependent transcriptional activation. *J Clin Invest* 1996;97(2):421-430.
149. Grunberg K, Smits HH, Timmers MC et al. Experimental rhinovirus 16 infection. Effects on cell differentials and soluble markers in sputum in asthmatic subjects. *Am J Respir Crit Care Med* 1997;156(2 Pt 1):609-616.

150. Fleming HE, Little FF, Schnurr D et al. Rhinovirus-16 colds in healthy and in asthmatic subjects: similar changes in upper and lower airways. *Am J Respir Crit Care Med* 1999;160(1):100-108.
151. Kicic A, Sutanto EN, Stevens PT, Knight DA, Stick SM. Intrinsic biochemical and functional differences in bronchial epithelial cells of children with asthma. *Am J Respir Crit Care Med* 2006;174(10):1110-1118.
152. Kotloff RM, Little J, Elias JA. Human alveolar macrophage and blood monocyte interleukin-6 production. *Am J Respir Cell Mol Biol* 1990;3(5):497-505.
153. Wark PA, Bucchieri F, Johnston SL et al. IFN-gamma-induced protein 10 is a novel biomarker of rhinovirus-induced asthma exacerbations. *J Allergy Clin Immunol* 2007;120(3):586-593.
154. Zhu Z, Tang W, Gwaltney JM, Jr., Wu Y, Elias JA. Rhinovirus stimulation of interleukin-8 in vivo and in vitro: role of NF-kappaB. *Am J Physiol* 1997;273(4 Pt 1):L814-L824.
155. Bentley JK, Newcomb DC, Goldsmith AM, Jia Y, Sajjan US, Hershenson MB. Rhinovirus activates interleukin-8 expression via a Src/p110beta phosphatidylinositol 3-kinase/Akt pathway in human airway epithelial cells. *J Virol* 2007;81(3):1186-1194.
156. Johnston SL, Papi A, Monick MM, Hunninghake GW. Rhinoviruses induce interleukin-8 mRNA and protein production in human monocytes. *J Infect Dis* 1997;175(2):323-329.
157. DeMore JP, Weisshaar EH, Vrtis RF et al. Similar colds in subjects with allergic asthma and nonatopic subjects after inoculation with rhinovirus-16. *J Allergy Clin Immunol* 2009;124(2):245-52, 252.
158. Morris GE, Whyte MK, Martin GF, Jose PJ, Dower SK, Sabroe I. Agonists of toll-like receptors 2 and 4 activate airway smooth muscle via mononuclear leukocytes. *Am J Respir Crit Care Med* 2005;171(8):814-822.
159. Proud D, Gwaltney JM, Jr., Hendley JO, Dinarello CA, Gillis S, Schleimer RP. Increased levels of interleukin-1 are detected in nasal secretions of volunteers during experimental rhinovirus colds. *J Infect Dis* 1994;169(5):1007-1013.
160. de Kluijver J, Grunberg K, Pons D et al. Interleukin-1beta and interleukin-1ra levels in nasal lavages during experimental rhinovirus infection in asthmatic and non-asthmatic subjects. *Clin Exp Allergy* 2003;33(10):1415-1418.
161. Terajima M, Yamaya M, Sekizawa K et al. Rhinovirus infection of primary cultures of human tracheal epithelium: role of ICAM-1 and IL-1beta. *Am J Physiol* 1997;273(4 Pt 1):L749-L759.
162. Stokes CA, Ismail S, Dick EP et al. Role of interleukin-1 and MyD88-dependent signaling in rhinovirus infection. *J Virol* 2011;85(15):7912-7921.
163. Mitsuta K, Matsuse H, Fukushima C et al. Production of TNF-alpha by peripheral blood mononuclear cells through activation of nuclear factor kappa B by specific allergen stimulation in patients with atopic asthma. *Allergy Asthma Proc* 2003;24(1):19-26.
164. Uhl EW, Clarke TJ, Hogan RJ. Differential expression of nuclear factor-kappaB mediates increased pulmonary expression of tumor necrosis factor-alpha and virus-induced asthma. *Viral Immunol* 2009;22(2):79-89.

165. Tosi MF, Stark JM, Smith CW, Hamedani A, Gruenert DC, Infeld MD. Induction of ICAM-1 expression on human airway epithelial cells by inflammatory cytokines: effects on neutrophil-epithelial cell adhesion. *Am J Respir Cell Mol Biol* 1992;7(2):214-221.
166. Cromwell O, Hamid Q, Corrigan CJ et al. Expression and generation of interleukin-8, IL-6 and granulocyte-macrophage colony-stimulating factor by bronchial epithelial cells and enhancement by IL-1 beta and tumour necrosis factor-alpha. *Immunology* 1992;77(3):330-337.
167. Newcomb DC, Sajjan US, Nagarkar DR, Goldsmith AM, Bentley JK, Hershenson MB. Cooperative effects of rhinovirus and TNF- α on airway epithelial cell chemokine expression. *Am J Physiol Lung Cell Mol Physiol* 2007;293(4):L1021-L1028.
168. Gosset P, Tscopoulos A, Wallaert B et al. Increased secretion of tumor necrosis factor alpha and interleukin-6 by alveolar macrophages consecutive to the development of the late asthmatic reaction. *J Allergy Clin Immunol* 1991;88(4):561-571.
169. Thomas PS. Tumour necrosis factor-alpha: the role of this multifunctional cytokine in asthma. *Immunol Cell Biol* 2001;79(2):132-140.
170. Cazzola M, Polosa R. Anti-TNF-alpha and Th1 cytokine-directed therapies for the treatment of asthma. *Curr Opin Allergy Clin Immunol* 2006;6(1):43-50.
171. Gosset P, Tscopoulos A, Wallaert B, Joseph M, Capron A, Tonnel AB. Tumor necrosis factor alpha and interleukin-6 production by human mononuclear phagocytes from allergic asthmatics after IgE-dependent stimulation. *Am Rev Respir Dis* 1992;146(3):768-774.
172. Spurrell JC, Wiehler S, Zaheer RS, Sanders SP, Proud D. Human airway epithelial cells produce IP-10 (CXCL10) in vitro and in vivo upon rhinovirus infection. *Am J Physiol Lung Cell Mol Physiol* 2005;289(1):L85-L95.
173. Donninger H, Glashoff R, Haitchi HM et al. Rhinovirus induction of the CXC chemokine epithelial-neutrophil activating peptide-78 in bronchial epithelium. *J Infect Dis* 2003;187(11):1809-1817.
174. Tsuchiya K, Toyama K, Tsuprun V et al. Pneumococcal peptidoglycan-polysaccharides induce the expression of interleukin-8 in airway epithelial cells by way of nuclear factor-kappaB, nuclear factor interleukin-6, or activation protein-1 dependent mechanisms. *Laryngoscope* 2007;117(1):86-91.
175. Malley R, Henneke P, Morse SC et al. Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection. *Proc Natl Acad Sci U S A* 2003;100(4):1966-1971.
176. Dogan S, Zhang Q, Pridmore AC, Mitchell TJ, Finn A, Murdoch C. Pneumolysin-induced CXCL8 production by nasopharyngeal epithelial cells is dependent on calcium flux and MAPK activation via Toll-like receptor 4. *Microbes Infect* 2011;13(1):65-75.
177. Yoo IH, Shin HS, Kim YJ, Kim HB, Jin S, Ha UH. Role of pneumococcal pneumolysin in the induction of an inflammatory response in human epithelial cells. *FEMS Immunol Med Microbiol* 2010;60(1):28-35.

178. Cao J, Gong Y, Cai B et al. Modulation of human bronchial epithelial cells by pneumococcal choline binding protein A. *Hum Immunol* 2011;72(1):37-46.
179. Marriott HM, Gascoyne KA, Gowda R et al. Interleukin-1beta regulates CXCL8 release and influences disease outcome in response to *Streptococcus pneumoniae*, defining intercellular cooperation between pulmonary epithelial cells and macrophages. *Infect Immun* 2012;80(3):1140-1149.
180. Muller-Alouf H, Alouf JE, Gerlach D, Ozegowski JH, Fitting C, Cavaillon JM. Comparative study of cytokine release by human peripheral blood mononuclear cells stimulated with *Streptococcus pyogenes* superantigenic erythrogenic toxins, heat-killed streptococci, and lipopolysaccharide. *Infect Immun* 1994;62(11):4915-4921.
181. Riesenfeld-Orn I, Wolpe S, Garcia-Bustos JF, Hoffmann MK, Tuomanen E. Production of interleukin-1 but not tumor necrosis factor by human monocytes stimulated with pneumococcal cell surface components. *Infect Immun* 1989;57(7):1890-1893.
182. Takashima K, Tateda K, Matsumoto T, Iizawa Y, Nakao M, Yamaguchi K. Role of tumor necrosis factor alpha in pathogenesis of pneumococcal pneumonia in mice. *Infect Immun* 1997;65(1):257-260.
183. Kerr AR, Irvine JJ, Search JJ et al. Role of inflammatory mediators in resistance and susceptibility to pneumococcal infection. *Infect Immun* 2002;70(3):1547-1557.
184. Isaacs A, Lindenmann J. Virus interference. I. The interferon. *Proc R Soc Lond B Biol Sci* 1957;147(927):258-267.
185. Fensterl V, Sen GC. Interferons and viral infections. *Biofactors* 2009;35(1):14-20.
186. Sheppard P, Kindsvogel W, Xu W et al. IL-28, IL-29 and their class II cytokine receptor IL-28R. *Nat Immunol* 2003;4(1):63-68.
187. Takaoka A, Yanai H. Interferon signalling network in innate defence. *Cell Microbiol* 2006;8(6):907-922.
188. Roberts RM, Liu L, Guo Q, Leaman D, Bixby J. The evolution of the type I interferons. *J Interferon Cytokine Res* 1998;18(10):805-816.
189. Pestka S, Krause CD, Walter MR. Interferons, interferon-like cytokines, and their receptors. *Immunol Rev* 2004;202:8-32.
190. Trent JM, Olson S, Lawn RM. Chromosomal localization of human leukocyte, fibroblast, and immune interferon genes by means of in situ hybridization. *Proc Natl Acad Sci U S A* 1982;79(24):7809-7813.
191. Kim SH, Cohen B, Novick D, Rubinstein M. Mammalian type I interferon receptors consists of two subunits: IFNAR1 and IFNAR2. *Gene* 1997;196(1-2):279-286.
192. Uze G, Schreiber G, Piehler J, Pellegrini S. The receptor of the type I interferon family. *Curr Top Microbiol Immunol* 2007;316:71-95.
193. Novick D, Cohen B, Rubinstein M. The human interferon alpha/beta receptor: characterization and molecular cloning. *Cell* 1994;77(3):391-400.

