The Effect of Live Attenuated Influenza Vaccine and Experimental Human Pneumococcal Carriage on Human Immunity

Thesis submitted in accordance with the requirements of the Liverpool School of Tropical Medicine for the degree of Doctor of Philosophy

by

Beatriz de Faria Carniel

December 2019

This PhD thesis is dedicated to my amazing parents

Emilia and Fernando Carniel

"What you get by achieving your goals is not as important as what you become by achieving your goals"

Henry David Thoreau

Declaration

This thesis is the result of the original work of the author. Recruitment of volunteers, clinical procedures and experiments were conducted in collaboration with internal colleagues outlined in Table 1.

The laboratory experiments presented in this thesis were carried out at the Liverpool School of Tropical Medicine and clinical procedures were conducted at the Royal Liverpool University Hospital, Liverpool, United Kingdom.

The contents of this thesis have not yet been presented, nor are currently being presented, wholly or in part, for any other degree or qualification.

Beatriz de Faria Carniel

December 2019

w

Table 1: Collaboration and support from the Liverpool School of Tropical Medicine staff and researchers to the work presented in this thesis.

Procedure	Collaborator
BAL isolation of cells	Elena Mitsi
BAL cell stimulation	Simon Jochems
Statistical analysis	Simon Jochems
Scoring tolerability of nasal sampling procedures	Katherine Piddock
ELISA of BAL cells	Elena Mitsi
Nasal cell processing	Simon Jochems, Carla Solorzano
Nasal wash processing	Sherin Pojar, Elena Mitsi, Esther German, Elissavet Nikolaou, Carla Solorzano
Spn inoculum preparation	Elena Mitsi, Esther German, Elissavet Nikolaou, Sherin Pojar
Luminex analysis	Mark Holloway, Simon Jochems, Elissavet Nikolaou
Flow cytometer analysis	Jesus Reine, Simon Jochems
ELISA optimisation	Elena Mitsi
Nasal mucosa methodologies assessment	Simon Jochems

Abstract

Live Attenuated Influenza Vaccine (LAIV) is used in immunisation campaigns but may alter the dynamics of naturally occurring nasal colonisation by *Streptococcus pneumoniae* (Spn), a common human pathogen. We tested how the attenuated influenza viruses contained in the vaccine and Spn interact in the host's nasopharynx using for the first time an Experimental Human Pneumococcal Challenge model (EHPC) with multiple live pathogens: LAIV and Spn of serotype 6B. Two double blinded randomised clinical trials represented two scenarios of controlled co-infection: 1) Antecedent LAIV administration followed by nasopharyngeal Spn inoculation or 2) Concurrent LAIV administration during established Spn colonisation, separated by a 3 day interval. We validated non-invasive micro-sampling techniques for mucosal immunity analysis by comparing reliability and reproducibility of available methods. Absorptive matrices and nasal curettes were established as the preferred techniques to investigate lining fluid and immune cells in the nasal mucosa. In addition, we collected nasal wash, BAL and serum from healthy adults to investigate immune cell recruitment, cytokine and influenza-specific antibody responses using flow cytometer, human cytokine 30-plex panel and ELISA analysis. Here, we showed that LAIV-induced inflammation in the nasopharynx was associated with Spn colonisation. Immune responses to Spn and to the attenuated influenza virus were impaired by LAIV, reducing chemoattractant cytokines, recruitment of monocytes, and activation of T-cells and neutrophils. In the lung, our results demonstrated that LAIV induces inflammatory cytokines produced by T-cells and that tissue-resident memory T-cells have an important role in producing specific

cytokines against the attenuated influenza virus. In short, LAIV was shown to be immunogenic in healthy adults, but less in Spn colonised individuals, highlighting the significance of nasal microbiota when developing vaccines and assessing its efficacy.

Papers published with work presented in this thesis

- Jochems, Simon P. ; Marcon, Fernando ; Carniel, Beatriz F. ; Holloway, Mark ; Mitsi, Elena ; Smith, Emma ; Gritzfeld, Jenna F. ; Solórzano, Carla ; Reiné, Jesús ; Pojar, Sherin ; Nikolaou, Elissavet ; German, Esther L. ; Hyder-Wright, Angie ; Hill, Helen ; Hales, Caz ; De Steenhuijsen Piters, Wouter A. A. ; Bogaert, Debby ; Adler, Hugh ; Zaidi, Seher ; Connor, Victoria ; Gordon, Stephen B. ; Rylance, Jamie ; Nakaya, Helder I. ; Ferreira, Daniela M**. Inflammation induced by influenza virus impairs human innate immune control of pneumococcus**. Nature Immunology, v. 19, p. 1, 2018.

- Jochems, Simon P. ; Piddock, Katherine ; Rylance, Jamie ; Adler, Hugh ; Carniel, Beatriz F. ; Collins, Andrea ; Gritzfeld, Jenna F. ; Hancock, Carole ; Hill, Helen ; Reiné, Jesus ; Seddon, Alexandra ; Solórzano, Carla ; Sunny, Syba ; Trimble, Ashleigh ; Wright, Angela D. ; Zaidi, Seher; Gordon, Stephen B. ; Ferreira, Daniela M. **Novel Analysis of Immune Cells from Nasal Microbiopsy Demonstrates Reliable, Reproducible Data for Immune Populations, and Superior Cytokine Detection Compared to Nasal Wash**. Plos One, v. 12, p. e0169805-15, 2017.

- Jochems, Simon P. De Ruiter, Karin Solórzano, Carla Voskamp, Astrid Mitsi, Elena Nikolaou, Elissavet Carniel, Beatriz F. Pojar, Sherin German, Esther L. Reiné, Jesús Soares-Schanoski, Alessandra Hill, Helen Robinson, Rachel Hyder-Wright, Angela D. Weight, Caroline M. Durrenberger, Pascal F. Heyderman, Robert S. Gordon, Stephen B. Smits, Hermelijn H. Urban, Britta C. Rylance, Jamie Collins, Andrea M. Wilkie, Mark D. Lazarova, Lepa Leong, Samuel C. , Yazdanbakhsh, Maria Ferreira, Daniela M. **Innate and adaptive nasal mucosal immune responses following experimental human pneumococcal colonisation.** Journal of Clinical Investigation, v. 2019, p. 1-42, 2019.

Contribution to papers during the thesis

- De Steenhuijsen Piters, Wouter A. A. ; Jochems, Simon P. ; Mitsi, Elena ; Rylance, Jamie ; Pojar, Sherin ; Nikolaou, Elissavet ; German, Esther L.; Holloway, Mark ; Carniel, Beatriz F. ; Chu, Mei Ling J. N. ; Arp, Kayleigh; Sanders, Elisabeth A. M. ; Ferreira, Daniela M. ; Bogaert, Debby. **Interaction between the nasal microbiota and *S. pneumoniae* in the context of live-attenuated influenza vaccine**. Nature Communications, v. 10, p. 2981, 2019.