194. Muller U, Steinhoff U, Reis LF et al. Functional role of type I and type II interferons in antiviral defense. *Science* 1994;264(5167):1918-1921.
195. Mertens C, Zhong M, Krishnaraj R, Zou W, Chen X, Darnell JE, Jr. Dephosphorylation of phosphotyrosine on STAT1 dimers requires extensive spatial reorientation of the monomers facilitated by the N-terminal domain. *Genes Dev* 2006;20(24):3372-3381.
196. Stark GR, Kerr I.M., Williams B.R., Silverman R.H., Schreiber R.D. How cells respond to interferons. *Annu Rev Biochem* 1998;67:227-264.
197. Schindler C, Levy DE, Decker T. JAK-STAT signaling: from interferons to cytokines. *J Biol Chem* 2007;282(28):20059-20063.
198. Kolenko SV, Gallagher G, Baurin VV et al. IFN-lambdas mediate antiviral protection through a distinct class II cytokine receptor complex. *Nat Immunol* 2003;4(1):69-77.
199. Kolenko SV. IFN-lambdas. *Curr Opin Immunol* 2011;23(5):583-590.
200. Pott J, Mahlakoiv T, Mordstein M et al. IFN-lambda determines the intestinal epithelial antiviral host defense. *Proc Natl Acad Sci U S A* 2011;108(19):7944-7949.
201. Wark PA, Grissell T, Davies B, See H, Gibson PG. Diversity in the bronchial epithelial cell response to infection with different rhinovirus strains. *Respirology* 2009;14(2):180-186.
202. Cakebread JA, Xu Y, Grainge C et al. Exogenous IFN-beta has antiviral and anti-inflammatory properties in primary bronchial epithelial cells from asthmatic subjects exposed to rhinovirus. *J Allergy Clin Immunol* 2011;127(5):1148-1154.
203. Lopez-Souza N, Favoreto S, Wong H et al. In vitro susceptibility to rhinovirus infection is greater for bronchial than for nasal airway epithelial cells in human subjects. *J Allergy Clin Immunol* 2009;123(6):1384-1390.
204. Bochkov YA, Hanson KM, Keles S, Brockman-Schneider RA, Jarjour NN, Gern JE. Rhinovirus-induced modulation of gene expression in bronchial epithelial cells from subjects with asthma. *Mucosal Immunol* 2010;3(1):69-80.
205. Gill MA, Bajwa G, George TA et al. Counterregulation between the FcepsilonRI pathway and antiviral responses in human plasmacytoid dendritic cells. *J Immunol* 2010;184(11):5999-6006.
206. Bufe A, Gehlhar K, Grage-Griebenow E, Ernst M. Atopic phenotype in children is associated with decreased virus-induced interferon-alpha release. *Int Arch Allergy Immunol* 2002;127(1):82-88.
207. Edwards MR, Regamey N, Vareille M et al. Impaired innate interferon induction in severe therapy resistant atopic asthmatic children. *Mucosal Immunol* 2012.
208. Sykes A, Edwards MR, Macintyre J et al. Rhinovirus 16-induced IFN-alpha and IFN-beta are deficient in bronchoalveolar lavage cells in asthmatic patients. *J Allergy Clin Immunol* 2012;129(6):1506-1514.
209. Baraldo S, Contoli M, Bazzan E et al. Deficient antiviral immune responses in childhood: distinct roles of atopy and asthma. *J Allergy Clin Immunol* 2012;130(6):1307-1314.

210. Rodel J, Assefa S, Prochnau D et al. Interferon-beta induction by *Chlamydia pneumoniae* in human smooth muscle cells. *FEMS Immunol Med Microbiol* 2001;32(1):9-15.
211. Trumstedt C, Eriksson E, Lundberg AM et al. Role of IRAK4 and IRF3 in the control of intracellular infection with *Chlamydia pneumoniae*. *J Leukoc Biol* 2007;81(6):1591-1598.
212. Rothfuchs AG, Trumstedt C, Mattei F et al. STAT1 regulates IFN-alpha beta- and IFN-gamma-dependent control of infection with *Chlamydia pneumoniae* by nonhemopoietic cells. *J Immunol* 2006;176(11):6982-6990.
213. O'Connell RM, Vaidya SA, Perry AK, Saha SK, Dempsey PW, Cheng G. Immune activation of type I IFNs by *Listeria monocytogenes* occurs independently of TLR4, TLR2, and receptor interacting protein 2 but involves TNFR-associated NF kappa B kinase-binding kinase 1. *J Immunol* 2005;174(3):1602-1607.
214. Radtke AL, Delbridge LM, Balachandran S, Barber GN, O'Riordan MX. TBK1 protects vacuolar integrity during intracellular bacterial infection. *PLoS Pathog* 2007;3(3):e29.
215. Stockinger S, Reutterer B, Schaljo B et al. IFN regulatory factor 3-dependent induction of type I IFNs by intracellular bacteria is mediated by a TLR- and Nod2-independent mechanism. *J Immunol* 2004;173(12):7416-7425.
216. Charrel-Dennis M, Latz E, Halmen KA et al. TLR-independent type I interferon induction in response to an extracellular bacterial pathogen via intracellular recognition of its DNA. *Cell Host Microbe* 2008;4(6):543-554.
217. Xiao N, Eidenschenk C, Krebs P et al. The Tpl2 mutation Sluggish impairs type I IFN production and increases susceptibility to group B streptococcal disease. *J Immunol* 2009;183(12):7975-7983.
218. Gratz N, Hartweger H, Matt U et al. Type I interferon production induced by *Streptococcus pyogenes*-derived nucleic acids is required for host protection. *PLoS Pathog* 2011;7(5):e1001345.
219. Gratz N, Siller M, Schaljo B et al. Group A streptococcus activates type I interferon production and MyD88-dependent signaling without involvement of TLR2, TLR4, and TLR9. *J Biol Chem* 2008;283(29):19879-19887.
220. Miettinen M, Veckman V, Latvala S, Sareneva T, Matikainen S, Julkunen I. Live *Lactobacillus rhamnosus* and *Streptococcus pyogenes* differentially regulate Toll-like receptor (TLR) gene expression in human primary macrophages. *J Leukoc Biol* 2008;84(4):1092-1100.
221. Joyce EA, Popper SJ, Falkow S. *Streptococcus pneumoniae* nasopharyngeal colonization induces type I interferons and interferon-induced gene expression. *BMC Genomics* 2009;10:404.
222. Nakamura S, Davis KM, Weiser JN. Synergistic stimulation of type I interferons during influenza virus coinfection promotes *Streptococcus pneumoniae* colonization in mice. *J Clin Invest* 2011;121(9):3657-3665.
223. Parker D, Martin FJ, Soong G et al. *Streptococcus pneumoniae* DNA initiates type I interferon signaling in the respiratory tract. *MBio* 2011;2(3):e00016-11.

224. Koppe U, Hogner K, Doebe JM et al. Streptococcus pneumoniae Stimulates a STING- and IFN regulatory factor 3-dependent type I IFN production in macrophages, which regulates RANTES production in macrophages, cocultured alveolar epithelial cells, and mouse lungs. *J Immunol* 2011;188(2):811-817.
225. Shahangian A, Chow EK, Tian X et al. Type I IFNs mediate development of postinfluenza bacterial pneumonia in mice. *J Clin Invest* 2009;119(7):1910-1920.
226. Dietrich N, Rohde M, Geffers R et al. Mast cells elicit proinflammatory but not type I interferon responses upon activation of TLRs by bacteria. *Proc Natl Acad Sci U S A* 2010;107(19):8748-8753.
227. Custovic A, Simpson A, Bardin PG, Le SP. Allergy is an important factor in asthma exacerbation: a pro/con debate. *Respirology* 2010;15(7):1021-1027.
228. Simpson A, Soderstrom L, Ahlstedt S, Murray CS, Woodcock A, Custovic A. IgE antibody quantification and the probability of wheeze in preschool children. *J Allergy Clin Immunol* 2005;116(4):744-749.
229. Sporik R, Holgate ST, Platts-Mills TA, Cogswell JJ. Exposure to house-dust mite allergen (Der p1) and the development of asthma in childhood. A prospective study. *N Engl J Med* 1990;323(8):502-507.
230. Peat JK, Salome CM, Woolcock AJ. Longitudinal changes in atopy during a 4-year period: relation to bronchial hyperresponsiveness and respiratory symptoms in a population sample of Australian schoolchildren. *J Allergy Clin Immunol* 1990;85(1 Pt 1):65-74.
231. Rosas I, McCartney HA, Payne RW et al. Analysis of the relationships between environmental factors (aeroallergens, air pollution, and weather) and asthma emergency admissions to a hospital in Mexico City. *Allergy* 1998;53(4):394-401.
232. Sporik R, Platts-Mills TA, Cogswell JJ. Exposure to house dust mite allergen of children admitted to hospital with asthma. *Clin Exp Allergy* 1993;23(9):740-746.
233. Dolen JG, Mathur A. Undetectable interferon-alpha serum levels in a patient with atopic dermatitis. *J Interferon Cytokine Res* 1995;15(11):973-975.
234. Kimata H, Akiyama Y, Kubota M, Furusho K. Interferon-alpha treatment for severe atopic dermatitis. *Allergy* 1995;50(10):837-840.
235. Chan A, Newman DL, Shon AM, Schneider DH, Kuldane S, Ober C. Variation in the type I interferon gene cluster on 9p21 influences susceptibility to asthma and atopy. *Genes Immun* 2006;7(2):169-178.
236. Larche M, Robinson DS, Kay AB. The role of T lymphocytes in the pathogenesis of asthma. *J Allergy Clin Immunol* 2003;111(3):450-463.
237. Kaplan MH, Wurster AL, Smiley ST, Grusby MJ. Stat6-dependent and -independent pathways for IL-4 production. *J Immunol* 1999;163(12):6536-6540.
238. Kurata H, Lee HJ, O'Garra A, Arai N. Ectopic expression of activated Stat6 induces the expression of Th2-specific cytokines and transcription factors in developing Th1 cells. *Immunity* 1999;11(6):677-688.

239. Pai SY, Truitt ML, Ho IC. GATA-3 deficiency abrogates the development and maintenance of T helper type 2 cells. *Proc Natl Acad Sci U S A* 2004;101(7):1993-1998.
240. Huber JP, Ramos HJ, Gill MA, Farrar JD. Cutting edge: Type I IFN reverses human Th2 commitment and stability by suppressing GATA3. *J Immunol* 2010;185(2):813-817.
241. Huber JP, Farrar JD. Regulation of effector and memory T-cell functions by type I interferon. *Immunology* 2011;132(4):466-474.
242. Sriram U, Biswas C, Behrens EM et al. IL-4 suppresses dendritic cell response to type I interferons. *J Immunol* 2007;179(10):6446-6455.
243. Tversky JR, Le TV, Bieneman AP, Chichester KL, Hamilton RG, Schroeder JT. Human blood dendritic cells from allergic subjects have impaired capacity to produce interferon-alpha via Toll-like receptor 9. *Clin Exp Allergy* 2008;38(5):781-788.
244. Pauwels R, Verschraegen G, van der Straeten M. IgE antibodies to bacteria in patients with bronchial asthma. *Allergy* 1980;35(8):665-669.
245. Tee RD, Pepys J. Specific serum IgE antibodies to bacterial antigens in allergic lung disease. *Clin Allergy* 1982;12(5):439-450.
246. Hollams EM, Hales BJ, Bachert C et al. Th2-associated immunity to bacteria in teenagers and susceptibility to asthma. *Eur Respir J* 2010;36(3):509-516.
247. Meylan E, Tschopp J, Karin M. Intracellular pattern recognition receptors in the host response. *Nature* 2006;442(7098):39-44.
248. Meylan E, Tschopp J. Toll-like receptors and RNA helicases: two parallel ways to trigger antiviral responses. *Mol Cell* 2006;22(5):561-569.
249. Thompson AJ, Locarnini SA. Toll-like receptors, RIG-I-like RNA helicases and the antiviral innate immune response. *Immunol Cell Biol* 2007;85(6):435-445.
250. Kumagai Y, Akira S. Identification and functions of pattern-recognition receptors. *J Allergy Clin Immunol* 2010;125(5):985-992.
251. Kawai T, Akira S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity* 2011;34(5):637-650.
252. Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol* 2010;11(5):373-384.
253. Kumar H, Kawai T, Akira S. Pathogen recognition in the innate immune response. *Biochem J* 2009;420(1):1-16.
254. Iwamura C, Nakayama T. Toll-like receptors in the respiratory system: their roles in inflammation. *Curr Allergy Asthma Rep* 2008;8(1):7-13.
255. Govindaraj RG, Manavalan B, Lee G, Choi S. Molecular modeling-based evaluation of hTLR10 and identification of potential ligands in Toll-like receptor signaling. *PLoS One* 2010;5(9):e12713.

256. Jin MS, Kim SE, Heo JY et al. Crystal structure of the TLR1-TLR2 heterodimer induced by binding of a tri-acylated lipopeptide. *Cell* 2007;130(6):1071-1082.
257. Dietrich N, Lienenklaus S, Weiss S, Gekara NO. Murine toll-like receptor 2 activation induces type I interferon responses from endolysosomal compartments. *PLoS One* 2010;5(4):e10250.
258. Liljeroos M, Vuolteenaho R, Rounioja S, Henriques-Normark B, Hallman M, Ojaniemi M. Bacterial ligand of TLR2 signals Stat activation via induction of IRF1/2 and interferon-alpha production. *Cell Signal* 2008;20(10):1873-1881.
259. Barbalat R, Lau L, Locksley RM, Barton GM. Toll-like receptor 2 on inflammatory monocytes induces type I interferon in response to viral but not bacterial ligands. *Nat Immunol* 2009;10(11):1200-1207.
260. Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* 2001;413(6857):732-738.
261. Zhang SY, Jouanguy E, Ugolini S et al. TLR3 deficiency in patients with herpes simplex encephalitis. *Science* 2007;317(5844):1522-1527.
262. Oshiumi H, Okamoto M, Fujii K et al. The TLR3/TICAM-1 pathway is mandatory for innate immune responses to poliovirus infection. *J Immunol* 2011;187(10):5320-5327.
263. Matsukura S, Kokubu F, Kurokawa M et al. Synthetic double-stranded RNA induces multiple genes related to inflammation through Toll-like receptor 3 depending on NF-kappaB and/or IRF-3 in airway epithelial cells. *Clin Exp Allergy* 2006;36(8):1049-1062.
264. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. *Cell* 2006;124(4):783-801.
265. Akashi-Takamura S, Miyake K. TLR accessory molecules. *Curr Opin Immunol* 2008;20(4):420-425.
266. Means TK, Hayashi F, Smith KD, Aderem A, Luster AD. The Toll-like receptor 5 stimulus bacterial flagellin induces maturation and chemokine production in human dendritic cells. *J Immunol* 2003;170(10):5165-5175.
267. Hayashi F, Smith KD, Ozinsky A et al. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature* 2001;410(6832):1099-1103.
268. Ha H, Lee JH, Kim HN et al. Stimulation by TLR5 modulates osteoclast differentiation through STAT1/IFN-beta. *J Immunol* 2008;180(3):1382-1389.
269. Kim YM, Brinkmann MM, Paquet ME, Ploegh HL. UNC93B1 delivers nucleotide-sensing toll-like receptors to endolysosomes. *Nature* 2008;452(7184):234-238.
270. Albiger B, Dahlberg S, Sandgren A et al. Toll-like receptor 9 acts at an early stage in host defence against pneumococcal infection. *Cell Microbiol* 2007;9(3):633-644.
271. Forsbach A, Samulowitz U, Volp K et al. Dual or Triple Activation of TLR7, TLR8, and/or TLR9 by Single-Stranded Oligoribonucleotides. *Nucleic Acid Ther* 2011;21(6):423-436.
272. Barber GN. Cytoplasmic DNA innate immune pathways. *Immunol Rev* 2011;243(1):99-108.

273. Yu HR, Huang HC, Kuo HC et al. IFN- α production by human mononuclear cells infected with varicella-zoster virus through TLR9-dependent and -independent pathways. *Cell Mol Immunol* 2011;8(2):181-188.
274. Uematsu S, Akira S. Toll-like receptors and Type I interferons. *J Biol Chem* 2007;282(21):15319-15323.
275. O'Neill LA, Bowie AG. The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. *Nat Rev Immunol* 2007;7(5):353-364.
276. von Bernuth H, Picard C, Jin Z et al. Pyogenic bacterial infections in humans with MyD88 deficiency. *Science* 2008;321(5889):691-696.
277. Yamamoto M, Sato S, Hemmi H et al. Essential role for TIRAP in activation of the signalling cascade shared by TLR2 and TLR4. *Nature* 2002;420(6913):324-329.
278. Karin M, Greten FR. NF- κ B: linking inflammation and immunity to cancer development and progression. *Nat Rev Immunol* 2005;5(10):749-759.
279. Hess J, Angel P, Schorpp-Kistner M. AP-1 subunits: quarrel and harmony among siblings. *J Cell Sci* 2004;117(Pt 25):5965-5973.
280. Honda K, Taniguchi T. IRFs: master regulators of signalling by Toll-like receptors and cytosolic pattern-recognition receptors. *Nat Rev Immunol* 2006;6(9):644-658.
281. Takaoka A, Yanai H, Kondo S et al. Integral role of IRF-5 in the gene induction programme activated by Toll-like receptors. *Nature* 2005;434(7030):243-249.
282. Sakaguchi S, Negishi H, Asagiri M et al. Essential role of IRF-3 in lipopolysaccharide-induced interferon- β gene expression and endotoxin shock. *Biochem Biophys Res Commun* 2003;306(4):860-866.
283. Douagi I, McInerney GM, Hidmark AS et al. Role of interferon regulatory factor 3 in type I interferon responses in rotavirus-infected dendritic cells and fibroblasts. *J Virol* 2007;81(6):2758-2768.
284. Yamamoto M, Sato S, Hemmi H et al. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science* 2003;301(5633):640-643.
285. Fitzgerald KA, Rowe DC, Barnes BJ et al. LPS-TLR4 signaling to IRF-3/7 and NF- κ B involves the toll adapters TRAM and TRIF. *J Exp Med* 2003;198(7):1043-1055.
286. Oganessian G, Saha SK, Guo B et al. Critical role of TRAF3 in the Toll-like receptor-dependent and -independent antiviral response. *Nature* 2006;439(7073):208-211.
287. Schmeck B, Huber S, Moog K et al. Pneumococci induced TLR- and Rac1-dependent NF- κ B-recruitment to the IL-8 promoter in lung epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 2006;290(4):L730-L737.
288. Schroder NW, Morath S, Alexander C et al. Lipoteichoic acid (LTA) of *Streptococcus pneumoniae* and *Staphylococcus aureus* activates immune cells via Toll-like receptor (TLR)-2, lipopolysaccharide-binding protein (LBP), and CD14, whereas TLR-4 and MD-2 are not involved. *J Biol Chem* 2003;278(18):15587-15594.

289. Letiembre M, Echchannaoui H, Bachmann P et al. Toll-like receptor 2 deficiency delays pneumococcal phagocytosis and impairs oxidative killing by granulocytes. *Infect Immun* 2005;73(12):8397-8401.
290. Han SH, Kim JH, Martin M, Michalek SM, Nahm MH. Pneumococcal lipoteichoic acid (LTA) is not as potent as staphylococcal LTA in stimulating Toll-like receptor 2. *Infect Immun* 2003;71(10):5541-5548.
291. Mogensen TH, Paludan SR, Kilian M, Ostergaard L. Live *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Neisseria meningitidis* activate the inflammatory response through Toll-like receptors 2, 4, and 9 in species-specific patterns. *J Leukoc Biol* 2006;80(2):267-277.
292. Santos-Sierra S, Golenbock DT, Henneke P. Toll-like receptor-dependent discrimination of streptococci. *J Endotoxin Res* 2006;12(5):307-312.
293. Branger J, Knapp S, Weijer S et al. Role of Toll-like receptor 4 in gram-positive and gram-negative pneumonia in mice. *Infect Immun* 2004;72(2):788-794.
294. Albiger B, Sandgren A, Katsuragi H et al. Myeloid differentiation factor 88-dependent signalling controls bacterial growth during colonization and systemic pneumococcal disease in mice. *Cell Microbiol* 2005;7(11):1603-1615.
295. Ratner AJ, Aguilar JL, Shchepetov M, Lysenko ES, Weiser JN. Nod1 mediates cytoplasmic sensing of combinations of extracellular bacteria. *Cell Microbiol* 2007;9(5):1343-1351.
296. Opitz B, Puschel A, Schmeck B et al. Nucleotide-binding oligomerization domain proteins are innate immune receptors for internalized *Streptococcus pneumoniae*. *J Biol Chem* 2004;279(35):36426-36432.
297. Davis KM, Nakamura S, Weiser JN. Nod2 sensing of lysozyme-digested peptidoglycan promotes macrophage recruitment and clearance of *S. pneumoniae* colonization in mice. *J Clin Invest* 2011;121(9):3666-3676.
298. Schroder K, Tschopp J. The inflammasomes. *Cell* 2010;140(6):821-832.
299. Harder J, Franchi L, Munoz-Planillo R, Park JH, Reimer T, Nunez G. Activation of the Nlrp3 inflammasome by *Streptococcus pyogenes* requires streptolysin O and NF-kappa B activation but proceeds independently of TLR signaling and P2X7 receptor. *J Immunol* 2009;183(9):5823-5829.
300. Witzenrath M, Pache F, Lorenz D et al. The NLRP3 inflammasome is differentially activated by pneumolysin variants and contributes to host defense in pneumococcal pneumonia. *J Immunol* 2011;187(1):434-440.
301. Guidelines for the measurement of respiratory function. Recommendations of the British Thoracic Society and the Association of Respiratory Technicians and Physiologists. *Respir Med* 1994;88(3):165-194.
302. Sterk PJ, Fabbri LM, Quanjer PH et al. Airway responsiveness. Standardized challenge testing with pharmacological, physical and sensitizing stimuli in adults. Report Working Party Standardization of Lung Function Tests, European Community for Steel and Coal. Official Statement of the European Respiratory Society. *Eur Respir J Suppl* 1993;16:53-83.