- Elena Mitsi, Beatriz Carniel, Jesús Reiné, Jamie Rylance, Seher Zaidi, Alessandra Soares-Schanoski, Victoria Connor, Andrea Collins, Andreas Schlitzer, Elissavet Nikolaou, Carla Solórzano, Sherin Pojar, Helen Hill, Angela Hyder-Wright, Kondwani C. Jambo, Stephen B. Gordon, Simon P. Jochems And Daniela M. Ferreira. **Nasal pneumococcal colonisation confers increased responsiveness to human alveolar macrophages against heterologous respiratory pathogens.** American Journal of Respiratory and Critical Care Medicine, v. 10, 2019.

- Simon P. Jochems, Karin De Ruijter, Carla Solórzano, Astrid Voskamp, Elena Mitsi, Elissavet Nikolaou, Beatriz F Carniel, Sherin Pojar, Esther L. German, Jesús Reiné, Alessandra Schanoski, Helen Hill, Rachel Robinson, Caroline Weight, Pascal F. Durrenberger, Rob S. Heyderman, Stephen Gordon, Hermelijn H. Smits, Britta Urban, Jamie Rylance, Andrea Collins, Mark Wilkie, Lepa Lazarova, Sam Leong, Maria Yazdanbakhsh, Daniela M. Ferreira. **Defining mucosal immunity using mass cytometry following experimental human pneumococcal challenge.** BioRxiv [Preprint] February 11, 2019.

Papers arising from the work presented in this thesis

- Beatriz Carniel, Elena Mitsi, Jamie Rylance, Seher Zaidi, Andrea Collins, Jesus Reine, Helen Hill, Victoria Connor, Katerina Cheliotis, Elissavet Nikolaou, Sherin Pojar, S. Alessandra S. Schanoski, Edessa Negera, Simon P. Jochems, Daniela M. Ferreira. ***Streptococcus pneumoniae*** **Colonisation Affects Mucosal Responses To Influenza Vaccination In Adults (in preparation).**

- Elissavet Nikolaou, Simon P. Jochems, Elena Mitsi, Sherin Pojar, Edessa Negera, Jesus Reine, Beatriz Carniel, Alessandra Soares Schanoski, Victoria Connor, Hugh Adler, Caz Hales, Helen Hill, Seher Raza Zaidi, Angela Hyder-Wright, Jamie Rylance And Daniela M. Ferreira. **Experimental Human Challenge Reveals Mechanisms of acquisition or Protection Against Pneumococcal Colonisation.**

Contents

Acknowledgments 14
Abbreviations 16
List of Figures 18
List of Tables 21
CHAPTER ONE
GENERAL INTRODUCTION 22
1.1 Streptococcus pneumoniae 23
1.1.1 Spn colonisation and transmission 24
1.1.2 Pneumococcal epidemiology and disease 25
1.1.3 Immunity to Spn 26
1.2 Influenza virus 29
1.2.1 Influenza epidemiology, transmission and disease 30
1.2.2 Immunity to influenza virus 32
1.2.3 Vaccination against influenza virus 33
1.3 Spn and wild-type influenza virus co-infection 36
1.3.1 Epidemiology and disease 36
1.3.2 Window of susceptibility to co-infection 36
1.3.3 Established Spn and wild-type influenza virus co-infection 38
1.4 Murine Spn and live attenuated influenza virus (LAIV) co-infection 41
1.5 Experimental Human Pneumococcal Challenge model for study of Spn and attenuated influenza virus co-infection 42
1.6 Thesis Aims 43
CHAPTER TWO
MATERIALS AND METHODS 45
2.1 Funding and study approvals 46
2.2 Recruitment and ethics/ consent 47
2.3 Studies design 48
2.4 Study schedules 51
2.5 Clinical procedures 52
2.5.1 Clinical consumables 52
2.5.2 Volunteers symptoms log and evaluation of procedures 53
2.5.3 Experimental Human Pneumococcal Carriage (EHPC) model 53
2.5.4 Vaccination 54

2.5.5 Nasal wash collection....54
2.5.6 Nasal cells collection....54
2.5.7 Nasosorption collection....56
2.5.8 Serum collection....56
2.5.9 Bronchoalveolar lavage collection....56
2.5.10 Safety monitoring....57
2.6 Laboratory procedures....58
2.6.1 Laboratory consumables....58
2.6.2 Reagent preparations....62
2.6.2.1 For Experimental Human Spn Inoculation....62
2.6.2.2 For nasal wash processing....62
2.6.2.3 For nasal cells processing and staining....63
2.6.2.4 For Luminex human cytokine 30-plex....64
2.6.2.5 For staining of non-adherent cells from BAL tissue....65
2.6.3 Bacterial stock preparation....66
2.6.3.1 Bacterial stock quantification....67
2.6.4 Experimental Human Spn Inoculation....68
2.6.5 Nasal Wash Processing....68
2.6.6 Nasal cells processing....69
2.6.6.1 Nasal cells staining....69
2.6.7 Luminex Human Cytokine 30-plex of nasal fluid....74
2.6.8 BAL Processing....75
2.6.8.1 Isolation of non-adherent cells from BAL tissue....75
2.6.8.2 Intracellular staining of non-adherent cells from BAL tissue....76
2.6.9 ELISA procedures....81
2.6.9.1 IgG and IgA ELISA on serum, nasal wash and BAL....81
2.7 Statistical analysis....82
CHAPTER THREE
DEVELOPMENT OF METHODOLOGY FOR NASAL MUCOSA ANALYSIS....83
3.1 Introduction....84
3.2 Methods....86
3.2.1 Volunteers recruitment....86
3.2.2 Collection of nasal samples....86
3.2.3 Flow cytometry analysis....87
3.2.4 Cytokine detection....87

3.2.5 Scoring tolerability of nasal sampling procedures 88
3.2.6 Multi-dimensional scaling and heat map generation 88
3.2.7 Statistical analysis....88
3.3 Results....89
Nasal curettage yields robust and reproducible data 89
Nasal curettage and nasal wash yield different cell populations 93
Cytokine detection from nasal lining fluid using nasosorption devices....95
Methods of nasal micro-sampling are well tolerated by volunteers and do not lead to symptoms....97
3.4 Discussion 101
CHAPTER FOUR
EFFECT OF SPN AND LAIV ON RECRUITMENT OF IMMUNE CELLS TO NASAL MUCOSA....103
4.1 Introduction....104
4.2 Methods....106
4.2.1 Volunteer recruitment, vaccination and inoculation....106
4.2.2 Nasal cells collection, processing and staining 106
4.2.3 Flow cytometry and statistical analysis 107
4.3 Results....108
4.3.1 Antecedent LAIV 108
Induction of monocyte recruitment to the nose by Spn colonisation is impaired by antecedent LAIV vaccination....109
Monocyte recruitment is associated with density of Spn only in TIV vaccinated. 111
Spn-induced activation of neutrophils in the nose is impaired by antecedent LAIV. 113
Antecedent LAIV induces activation of T-cells in the human nose. 115
4.3.2 Concurrent LAIV 118
Spn colonisation of the nasopharynx induce neutrophil activation. 119
LAIV in Spn colonised alters T-cell subsets induction and T-cell activation. 123
4.4 Discussion 129
CHAPTER FIVE
EFFECT OF SPN AND LAIV ON THE NASOPHARYNX CYTOKINE PRODUCTION 133
5.1 Introduction....134
5.2 Material and methods 135