303. Juniper EF, O'Byrne PM, Guyatt GH, Ferrie PJ, King DR. Development and validation of a questionnaire to measure asthma control. *Eur Respir J* 1999;14(4):902-907.
304. van den Nieuwenhof L, Schermer T, Eysink P et al. Can the Asthma Control Questionnaire be used to differentiate between patients with controlled and uncontrolled asthma symptoms? A pilot study. *Fam Pract* 2006;23(6):674-681.
305. British Thoracic Society guidelines on diagnostic flexible bronchoscopy. *Thorax* 2001;56 Suppl 1:i1-21.
306. Corne JM, Marshall C, Smith S et al. Frequency, severity, and duration of rhinovirus infections in asthmatic and non-asthmatic individuals: a longitudinal cohort study. *Lancet* 2002;359(9309):831-834.
307. Dolen JG, Mathur A. Undetectable interferon-alpha serum levels in a patient with atopic dermatitis. *J Interferon Cytokine Res* 1995;15(11):973-975.
308. Wang Q, Nagarkar DR, Bowman ER et al. Role of double-stranded RNA pattern recognition receptors in rhinovirus-induced airway epithelial cell responses. *J Immunol* 2009;183(11):6989-6997.
309. Daley D, Park JE, He JQ et al. Associations and interactions of genetic polymorphisms in innate immunity genes with early viral infections and susceptibility to asthma and asthma-related phenotypes. *J Allergy Clin Immunol* 2012;130(6):1284-1293.
310. Agrawal S, Kandimalla ER. Synthetic agonists of Toll-like receptors 7, 8 and 9. *Biochem Soc Trans* 2007;35(Pt 6):1461-1467.
311. Roponen M, Yerkovich ST, Hollams E, Sly PD, Holt PG, Upham JW. Toll-like receptor 7 function is reduced in adolescents with asthma. *Eur Respir J* 2010;35(1):64-71.
312. Moller-Larsen S, Nyegaard M, Haagerup A, Vestbo J, Kruse TA, Borglum AD. Association analysis identifies TLR7 and TLR8 as novel risk genes in asthma and related disorders. *Thorax* 2008;63(12):1064-1069.
313. Moisan J, Camateros P, Thuraisingam T et al. TLR7 ligand prevents allergen-induced airway hyperresponsiveness and eosinophilia in allergic asthma by a MYD88-dependent and MK2-independent pathway. *Am J Physiol Lung Cell Mol Physiol* 2006;290(5):L987-L995.
314. Camateros P, Tamaoka M, Hassan M et al. Chronic asthma-induced airway remodeling is prevented by toll-like receptor-7/8 ligand S28463. *Am J Respir Crit Care Med* 2007;175(12):1241-1249.
315. Durrani SR, Montville DJ, Pratt AS et al. Innate immune responses to rhinovirus are reduced by the high-affinity IgE receptor in allergic asthmatic children. *J Allergy Clin Immunol* 2012;130(2):489-495.
316. Edwards MR, Regamey N, Vareille M et al. Impaired innate interferon induction in severe therapy resistant atopic asthmatic children. *Mucosal Immunol* 2013;6(4):797-806.
317. Mayer AK, Muehmer M, Mages J et al. Differential recognition of TLR-dependent microbial ligands in human bronchial epithelial cells. *J Immunol* 2007;178(5):3134-3142.

318. Xirakia C, Koltsida O, Stavropoulos A et al. Toll-like receptor 7-triggered immune response in the lung mediates acute and long-lasting suppression of experimental asthma. *Am J Respir Crit Care Med* 2010;181(11):1207-1216.
319. Talbot TR, Hartert TV, Mitchel E et al. Asthma as a risk factor for invasive pneumococcal disease. *N Engl J Med* 2005;352(20):2082-2090.
320. Kandane-Rathnayake RK, Tang ML, Simpson JA et al. Adult Serum Cytokine Concentrations and the Persistence of Asthma. *Int Arch Allergy Immunol* 2013;161(4):342-350.
321. Schmeck B, Moog K, Zahlten J et al. Streptococcus pneumoniae induced c-Jun-N-terminal kinase- and AP-1 -dependent IL-8 release by lung epithelial BEAS-2B cells. *Respir Res* 2006;7:98.
322. Strieter RM, Belperio JA, Keane MP. Host innate defenses in the lung: the role of cytokines. *Curr Opin Infect Dis* 2003;16(3):193-198.
323. Arva E, Andersson B. Induction of phagocyte-stimulating and Th1-promoting cytokines by in vitro stimulation of human peripheral blood mononuclear cells with Streptococcus pneumoniae. *Scand J Immunol* 1999;49(4):417-423.
324. Hiemstra PS. The role of epithelial beta-defensins and cathelicidins in host defense of the lung. *Exp Lung Res* 2007;33(10):537-542.
325. Scharf S, Zahlten J, Szymanski K, Hippenstiel S, Suttorp N, N'Guessan PD. Streptococcus pneumoniae induces human beta-defensin-2 and -3 in human lung epithelium. *Exp Lung Res* 2012;38(2):100-110.
326. Biscione GL, Corne J, Chauhan AJ, Johnston SL. Increased frequency of detection of Chlamydomphila pneumoniae in asthma. *Eur Respir J* 2004;24(5):745-749.
327. Patel M, Xu D, Kewin P et al. TLR2 agonist ameliorates established allergic airway inflammation by promoting Th1 response and not via regulatory T cells. *J Immunol* 2005;174(12):7558-7563.
328. Fransson M, Adner M, Erjefalt J, Jansson L, Uddman R, Cardell LO. Up-regulation of Toll-like receptors 2, 3 and 4 in allergic rhinitis. *Respir Res* 2005;6:100.
329. Bjornvold M, Munthe-Kaas MC, Egeland T et al. A TLR2 polymorphism is associated with type 1 diabetes and allergic asthma. *Genes Immun* 2009;10(2):181-187.
330. Fageras BM, Hmani-Aifa M, Lindstrom A et al. A TLR4 polymorphism is associated with asthma and reduced lipopolysaccharide-induced interleukin-12(p70) responses in Swedish children. *J Allergy Clin Immunol* 2004;114(3):561-567.
331. Wilson RH, Maruoka S, Whitehead GS et al. The Toll-like receptor 5 ligand flagellin promotes asthma by priming allergic responses to indoor allergens. *Nat Med* 2012;18(11):1705-1710.
332. Broide D, Schwarze J, Tighe H et al. Immunostimulatory DNA sequences inhibit IL-5, eosinophilic inflammation, and airway hyperresponsiveness in mice. *J Immunol* 1998;161(12):7054-7062.

333. Creticos PS, Schroeder JT, Hamilton RG et al. Immunotherapy with a ragweed-toll-like receptor 9 agonist vaccine for allergic rhinitis. *N Engl J Med* 2006;355(14):1445-1455.
334. Sykes A. Characterising the molecular defect in interferon production in asthma. Pathogen recognition receptors and interferon responses to rhinovirus in asthma. 2010.
335. Lun SW, Wong CK, Ko FW, Hui DS, Lam CW. Expression and functional analysis of toll-like receptors of peripheral blood cells in asthmatic patients: implication for immunopathological mechanism in asthma. *J Clin Immunol* 2009;29(3):330-342.
336. Liu YC, Simmons DP, Li X, Abbott DW, Boom WH, Harding CV. TLR2 signaling depletes IRAK1 and inhibits induction of type I IFN by TLR7/9. *J Immunol* 2012;188(3):1019-1026.
337. Majcherczyk PA, Rubli E, Heumann D, Glauser MP, Moreillon P. Teichoic acids are not required for *Streptococcus pneumoniae* and *Staphylococcus aureus* cell walls to trigger the release of tumor necrosis factor by peripheral blood monocytes. *Infect Immun* 2003;71(7):3707-3713.
338. Sha Q, Truong-Tran AQ, Plitt JR, Beck LA, Schleimer RP. Activation of airway epithelial cells by toll-like receptor agonists. *Am J Respir Cell Mol Biol* 2004;31(3):358-364.
339. Tsutsumi-Ishii Y, Nagaoka I. Modulation of human beta-defensin-2 transcription in pulmonary epithelial cells by lipopolysaccharide-stimulated mononuclear phagocytes via proinflammatory cytokine production. *J Immunol* 2003;170(8):4226-4236.
340. Monick MM, Yarovinsky TO, Powers LS et al. Respiratory syncytial virus up-regulates TLR4 and sensitizes airway epithelial cells to endotoxin. *J Biol Chem* 2003;278(52):53035-53044.
341. Chun E, Lee SH, Lee SY et al. Toll-like receptor expression on peripheral blood mononuclear cells in asthmatics; implications for asthma management. *J Clin Immunol* 2010;30(3):459-464.
342. Parker D, Prince A. *Staphylococcus aureus* induces type I IFN signaling in dendritic cells via TLR9. *J Immunol* 2012;189(8):4040-4046.
343. Puig M, Tosh KW, Schramm LM et al. TLR9 and TLR7 agonists mediate distinct type I IFN responses in humans and nonhuman primates in vitro and in vivo. *J Leukoc Biol* 2012;91(1):147-158.
344. Asselin-Paturel C, Brizard G, Chemin K et al. Type I interferon dependence of plasmacytoid dendritic cell activation and migration. *J Exp Med* 2005;201(7):1157-1167.

Appendices

Appendix A: Supplementary figures

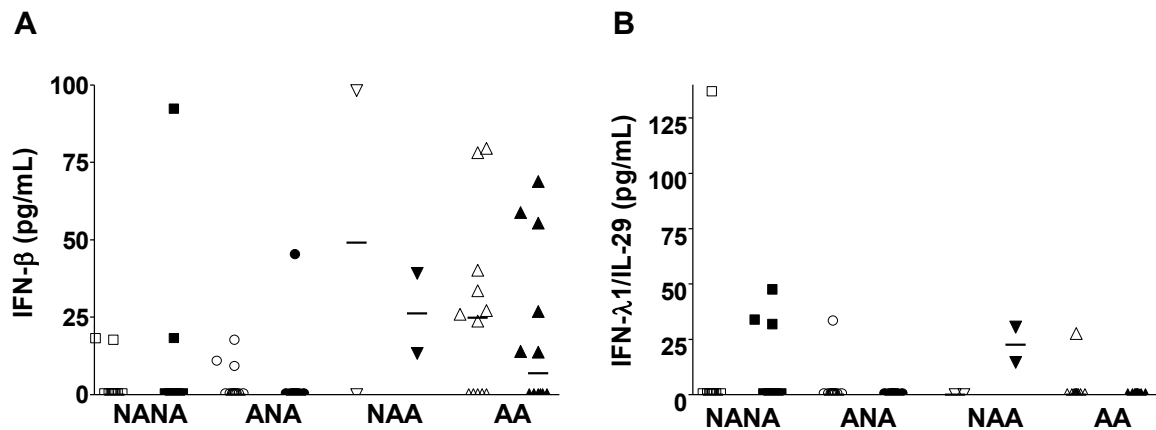


Figure A1. Interferon protein production in HBECs post stimulation with LTA at 24h. Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, black shapes denote LTA. IFN-β (A), IFN-λ1/IL-29 (B). No IFN protein production was observed.

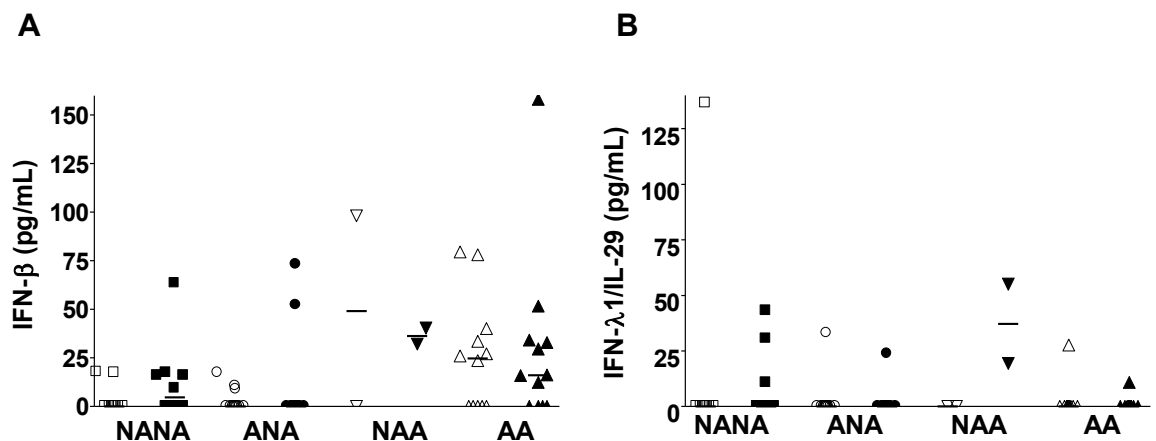


Figure A2. Interferon protein production in HBECs post stimulation with PGN at 24h. Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, black shapes denote PGN. IFN-β (A), IFN-λ1/IL-29 (B). No IFN-β protein production was observed. A non-significant weak IFN-λ1/IL-29 protein production in NAA was observed with no production in other groups. No differences were observed between groups.