5.2.1 Volunteer recruitment, vaccination and inoculation 135
5.2.2 Nasosorption collection 135
5.2.3 Luminex and statistical analysis 135
5.3 Results 136
LAIV induces pro-inflammatory cytokines in nasopharynx, especially in Spn colonised. 137
Antecedent LAIV-induction of cytokines that regulate adaptive immunity diverge between Spn colonised and non-colonised volunteers 152
LAIV induces cytokines that regulate cell growth in the nasopharynx especially in Spn colonised. 163
LAIV induction of anti-inflammatory cytokine response is impaired in the Spn colonised nasal mucosa 172
5.4 Discussion 176
CHAPTER SIX
EFFECT OF SPN AND LAIV ON INNATE AND ADAPTIVE IMMUNITY TO INFLUENZA 179
6.1 Introduction 180
6.2 Methods 183
6.2.1 Volunteer recruitment, vaccination and inoculation 183
6.2.2 Nasal wash, BAL and serum collection and processing 183
6.2.3 Intracellular cytokine staining of BAL cells 184
6.2.4 Enzyme-linked immunosorbent assay (ELISA) 184
6.2.5 Statistical analysis 184
6.3 Results 185
Concurrent LAIV increases the frequency of influenza-specific TNF-α and IFN-γ producing $CD4^+$ and TRM $CD4^+$ T-cells in the lung 186
Concurrent LAIV increases the frequency of TNF-α producing influenza-specific $CD8^+$ and TRM $CD8^+$ T-cells in the lungs 191
Concurrent LAIV increases frequency of influenza-responding IFN-γ producing TCR-$\gamma\delta^+$ in the lungs of non-colonised individuals 194
Concurrent LAIV increases frequency of $CD4^+$ regulatory T-cells in the lung of non-colonised individuals 196
TIV but not concurrent LAIV vaccination increases levels of IgG to influenza in serum 198
IgG but not IgA is induced by influenza vaccines in the lung, with concurrent LAIV mediated-responses being impaired by Spn colonisation 200

Spn colonisation impairs nasal IgA induction following LAIV but does not alter responses to TIV 201
6.4 Discussion 204
CHAPTER SEVEN
GENERAL DISCUSSION 209
7.1 Introduction 210
7.2 Summary and discussion of findings 211
7.3 Limitations of LAIV research in humans 214
7.4 Implications of this thesis and future research 215
7.5 Conclusion 219
8. References 221

Acknowledgments

There were a great number of people who helped make this journey possible and I would like to acknowledge the enormous support I have been given.

. First and foremost, I want to thank my supervisor Daniela Ferreira, it has been an honour to be her PhD student. I will forever appreciate the leap of faith in accepting me as a student and letting me tag along on countless research projects when I was just learning the ropes of a new field. Her confidence and willingness to speak her mind has been a huge influence, in this thesis and in my life. The joy and enthusiasm she has for her research and team was contagious and motivational to me, even during tough times. I am also thankful for the excellent example she provides as a successful woman in science and lecturer.

I also am immensely grateful to Simon Jochems, my second supervisor. I feel extremely lucky to be his student. He has inspired me with his intelligence, attention to detail and openness to questions and made my PhD an unique learning experience.

I want to thank both supervisors for the time invested in mentoring me with their expertise, providing helpful and constructive opinions which was instrumental for me to feel assured of all the work done during these 5 years. They have taught me - both consciously and unconsciously - how good science is done, supported my decisions and were understanding of my hardships.

The members of the EHPC group have contributed immensely to my personal and professional life. The team has been a source of friendship as well as good advice and collaboration. I want to thank them all for their confidence in me, encouraging support, and optimistic outlook at all times during this journey. I express my heartiest gratitude to my unofficial third supervisor, Elena Mitsi, who gave me guidance and enormous help during my project. I extend thanks to all team members especially Carla Solorzano and Esther German, for their companionship and important contributions in this doctoral thesis. Many thanks go to Sherin Pojar, Elissavet Nikolaou and Jesus Reine for their excellent technical help as well as to all the clinical team, whom this PhD would be impossible without. The time among this wonderful people has improved me greatly as a researcher and a person.

I gratefully acknowledge the funding sources that made my PhD work possible. I was funded by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil). I have been fortunate enough to have one of the last opportunities to be funded for higher education by the excellent Brazilian government program “Science Without Borders”. Today it would not have been possible to complete this PhD as the program is cancelled indefinitely as Brazil goes through a period of cuts and disbelief in research and science. I feel lucky to have had this opportunity and, at the same time, outraged that most people are denied the chance because of political, socio-cultural, or economic circumstances.

Lastly, I would like to thank my nuclear and extended family for all their love and encouragement. I dedicate this PhD to my parents Emilia and Fernando Carniel as they were the ones talking about it when I was only 15 years old! I thank them for raising me with a love for science and for supporting me in all my pursuits, providing me with a solid base from which to start this journey and later being sources of vital comfort during this journey, together with my brother Eduardo Carniel.

I have been very lucky to have my best friend from childhood Sarah Marconatto at my side, helping me with her confidence, patience, pride and nurturing love. I treasure the time I spent in Liverpool – the best city I’ve ever lived in (so far!) - and thank the amazing friendships I’ve built here, especially Adam Graff and Faris Khalifa. And most of all for my loving, supportive, encouraging, and patient partner Mateus whose support during all stages of this PhD is eternally appreciated.

Thank you everyone, you have all been unique guiding lights and pillars of support at all times. I will never forget my time here.

Abbreviations

Abbreviation	Meaning
µL	Microliter
Antecedent LAIV	Live attenuated influenza vaccine given before S*treptococcus pneumoniae* inoculation
BAL	Bronchoalveolar lavage
BSA	Bovine serum albumin
Concurrent LAIV	Live attenuated influenza vaccine given after S*treptococcus pneumoniae* inoculation
DC	Dendritic cell
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EHPC	Experimental human pneumococcal carriage model
Epcam	Epithelial cellular adhesion molecule
FGF-Basic	Basic fibroblast growth factor
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HA	Hemagglutinin
HGF	Hepatocyte growth factor
HLA-DR	Human leukocyte antigen-d related
IFN-α and IFN-γ	Interferon alpha and interferon gamma
IL-2R	IL-2 receptor
KC	Keratinocyte chemoattractant
LAIV	Live attenuated influenza vaccine
LOESS	Locally estimated scatterplot smoothing
MCP-1	Monocyte chemoattractant protein-1
MHC I and II	Major histocompatibility complex class i and class ii

MIG	Monokine induced by gamma
MIP-1α and MIP-1β	Macrophage inflammatory protein alpha and beta
mL	Millilitre
NA	Neuraminidase
Nod2	Nucleotide-binding oligomerisation domain containing 2
NS	Nasosorption
NW	Nasal wash
PAFR	Platelet-activating factor receptor
PBS	Phosphate buffered saline
PRRs	Pattern recognition receptors
PspA and PspC	*Streptococcus pneumoniae* protein a and c
RANTES	Regulated on activation, normal t cell expressed and secreted
Spn	*Streptococcus pneumoniae*
Spn colonised	Individuals who are nasally colonised by *Streptococcus pneumoniae*
T-cell	Thymus lymphocyte cell
Th17	T-helper 17
Th2	T-helper 2
TIV	Tetravalent inactivated influenza vaccine
TLR	Toll-receptor
TNF-α	Tumour necrosis factor alpha
TRM	Tissue resident memory cell
VEGF	Vascular endothelial growth factor

List of Figures

Figure 1. Design for Antecedent LAIV and Concurrent LAIV studies.. 49
Figure 2. Agar blood plate layout for quantification. 67
Figure 3. Representative flow plots of nasal ells from the Antecedent LAIV study analysis by flow cytometry.....72
Figure 4. Representative flow plots of nasal T-cells from the Concurrent LAIV study analysed by flow cytometry.....73
Figure 5. Representative flow plots of BAL T-cells and cytokine production analysed by flow cytometry using intracellular staining. 79
Figure 6. Representative plots of TRM T-cells identified by flow cytometry. 80
Figure 7. Repeatability of nasal curettage sampling.....90
Figure 8. Nasal curettage yields reproducible and consistent results over time. 92
Figure 9. Comparison of samples collected by nasal wash and nasal curette.. 94
Figure 10. Comparison of cytokine levels in samples collected by NW and using NS strips.....96
Figure 11.Tolerability of novel nasal sampling methods.....100
Figure 12. Induction of monocyte recruitment to the nose by Spn colonisation is impaired by antecedent LAIV vaccination. 110
Figure 13. Monocyte recruitment is associated with density of Spn only in TIV vaccinated group.....112
Figure 14. Spn-induced activation of active neutrophils in the nose is impaired by antecedent LAIV.....114
Figure 15. Antecedent influenza vaccines and Spn colonisation do not affect T-cell recruitment to the human nasal lumen.....116
Figure 16. Antecedent LAIV, but not TIV, induces activation of T-cells in the human nose.. 117
Figure 17. Induction of monocyte recruitment to the nose by Spn colonisation is affected by influenza vaccination. 120
Figure 18. Spn colonisation of the nasopharynx induces neutrophil activation. 122
Figure 19. Late increase of T-cells in Spn non-colonised.....124
Figure 20. LAIV and Spn colonisation alters late $CD4^+$ T-cells decrease and $CD8^+$ increase.. 125
Figure 21. LAIV and Spn colonisation alters T-cell activation.....126
Figure 22. Concurrent influenza vaccines and Spn colonisation do not significantly affect BDCA-1^+ and BDCA-2^+ DC levels in the human nasal mucosa. 128
Figure 23. TIV vaccinated group exhibits decrease in pro-inflammatory cytokines IL-1β and GM-CSF in the nasopharynx.....138
Figure 24. LAIV vaccinated group exhibits increase in pro-inflammatory cytokines in the nasopharynx.....139
Figure 25. LAIV vaccinated group exhibits increase in pro-inflammatory cytokines in the nasopharynx.....140

Figure 26. Antecedent LAIV induces pro-inflammatory cytokines IL-6 and MIG in nasopharynx of non-colonised volunteers. 144
Figure 27. Antecedent LAIV induces pro-inflammatory cytokines MCP-1 and TNF-α in nasopharynx of non-colonised volunteers. 145
Figure 28. Antecedent LAIV induces additional pro-inflammatory cytokines in nasopharynx of non-colonised volunteers. 146
Figure 29. Antecedent LAIV induces pro-inflammatory cytokines IL-6 and MIG in nasopharynx of Spn colonised volunteers. 147
Figure 30. Antecedent LAIV induces additional pro-inflammatory cytokines in nasopharynx of Spn colonised volunteers. 149
Figure 31. Antecedent LAIV induces pro-inflammatory cytokine IL-1β in nasopharynx of Spn colonised volunteers. 150
Figure 32. Non-colonised volunteers show induction of pro-inflammatory cytokines MIG and IP-10 in the nose 151
Figure 33. LAIV vaccinated show increase in cytokines IFN-γ and IL-15 in the nasopharynx. 153
Figure 34. LAIV vaccinated show increase in cytokines that regulate adaptive responses in the nasopharynx. 154
Figure 35. LAIV- and TIV-vaccinated show increase in the cytokine IL-4 in the nasopharynx. 155
Figure 36. Antecedent LAIV induce cytokines IL-2 and IL-4 in the nose. 158
Figure 37. Antecedent LAIV induce cytokines IFN-γ and IL-15 in the nose. 159
Figure 38. Antecedent LAIV induce cytokines IL-12 and IL-2R in the nose of non-colonised volunteers.. 160
Figure 39. Non-colonised volunteers show induction of IL-4 in the nose 161
Figure 40. Antecedent LAIV induce cytokine IL-12 and IL-17 in the nose of volunteers colonised by Spn.. 162
Figure 41. TIV- and LAIV- vaccinated show increase in cytokines EGF in the nasopharynx. 164
Figure 42. LAIV vaccinated show increase in cytokines VEGF in the nasopharynx. 165
Figure 43. LAIV vaccinated show increase in cytokine FGF-Basic in the nasopharynx. 166
Figure 44. Antecedent LAIV induce cytokine G-CSF in the nose of volunteers.. 168
Figure 45. LAIV induces FGF-Basic and VEGF in the nasopharynx of Spn colonised volunteers. 169
Figure 46. LAIV induces cytokine FGF-Basic and EGF in the nasopharynx of non-colonised volunteers.. 170
Figure 47. LAIV induces cytokine IL-7 in the nasopharynx of Spn colonised volunteers. 171
Figure 48. Spn colonised show increased levels of cytokine EGF in the nasopharynx. 171
Figure 49. TIV-vaccinated show increase in cytokine HGF in the nasopharynx.. 172

Figure 50. LAIV induces anti-inflammatory cytokines IL-10 and HGF in the nasopharynx of non-colonised volunteers. 174
Figure 51. Non-colonised volunteers show increased levels of IL-10 in the nose.. 175
Figure 52. Concurrent LAIV and TIV do not affect frequencies of total $CD4^+$, $CD8^+$ and $TCR\gamma\delta^+$ T-cell subsets in the lung. 187
Figure 53. Concurrent LAIV increases frequency of influenza-specific TNF-α and IFN-γ-producing $CD4^+$ and TRM $CD4^+$ T-cells in the lung. 189
Figure 54. Concurrent LAIV and TIV do not increase the frequency of influenza-specific, IL-17A-producing T-cells in the lung.. 190
Figure 55. Concurrent LAIV increases frequency of TNF-α producing influenza-specific $CD8^+$ and TRM $CD8^+$ T-cells in the lungs.....192
Figure 56 Concurrent LAIV and TIV do not increase the frequency of influenza-specific, IL-17A-producing T-cells in the lung.. 193
Figure 57. Concurrent LAIV increases frequency of IFN-γ producing influenza-specific TCR-$\gamma\delta^+$ in the lungs of Spn non-colonised individuals. 195
Figure 58. Concurrent LAIV increases frequency of $CD4^+$ regulatory T-cells in the lung of Spn non-colonised individuals. 197
Figure 59. TIV but not concurrent LAIV vaccination increases levels of IgG to influenza in serum.. 199
Figure 60. IgG but not IgA is induced by influenza vaccines in the lung, with concurrent LAIV responses being reduced during Spn colonisation.. 202
Figure 61. Spn colonisation impairs nasal IgA induction following concurrent LAIV but does not alter responses to TIV. 203