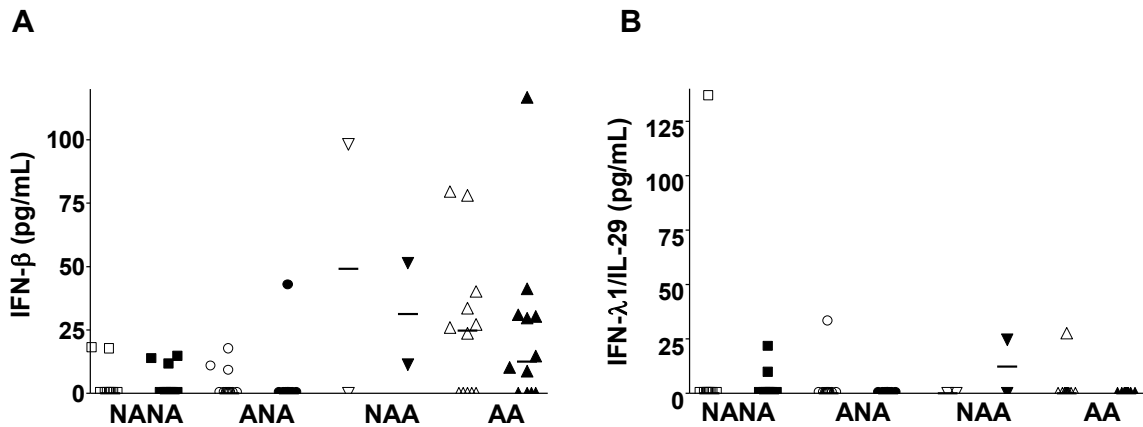


Figure A3. Interferon protein production in HBECs post stimulation with FSL at 24h. Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, black shapes denote FSL. IFN-β (A), IFN-λ1/IL-29 (B). No IFN protein production was observed.

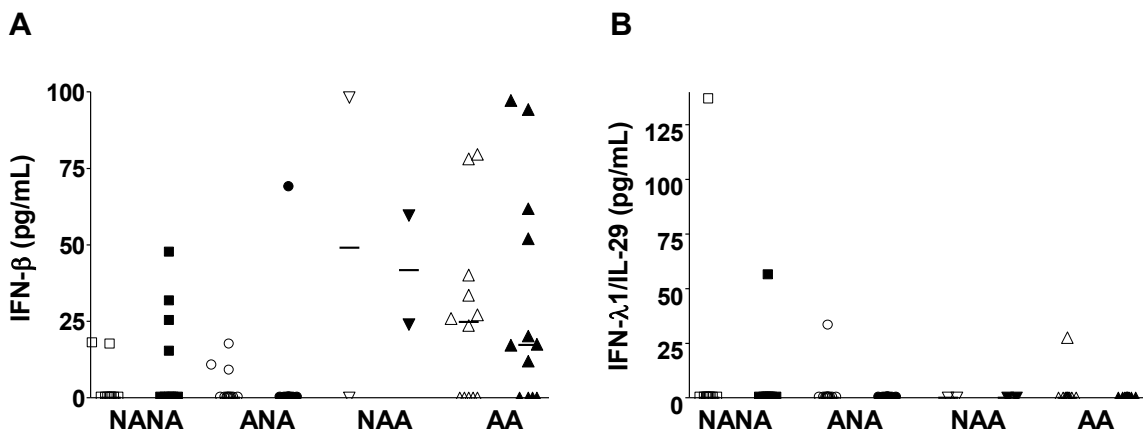


Figure A4. Interferon protein production in HBECs post stimulation with LPS at 24h. Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, black shapes denote LPS. IFN-β (A), IFN-λ1/IL-29 (B). No significant IFN protein production above that of medium treated cells was observed.

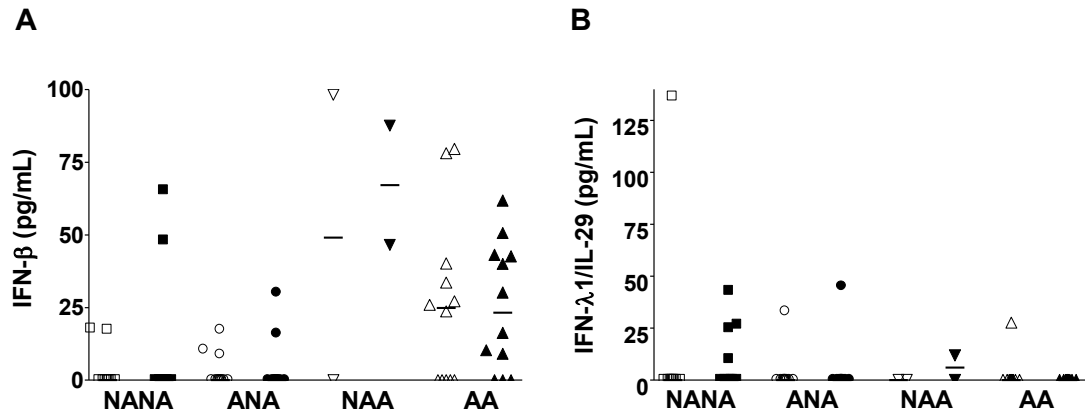


Figure A5. Interferon protein production in HBECs post stimulation with FLA at 24h. Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, black shapes denote FLA. IFN- β (A), IFN- λ 1/IL-29 (B). No significant IFN protein production above that of medium treated cells was observed.

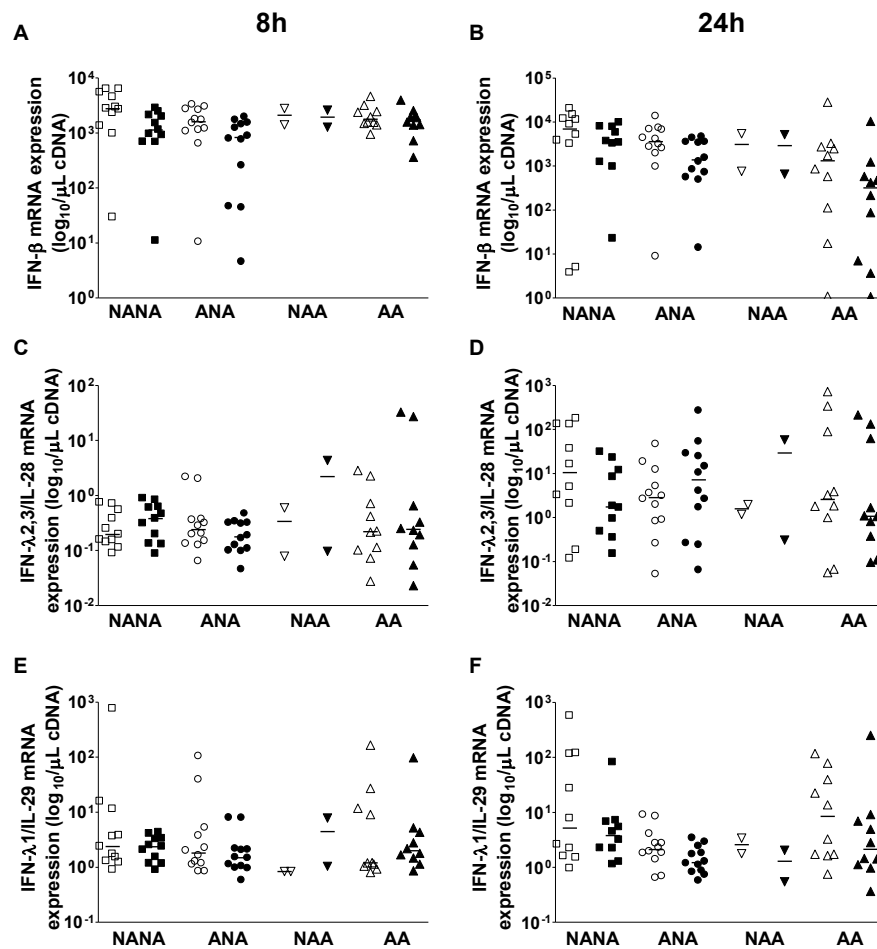


Figure A6. Interferon mRNA production in HBECs post stimulation with CpG-C at 8h (A, C and E) and 24h (B, D and F). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, black shapes denote CpG-C. IFN- β (A and B), IFN- λ 2,3/IL-28 (C and D), IFN- λ 1/IL-29 (E and F). No significant type I or III IFN mRNA induction was observed at 8 or 24h post stimulation with CpG-C.

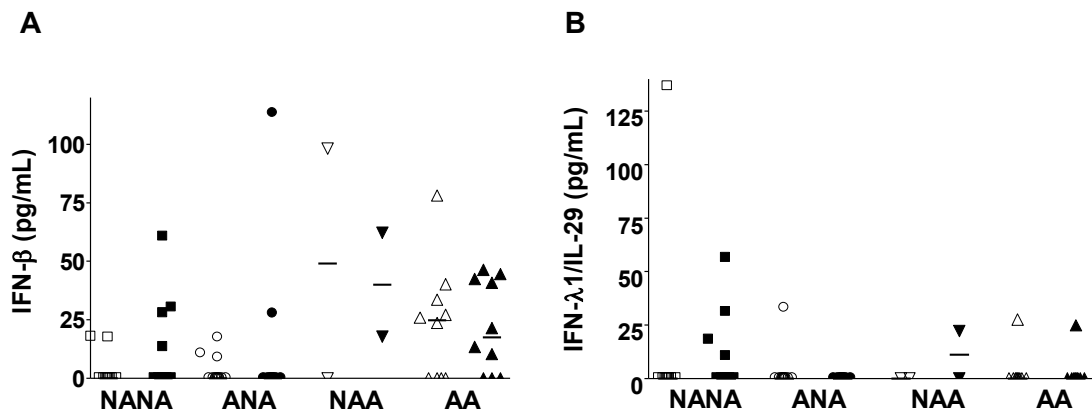


Figure A7. Interferon protein production in HBECs post stimulation with CpG-C at 24h. Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, black shapes denote CpG-C. IFN- β (A), IFN- λ 1/IL-29 (B). No significant IFN protein production above that of medium treated cells was observed.