List of Tables

Table 1: Collaboration and support from the Liverpool School of Tropical Medicine staff and researches to the work presented in this thesis. 3
Table 2 Ethical and clinical trial approvals for Concurrent LAIV and Antecedent LAIV studies. 46
Table 3. Demographics of volunteers enrolled in the Antecedent LAIV and Concurrent LAIV studies. 50
Table 4. Visit schedule for sample collections in the Antecedent LAIV study. 51
Table 5. Visit schedule for sample collections in the Concurrent LAIV study. 51
Table 6. Surface antibody markers applied to nasal cells in each study as well as volume used for each sample. 71
Table 7 Surface antibody markers applied to BAL cells as well as volume used for each sample. 78
Table 8. Levels of pain, discomfort and lacrimation associated with nasal curettage and NS. 99

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Streptococcus pneumoniae

Streptococcus pneumoniae (Spn) is a gram-positive cocos of approximately 1µm of diameter. This bacteria is a facultative anaerobe, generating energy by carbohydrate fermentation. They are generally grouped in pairs (diplococci) or in short chains and are able to replicate outside host cells, for example in circulation, connective tissues, and tissue spaces such as the airway lumen.

The Spn are coated with a polysaccharide capsule which is its main virulence factor [1,2] and protects the bacteria from phagocytosis by host immune cells [3,4] as well as reduces the autolysis process [5]. Spn capsules vary in size, composition, and antiphagocytic properties [6–8] and bacteria presenting different types of capsule can colonise hosts simultaneously [9]. Moreover, its cytoplasmic membrane is composed of lipoprotein bound to lipoteichoic acid by hydrophobic interactions. Penicillin-binding proteins represent a small percentage of the membrane and are the primary targets of anti-microbials [10]. In addition, the capsule's chemical composition is used to categorise the pathogen into serotypes, with over 95 serotypes identified [6–8,11–13]. Notably, the distribution of serotypes varies according to population, geographic region, time of year and age of the patient [7].

Furthermore, many factors contribute to Spn pathogenicity, such as pneumolysin, autolysin A, neuroaminidases (NA), choline binding proteins, Spn protein C (PspC), Spn surface protein A (PspA) and, especially, pilus subunits [14–18]. Importantly, pneumolysin is a multifunctional toxin that acts as a protective antigen [19,20], showing cytotoxic action and activation of the host complement system [18] while bacterial pili contributes to Spn adherence [21]. Furthermore, Spn

can form biofilms that aid growth during colonisation and contributes to the development of invasive diseases [17,22].

1.1.1 Spn colonisation and transmission

Spn is part of the common children's upper respiratory tract microbiota, colonising nostrils, pharynx and larynx [23] for up to 2 weeks, although some studies have observed events lasting up to 30 weeks [3,24–28]. The nasopharynx is the natural site of colonisation [29] and prevalence in healthy individuals varies mainly with age, with colonisation rates higher in children than adults and elderly. Importantly, Spn colonisation events start soon after birth and prevalence increases between 1 and 2 years of age, followed by rate reduction to below 10% in adults [27,30–32]. In addition, colonisation rates are variable depending on location [9,33,34]. In England, Spn colonisation was observed in 52% of children under 2 years and 45% in 3 to 4 year old [30], whereas in Gambia and Kenya, the prevalence is 80% and 66%, respectively [35,36].

However, it is important to notice that colonisation rates may vary according to sampling techniques and devices used as well as the site and frequency of collection in humans. Various methods have been used for quantification of Spn colonisation density through nasal lavage such as nasal pool devices [37], micro-suction [38], aspiration [39], sponges [40], nasal secretion collectors [41], gauzes [42] and swabs [43,44]. The nasal lavage technique selected in this thesis was nasal wash based on the methodology published by Naclerio et al. [45] and was previously used successfully in several clinical trials [46–53]. This method combined with cell culture have been proven effective for assessing colonisation

[47], cytological analysis [54] and quantification of inflammatory markers even in children [55].

Nevertheless, regarding increased Spn colonisation rates, the host can show susceptibility characteristics such as sickle cell anaemia, HIV infection, neoplasia and chronic degenerative conditions, for instance diabetes mellitus, chronic renal failure, nephrotic syndrome, chronic obstructive pulmonary disease and chronic liver diseases [56]. Additionally, the interaction between Spn and the resident microbiota in the upper respiratory tract affect colonisation rates as observed in studies with *Haemophylus influenzae* [57] and *Staphylococcus aureus* [17,58,59]. Importantly, the continuous use of antibiotics is shown to develop resistance in Spn and impact prevalence of bacteria in the population [60,61].

In healthy individuals, an asymptomatic colonisation triggers immunisation [9], providing benefits to the host as it develops cellular and adaptive immunity [49,62]. However, even when asymptomatic, colonisation is not entirely benign as it primes the host for transmission, tissue invasion or dissemination into the lower airways, a prerequisite for invasive disease [58]. Notably, the transmission of Spn between humans occurs through direct contact or dispersion of aerosol secretions by colonised people [27,58,63] with higher rates during the winter season [64]. Moreover, outbreaks of increased Spn transmission can occur in nursing homes, day care centres, military units, shelters, schools and prisons [65–71]

1.1.2 Pneumococcal epidemiology and disease

Previous to antibiotics development, pneumonia and invasive pneumococcal disease were the leading cause of death in humans [19,72] and currently - according to the World Health Organisation [73]- it is estimated that 1.6

million people die every year from pneumococcal disease worldwide, with nearly all these deaths occurring in children living in low income settings.

The vast majority of Spn disease are associated with a relatively small number of serotypes [74] and requires colonisation of the nasopharynx [58] as well as high bacterial load [75,76] and aspiration to the lungs [77] that elicits inflammatory pathology in the host [78]. Pathogenesis ranges from less severe infections such as conjunctivitis, otitis and sinusitis to severe disease such as pneumonia, bacteremia and meningitis [79,80]. Importantly, invasive pneumococcal disease have increased rates in elderly as well as people that present dementia, convulsive disorders, heart failure, cerebrovascular disease, chronic lung disease, HIV infection, alcoholism, smoking, malnutrition, diabetes, liver cirrhosis, renal failure, antibody deficiency and phagocytic function deficiency [81,82].

1.1.3 Immunity to Spn

After Spn entry in the nasopharynx, the host elicits immune responses against major infection events, that start with the recognition of bacteria by the host immune system which Spn can evade to colonise the nasal mucosa, with subsequent invasion into epithelial tissue resulting in damage to the host's cells [20,83]. Specifically, the innate immune response acts to defend against the bacteria by first recognising pathogen-associated molecular patterns (PAMPs) through pattern recognition receptors (PRRs) such as the Toll-like receptor (TRL) family [84,85]. In addition, surfactant proteins also play an important role in innate immunity phagocytosis of Spn by modulating cellular responses [85,86], activating macrophages and neutrophils as well as inducing cytokine production [87,88]. In short, the ability of the host to control epithelium damage, inflammatory

responses and bacteria multiplication without releasing excessive debris in the nasopharynx is essential for bacterial clearance and prevention of subsequent invasive Spn disease [89,90].

The innate immune responses against Spn consists of pre-infection protective host mechanisms, typically non-specific responses using physical (mucus ciliary clearance) and chemical barriers, blood proteins, resident phagocytes, neutrophils, macrophages, NK-cells, cytokines and dendritic cells (DCs) [91]. Indeed, in healthy individuals, asymptomatic colonisation responses include classic activation of macrophages, Spn-specific memory $CD4^+$ T-cells, as well as sustained TGF-β levels, responsible for increasing T-regulatory cells (T-regs) in the nasopharynx and protection of the host from exaggerated inflammation and tissue damage [49,92–95].

In addition, this immunising stable colonisation is maintained by a fine balance between pro-inflammatory and anti-inflammatory immune responses [96]. After Spn recognition, monocytes are attracted to the site of the infection and are considered one of the essential players in immunity against Spn. Moreover, monocytes are white blood cells that typically circulate through the blood for 1 to 3 days before migrating into other tissues, where they become macrophages or DCs [91]. Particularly, macrophages are monocytes that have migrated from the bloodstream into the tissue where they aid in Spn phagocytosis and cleaning of cellular debris [91].

Activation of macrophages in pneumococcal disease is signalled by TLR - a class of PRRs surface receptors expressed by phagocytes and other cell types - particularly TLR-2 and TLR-4 [97–102] which recognize Spn molecular structures such as surface lipoteichoic acid [103], pneumolysin [97,104] and lipoproteins [105], and

enables further host immune responses [106]. In mice, the recruitment of monocytes correlates with Spn clearance, dependent of TLR-2 and IL-17A [94]. Furthermore, early recruited monocytes differentiated into macrophages and produce MCP-1 [107]. In turn, this cytokine attracts more monocytes to the site. Studies in elderly mice have demonstrated that reduced monocyte recruitment to the nasopharynx results in decreased bacterial clearance [108].

Also part of the initial innate immune response are DCs, that play an important role in eliciting T-cell responses against Spn later in the infection [91]. When immature, DCs are located in the respiratory system epithelium and migrate to the lymph nodes where they maturate and reside [91]. Further, when at the infection site, DCs are activated via TLR [109] and decrease Spn-induced apoptosis of host's cells [110,111]. In addition, the complement system mediate protection from Spn in early infection [112]. This mechanism is subverted by the bacteria by altering expression of its surface proteins [113].

Subsequently, if the innate defence is not enough to control the bacteria, the adaptive responses armoury of humoral and cellular immunity will mediate clearance at the nasal mucosa [89,101]. Contrary to initial innate responses, adaptive immunity is pathogen specific and elicits memory cells that prevent future infections [91].

Firstly, humoral response develops with the production of antibodies by B-cells. Notably, induction of antibodies against Spn in human proteins occurs naturally throughout the life course as a result of continuous exposure to the bacteria, with reported increases in titres of lung and serum antibodies IgA and IgG, associated with protection from colonisation [114] and reduced bacteraemia [115,116]. IgA is known to bind to Spn in the nasopharynx in order to promote

bacterial agglutination and adherence to host cells [117] as well as phagocytosis [118,119]. Spn IgA protease can however be used to evade host IgA defences and instead facilitate internalisation of Spn in the epithelium [120].

Secondly, cellular immune responses have been demonstrated to modulate inflammatory responses to Spn, with production of cytokines by resident cells. Spn induces production of cytokines TNF-α, IL-6, IL-12, IL-4, IFN-γ and IL-10 [121,122]. This cytokine cascade then mediate the recruitment of neutrophils, monocytes and other effector cells to clear bacteria [78,123] as week as differentiate T-cells into T-helper 17 (Th17), T-helper 2 (Th2) and T-regs [124–132].

Notably, the cytokine TNF-α is essential for both innate and adaptive responses to Spn, specially for recruitment and activation of neutrophils [133] and monocytes [91] to the site of infection. Moreover, IL-4 elicits development of $CD4^+$ Th2 cells and IgG signalling [91]. whereas IFN-γ induces activation of T-cells [134], T-regs [135] and NK-cells [136,137]. Importantly, macrophages and DCs produce anti-inflammatory cytokine IL-10 during pneumococcal disease [3] and is associated with host protection against exaggerated pro-inflammation and tissue damage, in part because of its ability to inhibit DC activity [138,139].

In murine models, the bacteria can evade adaptive immunity by inducing T-cell death [140]. As expected, this mechanism is associated with increases in the disease severity [141] and susceptibility to pneumonia [142,143] as Spn colonisation control is dependent on $CD4^+$ T-cells [144,145].

1.2 Influenza virus

The influenza virus is an single-stranded, segmented RNA virus, of spherical or filamentous form and about 80 nanometers when observed in nature

[146]. The viral lipidic envelope derives from the host cell's plasma membrane and presents surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) [147] - the main targets of immune responses [146] - as well as nucleoprotein molecules that protect the RNA strand [148]. Both proteins are known to bind to sialic acid receptors on the surface of host's cells, mediating the fusion of the viral envelope to the cell membrane [146,149,150].

Importantly, influenza virus is unique among respiratory virus regarding their capability for antigenic variations, as it continuously undergoes mutation to escape host immune mechanisms [151]. The surface proteins HA and NA can mutate in two distinct ways: antigenic drift or antigenic shift. On one hand, antigenic drift occurs when there is an accumulation of point mutations due to the virus low ability to correct errors after RNA replication [152] and is a common result of the pressure exerted by the host immunity [153]. On the other hand, antigenic shift is the exchange of segments between different influenza virus [154].

Moreover, the genetic and antigenic differences in virus HAs and NAs are commonly used to categorise into types A, B and C [146,153,155]. Importantly, humans are mostly infected by types A and B, although type C can cause subclinical disease [146,156–159].

1.2.1 Influenza epidemiology, transmission and disease

The influenza virus is responsible for an acute infection, also called influenza, with global distribution and mostly associated with winter due to increased transmission [160,161]. The constant contact between humans and the various types of influenza virus allows the host to create immunological memory. Influenza virus often causes epidemics due to its unique ability to mutate and

adapt as well as, less frequently, pandemics [156–158,162]. The epidemic and pandemic outbreaks of infection results in significant morbidity in all age groups [163], and mortality in children [164–169], elderly [170,171], as well as in patients with chronic and autoimmune diseases [172,173], resulting in over 5 million cases of severe illness and approximately half a million deaths annually [174].