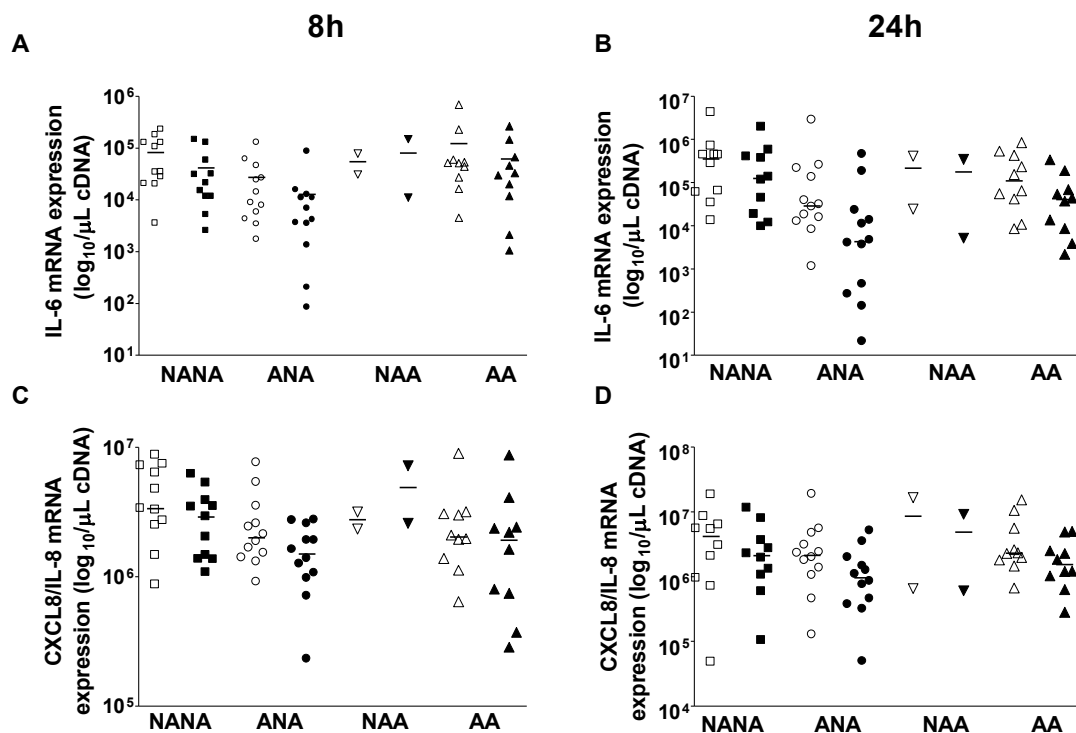


Figure A8. IL-6 and CXCL8/IL-8 mRNA production in HBECs post stimulation with CpG-C at 8h (A and C) and 24h (B and D). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, black shapes denote CpG-C. IL-6 (A and B), CXCL8/IL-8 (C and D). Induction of IL-6 and CXCL8/IL-8 mRNA was not observed at 8 or 24h

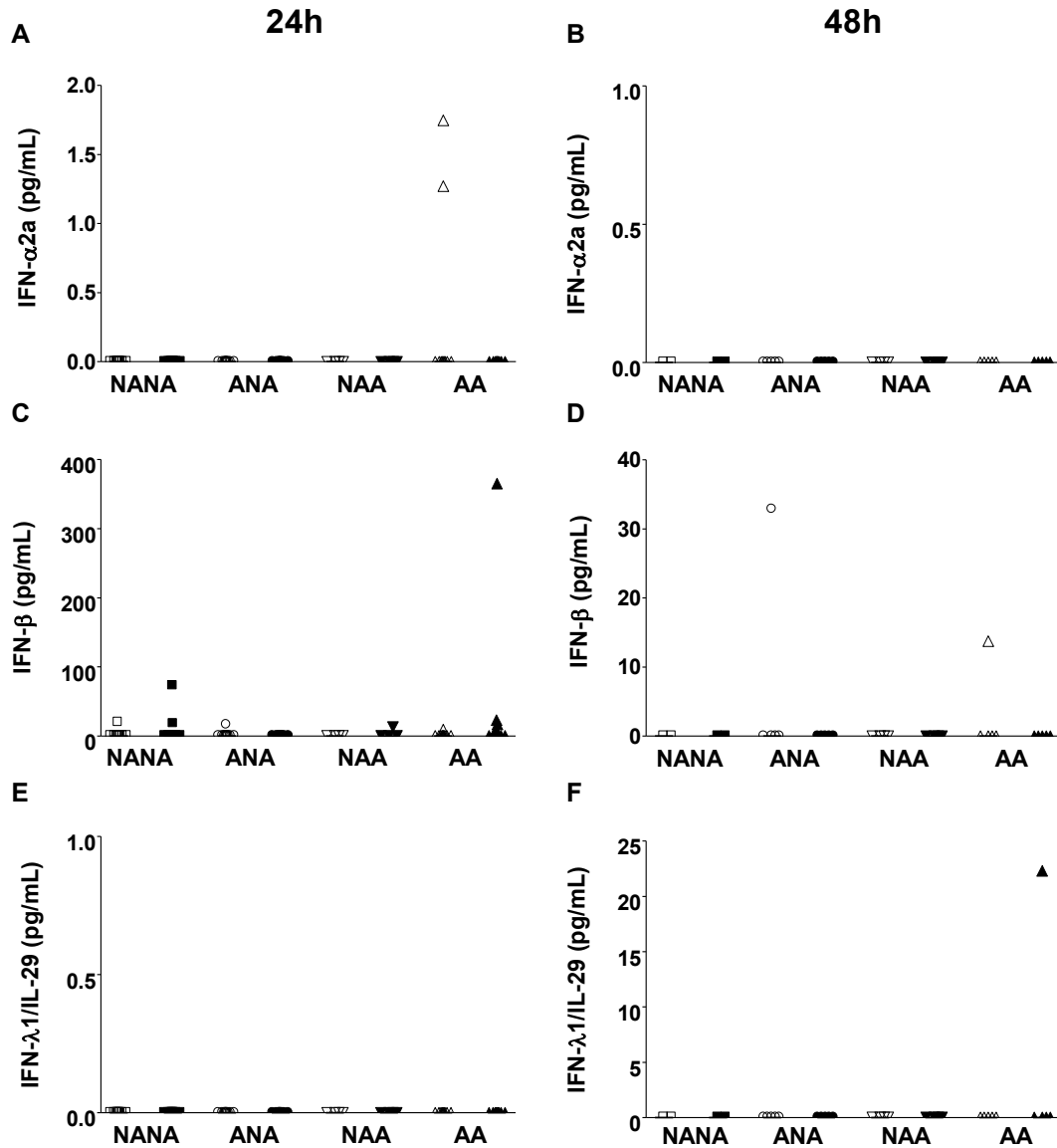


Figure A9. Interferon protein production in PBMCs post stimulation with FLA at 24h (A, C and E) and 48h (B, D and F). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, black shapes denote FLA. IFN- α 2a (A and B), IFN- β (C and D), IFN- λ 1/IL-29 (E and F). No significant IFN- α 2a production was observed. No IFN- β protein production was observed other than in a single AA participant's sample at 24h. No IFN- λ 1/IL-29 protein production was observed other than in a single AA participant's sample at 48h. These findings were from different participants.

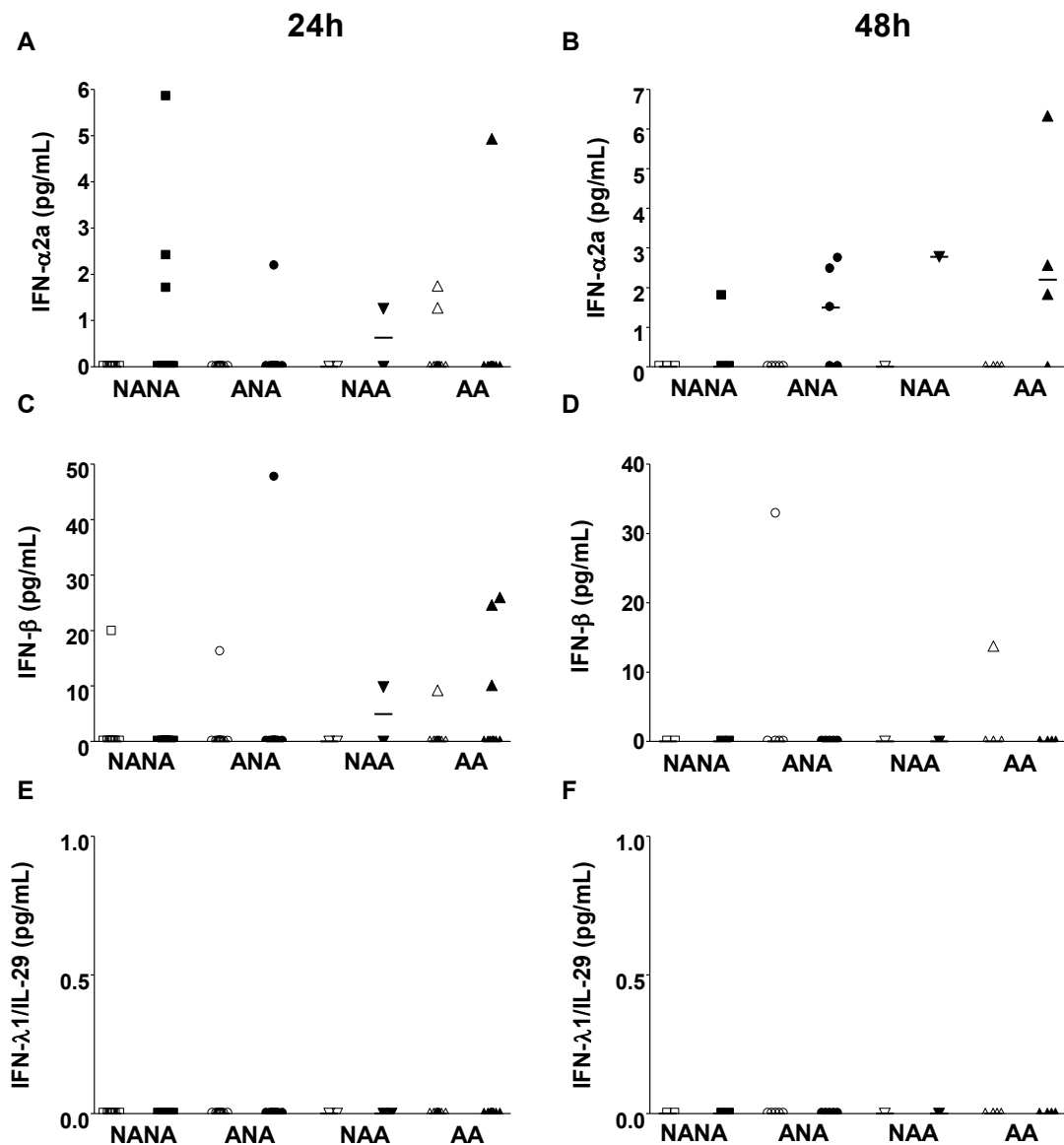


Figure A10. Interferon protein production in PBMCs post stimulation with CpG-C at 24h (A, C and E) and 48h (B, D and F). Squares denote NANA, circles denote ANA, downward triangles denote NAA, upward triangles denote AA; open shapes denote medium, black shapes denote CpG-C. IFN- α 2a (A and B), IFN- β (C and D), IFN- λ 1/IL-29 (E and F). No significant IFN- α 2a production was observed. No IFN- β protein production was observed other than in a single ANA participant's sample at 24h. No significant IFN- λ 1/IL-29 protein production was observed.

Appendix B: Patient information sheet, consent, screening proforma and ethics approval letter

Patient Information Sheet

Asthmatic Participants

(08/H0712/39, Version 5, 20/10/2009)

You are invited to take part in a research study which aims to determine the important differences in the response of asthmatic subjects to a cold compared to normal healthy people. This will help us to understand why cold viruses trigger asthma and if this can be treated better. This study is being funded by the Medical Research Council.

1. Name of the project:

Role of Atopy in Deficient Interferon Production in Response to Rhinovirus and Streptococcus pneumoniae Infection

2. The problem or question addressed by the research

Asthma is a disease characterised by inflammation of the airways in the lung (bronchi). People who suffer from it often have attacks when their breathing worsens and if very severe can even result in them being hospitalised. One of the commonest causes for such attacks is the common cold. Colds are caused by a number of different viruses and the commonest one is called the rhinovirus. The rhinovirus usually only causes a mild cold in healthy individuals but in people with airway diseases such as asthma it causes more severe symptoms. Asthmatics have been shown to have an association with the bacterium, Streptococcus pneumoniae. We wish to understand the mechanisms whereby the rhinovirus and Streptococcus pneumoniae causes attacks of asthma and by doing this we may be able to develop new treatments in the future that can prevent or treat such attacks. To do this we need to collect samples from blood and the airways of people who suffer from asthma and also a group of non-asthmatic people with which to make comparisons. From the samples we collect we will isolate the cells that are involved in fighting virus and bacterial infection and study their responses to rhinovirus and Streptococcus pneumoniae that we have in our laboratory. You have been approached as you have a diagnosis of asthma and therefore may be suitable for the above study.

3. Will you consider helping us?

We need to perform a number of procedures that will allow us to obtain these cells for our research.

4. The hoped-for benefits

Respiratory virus infections (most commonly rhinoviruses) cause around 80% of asthma attacks in children and 60% in adults. Despite being so common no specific therapy is available except steroids, which have potentially serious side effects. Asthma is very common in the UK and is responsible for much time off work, GP consultations, hospital admissions and death. Therefore finding an effective treatment for virus-induced asthma attacks has become a major priority. A clear understanding of the mechanisms of such attacks, and the differences from the response of a normal subject undergoing the same infection; will lead to the identification of such targets. There will be no immediate benefits of this study to you. However, it is hoped the study will help us to understand asthma and respiratory infections better, and everyone may eventually benefit from improved treatments or vaccination.

5. What the request involves: research practice

The project involves attending the outpatients department three times.

The first visit will last about 1.5 hours, and will involve visiting the hospital to have a brief medical examination, questions and investigations to determine your suitability as participant in the study. If suitable you will be asked to sign a consent form, have a chest X ray and lung function tests. If you have had any of these procedures in the last year they will not need to be repeated.

The second visit will involve having an ECG, skin prick test for allergy, lung function, histamine challenge test and a blood test. These are described in more detail below as you may not have had these tests before. If you have had a histamine challenge test before it will not need to be repeated.

Between visits you will be asked to do some simple lung measurements at home and will be given the equipment to do this at this visit. You should not have had a cold in the previous 6 weeks.

ECG

An ECG or electrocardiogram is a tracing of the electrical activity of the heart. It is painless, takes a few minutes and is measured by placing stickers on the chest wall. If you have had an ECG in the last year you will not need to have this repeated.

The skin prick test

We will test your skin to see if you are allergic to common allergens (e.g. cat, house dust and pollens), which involves tiny scratches on the forearm. A droplet of allergen substance is put onto the forearm and then a tiny scratch is made through it. Fifteen minutes later any substances that you are allergic to will have caused a small reaction in the skin. This is a small raised bump approx 1 -2 cm on the surface of the forearm which usually resolves over the next few hours. If you are not allergic we would expect you only to react to the control solution called histamine. The skin bump may be temporarily red and itchy and very rarely some people may have a more severe allergic reaction requiring antihistamines. If you have not had allergies before it is very unlikely that you will have a big reaction to skin prick testing. The vast majority of people who have this test do not have severe reactions and the procedure will be performed by a doctor who will be with you throughout. If you are taking antihistamines we will ask you to stop taking these for a short time (usually 3-5 days) before you have the skin prick test so that this medication does not interfere with the test results.

Lung function testing

We will also do a test to test the volumes of your lungs. This involves taking deep breaths in and breathing out through a tube attached to a machine. The machine measures how much and how fast you can breathe out and is usually performed three times and an average taken. Most people find this easy and it will not be uncomfortable. You will then use a salbutamol inhaler and go on to have the same measurements taken again to measure how much and how fast you can breathe out. This is to assess if using a salbutamol inhaler has any effect on your lung measurements.

Histamine challenge

Depending on the results of your lung function tests you may then go on to do the second part of the lung function test which is the histamine challenge. This measures how reactive your lungs are by asking you to breathe in a histamine solution and measuring your lung function to assess your response. Histamine can cause chest tightness in people who are susceptible. People who have a positive reaction to the histamine challenge are usually already known to be asthmatic however it is possible that people who are not known to be asthmatic may experience a positive reaction. The test involves checking your lung function as above and then asking you to breathe via a nebuliser. This is a loose fitting mask which blows air and aerosolised droplets of saline (salty water) and allows you to breathe this in. Your lung function test is checked afterwards to check if it has affected you. The procedure is then repeated with solutions of different strengths of a substance called histamine. This is a test often used in clinical practice to make a diagnosis of asthma so it is common for asthmatic people to experience some chest tightness during the procedure. In addition if you are on inhaled bronchodilator medication the study doctor may ask you to stop taking these up to 48 hours before the procedure. In the vast majority of people who have any chest tightness this is easily and quickly

reversed by using a salbutamol inhaler. Very rarely using the inhaler does not fully reverse the chest tightness and further medical attention may be required. Your lung function will be closely measured throughout the procedure and a doctor will be present throughout and will be able to ensure that in the rare case of problems they are dealt with promptly. The test will take place in a fully equipped outpatient department at St. Mary's Hospital.

Blood tests

We will take a total of 90 ml of venous blood throughout the study period. This is approximately 18 teaspoons. It is possible that a small bruise may appear at the skin surface where the needle was inserted in order to take blood from vein.

We have recently become aware of some research results which links certain genes to asthma. One of the blood tests would be to perform DNA analysis to look for genes associated with asthma. Your blood will be used to extract DNA which will be stored in a secure place for future research.

Will this blood test affect my ability to get insurance?

No. This blood test is not a "genetic test" as defined by insurance companies and your taking part in this project will have no effect on your eligibility for insurance.

Will I get any feedback on the results of the blood tests?

No. All genetic tests will be anonymous. We will not feed back any information as we will have no way of knowing which person donated which sample.

The third visit

If the above investigations indicate that you would be a good candidate to take part in the study then you will be invited back to have a further blood test and a bronchoscopy.

The bronchoscopy procedure

A bronchoscopy is a procedure that is commonly used to make a diagnosis of several different lung diseases. It is carried out with a bronchoscope, which is a narrow fibre-optic tube that is attached to a camera to look into the lungs. This allows us to see directly the airways of the lung and to take samples of airway fluids and cells of the lining of the airway for microscopic investigations

You will have to attend the Endoscopy Suite at the hospital on an empty stomach. You must have no food or drink for 6 hours or more prior to the procedure, if the procedure is in the morning most people do not eat after midnight. The procedure is usually a half day procedure with you attending in the morning and going home in the early afternoon. A small needle will be inserted into the vein of one arm and will be used to give you a light sedative. This will make you drowsy though you will remain conscious throughout the procedure. In addition the sedative often has the effect that you do not remember the procedure afterwards. This is not the same thing as a general anaesthetic. The sedation can affect your judgement so it is advised that you will not be able to drive for the rest of the day. We will also give you a local anaesthetic spray to your nose and the back of your throat that will minimise the discomfort when the bronchoscope passes into your lung. The bronchoscope passes into the lung via the mouth or nose. It is a flexible tube approx 0.75 cm in width, there is plenty of room to breathe around the scope and the team performing the test will be monitoring the oxygen in your blood via a small probe attached to one of your fingers. You will be able to ask any questions that you have prior to the procedure and you will be able to ask the doctor to stop at any point if you would like to, even after the test has started.

When the bronchoscope enters your lung more local anaesthetic is sprayed into the lungs so that the airways themselves will be anaesthetised. The procedure is not painful but does cause you to cough

until the local anaesthetic starts to take effect. Once the bronchoscope is in the lung we will pass a small brush down the bronchoscope and brush your airways to collect some of the cells that line your airways. The lining of your airways is insensitive and so you will not feel the brush. We will then insert some saline (salty water) through the bronchoscope, leave it for a few seconds in your lung and then suck it up again. We will also remove a small tissue specimen by removing a small sample of the lining of your airways (biopsy). In this way we will be able to collect the cells that are in your lungs and that we wish to study. Again, this procedure may cause some cough but will not be painful.

The bronchoscopy itself usually takes at the most 15 minutes but with preparation and administration of a light sedation, the whole procedure takes around an hour. After the procedure you will need to remain in hospital for about 4 hours until the effect of the sedation has worn off completely. Transport will be provided where needed, as you are not allowed to drive home. You may alternatively like to arrange transport with a family member or friend if you prefer.

Side-effects and risks of bronchoscopy

There may be some discomfort during the bronchoscopy but importantly it will not be painful. Most commonly the discomfort is cough. Approximately half of people undergoing bronchoscopy will experience some cough especially when the bronchoscope passes across the vocal cords. This is minimised by application of local anaesthetic to the airways and vocal cords prior to passing the bronchoscope into the lungs and any cough usually settles down during the procedure.

Bronchoscopy is a normally safe procedure that rarely gives rise to complications however there are some risks that you should be aware of.

On occasions (up to 5% in asthmatics) it may cause some narrowing of your airways, similar to that experienced during a mild asthma attack. This is more likely if you have asthma but the risk is still small. You will have a nebuliser prior to the bronchoscope to minimise the occurrence of chest tightness as much as possible. If this were to occur the bronchoscopy would be stopped and you would be given a salbutamol nebuliser in the same way as during an asthma attack which would reverse the narrowing. It will be carried out by an experienced doctor who will be following internationally evaluated and approved guidelines for bronchoscopy. There are full facilities on site if you require any more than this.

Another side-effect is that it can cause the level of oxygen in your blood to fall but we will be monitoring this continually by means of a probe attached to your finger so that if it does occur we will detect it immediately. As we will be giving you oxygen throughout the procedure the risk of this happening is very small.

Up to 10% of people may experience fever and flu-like symptoms within 24 hours after the investigation, but these are usually mild and transient. Should you experience fever we would like you to contact us on the telephone numbers indicated. If this does occur it usually resolves with paracetamol.

A very rare (less than 0.1% chance) but more serious risk is a pneumothorax. A pneumothorax is a condition in which air or gas collects in the space around the lungs. This can cause the lung(s) to collapse. This condition is easily treated and may go away on its own. If it significantly interferes with breathing, a tube may need to be placed in the space around the lungs to remove the air.

Should you feel discomfort of any kind at any time during the bronchoscopy and do not wish us to proceed then the procedure will be terminated.

PLEASE NOTE IT IS IMPORTANT NOT TO DRIVE A MOTOR VEHICLE FOR THE REST OF THE DAY AFTER SEDATION.

6. What if something goes wrong?

Imperial College London holds insurance policies which apply to this study. If you experience harm or injury as a result of taking part in this study, you will be eligible to claim compensation without having to prove that Imperial College is at fault. This does not affect your legal rights to seek compensation.