Importantly, since the 16th century, at least 30 pandemic episodes have been caused by various mutants and combinations of influenza virus. Noteworthy pandemics that occurred in 1918 and 2009 (known as spanish flu and swine flu, respectively) were both caused by H_1N_1 variant of influenza virus, whereas the 1957, 1968 and 2004 pandemics (known as asian flu, Hong-Kong flu and avian flu, respectively) were caused by the H_2N_2, H_3N_2 and H_5N_1 strains [156–158,162,175,176].

Notably, the spanish flu was known as the deadliest pandemic that ever affected humans, infecting 50% of the world's population and causing 40 to 50 million related deaths [177]. The pandemic occurred in two waves, with the first being mild and the second with higher mortality as infection spread even in remote areas and islands [178].

The influenza virus incubation period ranges from 1 to 3 days and recovery usually occurs between 4 to 7 days after infection [179]. Moreover, transmission occurs through direct contact with secretions or aerosols as well as surfaces contaminated with the virus [180]. In addition, it can remain viable for 8 to 10 hours on porous surfaces and for up to 48 hours on non-porous surfaces and hands [181]. Importantly, transmission is increased under low temperature, high humidity and decreased ultraviolet radiation. Furthermore, animal hosts can serve as reservoir for virus with transmission to humans after virus mutation [182].

Importantly, symptoms include fever accompanied by respiratory and systemic symptoms such as nasal obstruction, cough, muscle aches and fatigue [183,184]. In addition, the severity of the influenza infection varies greatly, ranging from mild rhinopharyngitis to fatal lung pathology [185].

1.2.2 Immunity to influenza virus

When wild-type influenza virus infects the host, it passes through the mucus layer of the upper respiratory tract and is recognised by epithelial cells' pattern recognition receptors TLR2 and Nod2 [107,186]. In the early stages of respiratory infection, T-cells move to the site independent of their specificity [187] which allows a cascade of adaptive immune responses following virus recognition.

Adaptive immunity elicited against influenza include $CD4^+$ and $CD8^+$ T-cell proliferation as well as the production of type I IFNs, IL-6 and MCP-1 [186,188 189]. This inflammatory process recruits neutrophils [189] that limit viral replication in the nasopharynx and eliminate infected cells [190]. Although influenza virus lung infection induces recruitment of monocytes and neutrophils, it decreases their ability to phagocytose pathogens as well as the ciliary action of the epithelium mucosa [124,191–193].

Notably, $CD8^+$ T-cells play a critical role in viral clearance through production of cytokines that regulate recruitment and function of a broad array of cells [194,195]. Furthermore, these cells contributes to lysis of influenza-infected cells [196] by releasing cytolytic granules and producing cytokine IFN-γ [197–199]. Interestingly, the absence of $CD8^+$ or IFN-γ-producing $CD8^+$ T-cells were associated with severe cases of influenza [199,200].

1.2.3 Vaccination against influenza virus

A World Health Organisation committee meets twice a year to formalize the recommendation of influenza virus strains to be included in the vaccine composition [201]. This committee uses epidemiological data collected throughout the year so that the appropriate formulation for the next circulating influenza virus is obtained [202,203]. Importantly, the benefits of influenza vaccination have been extensively proven flu reduction [204–206], with reduction in otitis media incidence and use of antibiotics against secondary bacterial infections, as well as school absence and transmission [207–209].

Currently, there are two types of influenza vaccines developed for immunisation, containing inactivated or attenuated influenza virus, showed to be effective in preventing the development of the disease in multiple placebo-controlled studies [210,211]. Since 1977, the influenza vaccine composition recommendation has included three viral strains: 2 type A strains, respectively of the H_1N_1 and H_3N_2 subtypes, and 1 type B, so the vaccine is considered trivalent. Recently, since 2012, licenced vaccines that contain H_1N_1, H_3N_2 and 2 type B are also used for immunisation and are called tetravalent inactivated influenza vaccine (TIV) [212], which is given intramuscularly, and live attenuated influenza vaccine (LAIV) [213], which is administered intranasally.

Inactivated vaccines are produced with the influenza virus inactivated with formaldehyde in embryonated chicken eggs [214] and are available in whole, fractional and subunit virus forms. On one hand, the whole virus vaccine is composed of the whole viral particle, including lipidic cell membrane that is highly immunogenic [215]. On the other hand, the fractionated vaccine contains viral

components such as HA, NA and nucleoproteins, that are fragmented and then purified [216]. Moreover, the subunit vaccine consists only of HA and NA, and also provides satisfactory protection rates [217]. After vaccination with inactivated vaccines, the most frequent adverse reactions occur at the application site, such as pain and redness in 15% of vaccinated and rarely (in 1 to 2%) fever and myalgia is observed [218].

Similarly, the attenuated vaccine is also produced from the infection of influenza virus into embryonic chicken eggs, but in the presence of antibodies against the strain's surface glycoproteins [219–221]. However, contrary to the inactivated vaccine, production of attenuated influenza virus vaccine takes advantage of the segmented nature of the viral genome to recombine genes that encode wild-type virus HA and NA as well as 6 internal segments from attenuated virus (segments PB1, PB2, PA, M, NP, and NS) [222]. Additionally, adverse effects from vaccination such as nasal congestion, rhinorrhoea, fever and muscle pain [223] are uncommon, whereas severe reactions are rare, although it has been reported cases of wheezing and post-vaccine hospitalisation [223].

While both vaccines protect against influenza infection [224], studies have shown differences in the immune responses elicited by the inactivated vaccine when compared to the live attenuated one. The primary response to inactivated vaccine is from B-cells [218,225] that are activated at the immunisation site and recruited into the lymph nodes where they proliferate and specialise to migrate to other host tissues [226–229]. B-cells produce specific antibodies, specially influenza-specific IgG antibodies in the serum, and lower concentrations of IgM and IgA [230,231]. Importantly, these antibodies stimulate host response against viral HA and

NA , and can be cross-reactive [232]. Further, antibody production elicits memory [233] and adaptive immunity, especially $CD4^+$ T-cells [234,235].

On the other hand, with attenuated vaccines, the goal is to induce similar immune response to a wild-type influenza infection in the upper respiratory tract, inducing local and systemic immunity by viral replication [236], especially influenza specific memory T-cells and B-cells [237,238]. Notably, only attenuated virus stimulates recruitment of CD8 and TCR-$\gamma\delta$ T-cells [14,239]. In addition, systemic immunity is elicited by the attenuated vaccine, however, serum antibodies IgA and IgM peak only after 2 weeks post-vaccination whereas IgG takes at least 4 weeks to reach its maximum levels [240] and titres are not as marked as with inactivated vaccines [217] .

Nevertheless, even with delayed antibody responses, studies have shown that the attenuated vaccine formulation present greater persistence [241,242] and efficacy against influenza virus in vitro and in children when compared to inactivated virus vaccines [210,225,243–247]. This suggests that the most appropriate way to investigate antibody production against influenza following vaccination is by measuring responses in the nasopharynx - the site of infection and replication of the wild-type virus [248]. Indeed, the attenuated influenza vaccine has been shown to induce antibody production in nasal wash, particularly IgA until 6 months after vaccination [240], not seen in inactivated vaccines.