If you are harmed due to someone's negligence, then you may have grounds for a legal action. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been treated during the course of this study then you should immediately inform the Investigators (Dr Jonathan Macintyre, j.macintyre@imperial.ac.uk, 020 7594 3763 or Prof Sebastian Johnston, s.johnston@imperial.ac.uk). The normal National Health Service complaint mechanisms are also available to you. If you are still not satisfied with the response, you may contact the Imperial College Clinical Research Office.

The above study has been reviewed by the ethics committee at St Mary's Hospital.

7. What about confidentiality?

By signing this form you consent to the collection and use of your personal data for the study (Study Data). This may include sensitive personal data about your age, gender, ethnic origin and health. Your consent to the use of your Study Data does not have a specific expiry date, but you may withdraw your consent at any time.

The Study Data will be used by Imperial College and are protected by the use of a code, which is a number specific to you. Imperial College controls the code 'key', which is needed to connect coded Study Data back to you. A person appointed by Imperial College, regulatory authorities or other supervisory bodies may review any of your Study Data at Imperial College to ensure the proper running of the Study.

Your Study Data will be used for research into the activity of Imperial College and may be used in research related to the development of pharmaceutical products, diagnostics or medical aids. Imperial College is responsible for its handling of your Study Data in accordance with applicable Data Protection law(s).

The results of the Study may be published in medical literature, but you will not be identified.

You have the right to request information about your Study Data and to be provided with a copy of them. You also have the right to request that any inaccuracies in such data be corrected. If you wish to make a request, then please contact Imperial College.

We would normally inform your GP or hospital consultant that you are involved in this study. If you do not wish us to inform them there is an option to do this on the consent form.

8. What happens if I don't want to take part?

Nothing. You simply don't sign the consent form. This will not affect your medical care or your legal rights.

9. Can I withdraw my permission?

You may withdraw your permission to the use of your data and samples at any time without giving reasons for doing so, even after you have agreed to take part. Your future medical care will not be affected if you do not want to enter the study or want to withdraw at any time during the study. The investigator may also withdraw you from the study at any time if he/she considers this to be

appropriate. If you withdraw your permission before your samples and data are used, we will not use the data and the samples will be destroyed. If you withdraw your consent after your samples have been analysed we will ensure that the remainder is destroyed. However, if analysis has already been performed we are not obliged to destroy results of this research.

If you require any **further information about this study**, please contact the following:

Dr **Jonathan Macintyre** or Professor **Sebastian Johnston**

Department of Respiratory Medicine,

Imperial College London,

Norfolk Place,

London

W2 1PG

Office phone no: 020 7594 3763 or 0759 025 0487

Email: Respiratory.Research01@imperial.ac.uk

Participant Consent Form

Imperial College London

Study Number:

Patient Identification Number for this trial:

CONSENT FORM

Title of Project: Role of Atopy in Deficient Interferon Production in Response to Rhinovirus and Chlamydophila pneumoniae Infection

Name of Investigators: Dr Jonathan Macintyre & Prof Sebastian Johnston

Please initial box

1. I confirm that I have read and understand the information sheet dated 20/10/2009 (version 5) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason and without my medical care or legal rights being affected. ☐
3. I consent to use the samples taken for research by Imperial College, London and I understand that full ethical approval will be sought for any future research project. ☐
4. I understand that I am consenting to attend St Mary's Hospital on three further occasions. The study includes a medical history and examination, lung function tests with reversibility, blood tests, skin prick tests and challenge testing. I understand that I am required to keep a peak flow chart between the second and third visit. I understand that the third visit involves further blood tests and fibre optic bronchoscopy. ☐

5. I consent to a blood sample being taken which will be used to obtain my DNA. I understand that this is not a diagnostic test and that I will not receive any results ☐
6. I consent to the processing of my personal information for the purposes of this study only, and understand that my information will not be used for any other purposes by the Investigators. I understand that my information will be treated as strictly confidential and handled in accordance with the provisions of the Data Protection Act 1998. ☐
7. I understand that relevant sections of any of my medical notes and data collected during the study may be looked at by responsible individuals from Imperial College, from regulatory authorities or from the Imperial College Healthcare NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐
8. I agree to my GP being informed of my participation in the study. ☐
9. I agree to take part in the above study. ☐

_____	_____	_____
Name of Patient	Date	Signature

_____	_____	_____
Name of Witness	Date	Signature

Investigator's statement

I have explained the nature, demands and foreseeable risks of the above research to the subject.

_____	_____	_____
Investigator	Date	Signature

Subject Screening Data

Date

Study no

Name

Hospital No.

Address

.....

.....

Tel no

Email

DOB (age)

Sex

GP

.....

.....

.....

.....

GP informed

☐

Occupation

Notes

.....

.....

.....

.....

.....

.....

.....

.....

.....

.....

.....

.....

.....

.....

.....

Subject Date.....

- Have you at any time in the last 12 months had whistling or wheezing in your chest?
- Have you at any time in the last 12 months woken up in the morning with a feeling of tightness in the chest?
- Have you at any time in the last 12 months had an attack of shortness of breath that came on during the day when you were not doing anything strenuous?
- Have you at any time in the last 12 months had an attack of shortness of breath that came on when you had stopped exercising?
- Have you at any time in the last 12 months been woken at night by an attack of shortness of breath?
- If you were in a dusty place, near animals or near feathers would you get short of breath?
- Have you ever been diagnosed with asthma?
- Have you had an attack in the last 12 months?
- Do you take any medication for asthma?

Medication

Drug allergies

Other allergies

Allergy history: hayfever
 eczema
 urticaria
 angioedema

Subject Date.....

FHx of asthma / allergic disease

Animal / pet exposure

Smoking status

current

Lifelong non smoker

Ex smoker date stopped pack years

Alcohol intake

Examination:

Subject Date.....

Asthmatics Only:

- Asthma control (Asthma control Questionnaire {ACQ})
 - On average, during the past week, how often were you woken by your asthma during the night?
 - Never (0)
 - Hardly ever (1)
 - A few times (2)
 - Several times (3)
 - Many times (4)
 - A great many times (5)
 - Unable to sleep because of asthma (6)
 - On average, during the past week, how bad were your asthma symptoms when you woke up in the morning?
 - No symptoms (0)
 - Very mild symptoms (1)
 - Mild symptoms (2)
 - Moderate symptoms (3)
 - Quite severe symptoms (4)
 - Severe symptoms (5)
 - Very severe symptoms (6)
 - In general, during the past week, how limited were you in your activities because of your asthma?
 - Not limited at all (0)
 - Very slightly limited (1)
 - Slightly limited (2)
 - Moderately limited (3)
 - Very limited (4)
 - Extremely limited (5)
 - Totally limited (6)
 - In general, during the past week, how much shortness of breath did you experience because of your asthma?
 - None (0)
 - A very little (1)
 - A little (2)
 - A moderate amount (3)
 - Quite a lot (4)
 - A great deal (5)
 - A very great deal (6)
 - In general, during the past week, how much of the time did you wheeze?
 - Not at all (0)
 - Hardly any of the time (1)
 - A little of the time (2)
 - A moderate amount of the time (3)
 - A lot of the time (4)
 - Most of the time (5)
 - All the time (6)

Subject Date.....

- On average, during the past week, how many puffs of short-acting bronchodilator (e.g., Ventolin) have you used each day?
 - None (0)
 - 1-2 puffs most days (1)
 - 3-4 puffs most days (2)
 - 5-8 puffs most days (3)
 - 9-12 puffs most days (4)
 - 13-16 puffs most days (5)
 - More than 16 puffs most days (6)

Questionnaire score:

Section 1: score (out of 9)

Precipitating factors

Seasonal variation

Frequency of inhaler use

Age of onset

Frequency of exacerbations

Last exacerbation

Hospital admissions

ITU admissions

Last course of oral steroids

Additional Medical History

Subject Date.....

Spirometry

Date:	absolute	% predicted	Post salbutamol
PEF (l/s)			
FEV1 (l)			
FVC (l)			
FEV1/FVC			
F50			
F25			

PEF diurnal variation (%) over 2 weeks

Date:

PC20 (histamine)

Date:

Histamine dose	1 min	3 min	notes
Saline			
0.03			
0.06			
0.12			
0.24			
0.48			
1			
2			
4			
8			
16			
Recovery			Inhaler given Y / N

CXR

Date:

ECG

Date:

Subject Date.....

Skin Prick Tests – Results Sheet

Investigator

Response (mm)

diluent neg control	
6 grass mix	
house dust mite (Der P)	
cladosporium herbarum	
alternaria alternata	
aspergillus fumigatus	
silver birch	
3 trees	
nettle pollen	
cat dander	
dog hair	
histamine pos control	

1 response with wheal 3mm greater than the negative control indicates atopy.

Atopic

☐

Non-atopic

☐

Subject Date.....

TEST	DATE	RESULT
FBC		
Biochemistry		
Coagulation screen		
IgE (total)		

Ethics Approval Letter

St Mary's REC
Royal Free Hospital
Research Ethics Committees
Pond Street
London
NW3 2QG
Telephone: 0207 794 0635

10 June 2008

Professor Sebastian Johnston
Professor of Respiratory Medicine
National Heart and Lung Institute
Department of Respiratory Medicine
Imperial College London
Norfolk Place
London
W2 1PG

Dear Professor Johnston

Full title of study: **Role of Atopy in Deficient Interferon Production in Response to Rhinovirus and Chlamydomphila pneumoniae Infection**

REG reference number: **08/H0712/39**

Thank you for your letter, responding to the Committee's request for further information on the above research and submitting revised documentation, subject to the conditions specified below.

The further information was considered at the meeting of the Sub-Committee of the REC held on 16 May 2008. Your further information was reviewed on behalf of the REC by the Chairman, Barrie Newton and Aidan Moss.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

Ethical review of research sites

The favourable opinion applies to the research sites listed on the attached form.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission at NHS sites ("R&D approval") should be obtained from the relevant care organisation(s) in accordance with NHS research governance arrangements. Guidance on applying for NHS permission is available in the Integrated Research Application System or at <http://www.rdforum.nhs.uk>.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

<i>Document</i>	<i>Version</i>	<i>Date</i>
Application		27 March 2008
Investigator CV		
Protocol	1	26 March 2008
Covering Letter		09 May 2008
Letter from Sponsor		18 March 2008
Peer Review		02 January 2007
Compensation Arrangements		27 March 2008
Questionnaire: Clinical Questionnaire	1	10 March 2008
Advertisement	1, Volunteers needed	26 March 2008
GP/Consultant Information Sheets	1	04 March 2008
Participant Information Sheet	2	09 May 2008
Participant Information Sheet	2	09 May 2008
Participant Consent Form	2	09 May 2008
Response to Request for Further Information		
Bronchoscopy protocol	1	10 March 2008
Protocol Skin Prick Testing to Common Allergens	1	26 March 2008
Chart for diurnal peak flow record & use of medication	1	11 March 2008
Cover letter for information pack	1	13 March 2008
Subject Screening Data: General Details	1	26 March 2008

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Website > After Review

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

The attached document "After ethical review - guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nres.npsa.nhs.uk.

08/H0712/39

Please quote this number on all correspondence

With the Committee's best wishes for the success of this project

Yours sincerely

Barrie Newton
Chairman

Email: adriana.fanigliulo@royalfree.nhs.uk

Enclosures: "After ethical review - guidance for researchers"
Site approval form

Copy to: Gary Roper, Imperial College London
Selvy Raju, R&D office for Imperial College Healthcare NHS Trust