Interestingly, attenuated vaccines have an advantage in those with no prior immunity to influenza virus as its efficacy depends on the attenuated virus replication in the nose to elicit host immune memory [249]. However, inactivated vaccines are superior in those with greater prior exposure such as elderly, who

do not elicit immune responses to influenza after vaccination with attenuated virus vaccines [250].

1.3 Spn and wild-type influenza virus co-infection

1.3.1 Epidemiology and disease

Secondary infection following pandemic and seasonal influenza virus infection is a significant cause of mortality worldwide and, of all influenza co-infections, Spn is the pathogen most commonly detected [251] - especially during influenza pandemics [177,252–255]. Spn and wild-type influenza virus co-infection is associated with 3 times increased odds of Spn colonisation [46 256] and it causes 1 in 3 cases of bacterial pneumonia following severe influenza infection [257]. In addition, higher susceptibility to co-infection has been demonstrated in elderly and immunosuppressed, with greater risk of mortality [258]

1.3.2 Window of susceptibility to co-infection

A fine balance of pro- and anti-inflammatory responses maintains a stable and asymptomatic Spn colonisation [96], however, this equilibrium is altered by wild-type influenza virus co-infections, affecting essential inflammatory mechanisms in the nasal mucosa [190]. The impact on Spn colonisation is due to complex interactions between virus, host and bacteria, associated with morphologic and immunological alterations of the upper respiratory tract [259]. For that reason, following host initial recognition and response to virus entry in the nasopharynx, influenza infection can cause a window of susceptibility to Spn that

generally starts 48 hours after virus infection [186,260,261]. Notably, studies have demonstrated this modified state can last several months after resolution of influenza [262].

Initially, the virus causes damage to the epithelium, the first line of defence against the Spn [196]. Viral NAs denude the host epithelial surface by removing sialic acid which increases its availability as nutrients for bacteria. The damaged epithelium exposes Spn to new receptors in the membrane such as platelet-activating factor receptor (PAFR) [263,264], increasing bacterial adherence mediated by Spn such as PspC [62]. Additionally, the virus reduces mucociliary bacterial clearance [196] and desensitizes epithelial cells to Spn pathogen-associated molecular pattern, impairing bacterial TLRs [193,196,265–267].

In order to clear the virus, the host elicits production of IFN-γ mainly by $CD4^+$, $CD8^+$ T- and NK-cells, however, this cytokine also modifies macrophages in its scavenger receptor, reducing innate defences against Spn by inhibiting its phagocytosis [124,186,191,192,262,268]. Regarding this aspect, Metzger and Sun [269] hypothesize that the mechanism of macrophage receptor alteration could have evolved to prioritise specific and efficient anti-influenza T-cell memory in acute infections. Moreover, reduction in macrophage efficacy consequently diminishes the overall production of TNF-α, a strong chemoattractant of neutrophils [188,270].

In addition, neutrophil influx is also impacted by the increased expression of cytokine IL-10, stimulated by rises in IFN-γ, and type I IFNs levels produced by $CD8^+$ T-cells [92,132,186,262,271]. Importantly, epidemiological studies in humans show that $CD8^+$ T-cell levels were increased by influenza in the lung [188], a critical aspect considering the strong correlation between higher levels of IFN-γ-producing $CD8^+$ T-cells and less severity of co-infection disease [199].

Furthermore, type I IFNs are responsible for inducing anti-viral cytokines and chemokines to stop virus replication and stimulate adaptive immunity. However, the exaggerated accumulative production of type I IFNs has been related to susceptibility and Spn colonisation, indicated by mice's resistance to co-infections in the absence of type I IFN signalling [186,262].

In short, wild-type influenza virus facilitates adhesion to cells and impairs host's innate and cellular protection, resulting in increased susceptibility to colonisation by Spn due to its higher levels in the nasopharynx. Importantly, bacterial transmission is increased by influenza infections, and driven by increased Spn density [256].

1.3.3 Established Spn and wild-type influenza virus co-infection

Following establishment of Spn and wild-type influenza virus co-infection, the presence of high levels of both pathogens in the nasopharynx leads to disbalance of inflammatory responses in an attempt to contain invasive virus and bacteria [193,196,265,266]. In murine models, pneumococcal disease prior to influenza virus leads to improved survival, with less morbidity and lung immunopathology [272]. On the other hand, if virus infection occurs before bacterial infection, as first shown in a ferret model by McCullers [273], the infection presents increased Spn density in the upper respiratory tract [76,92,274]. Additionally, there is evidence that viral load also increase during co-infection [275] although the unbalanced immune responses have no proven correlation to the augmented virus titers [276]. Importantly, in humans, clearance of co-infection requires a robust adaptive immunity [271] as innate defences alone are unable to provide protection to the host [188].

Firstly, the host's nasopharynx reacts to co-infection with Spn-induced TGF-β and T-reg levels increase [92]. Importantly, exaggerated production of this cytokine during co-infection suppresses NK-cells induction and macrophages activation [132] and is associated with decrease in IL-2 concentration which, in turn, impairs T-cell development [188] and reduces levels of $CD3^+$ and $CD4^+$ T-cells [270]. Further, the established co-infection express signals to the host from both pathogens, causing reducing of TCR-γδ and Th17 T-cells levels [270].

In addition, modulation of B-cell response is altered by inhibition of cell development and activation [188,270,277]. The reduced B-cell immunity is possibly due to the decreased numbers of $CD4^+$ T-cells, which reduces support of B-cell function and production of cytokines [270]. Interestingly, murine models demonstrate that the decrease in influenza-specific T-cell response correlates with the increase in T-regs [188] due to T-reg production of adenosine, perforin and granzymes that are toxic to T-cells [278]. Moreover, similarly to influenza virus infection alone, low levels of $CD8^+$ T-cells are associated with virus and Spn co-infection severity [188,199]. Importantly, in murine models of coinfection, $CD8^+$ T-cells are presented in lower levels [188], consequently reducing production of cytokines such as TNF-α [188] and recruitment of immune cells [188].

Interestingly, studies have shown contradictory results regarding how excess production of type I IFN affects Spn and wild-type-influenza co-infection. Although there are studies where type I IFNs do not affect neutrophil recruitment [186], the majority of researches demonstrate impairment in both neutrophils [192,279,280] and macrophages due to lower expression of keratinocyte chemoattractant (KC) and MCP-1, of critical role in control of co-infection [186]. Furthermore, in co-infection models, the increased production of type I IFN

suppresses expression of pro-inflammatory cytokine IL-17 - especially by TCR-γδ - associated with increased Spn density [262] due to its involvement in the early control of bacteria by recruiting neutrophils to the infection site.

In summary, Spn and wild-type influenza virus co-infection deregulates innate and adaptive immune defences and increases Spn colonisation rates and density which, in turn, increases Spn transmission [273], susceptibility to invasive disease [186] and bacterial density [260,261].

1.4 Murine Spn and live attenuated influenza virus (LAIV) co-infection

A controversial epidemiological aspect of Spn and wild-type influenza virus co-infection is the use of live attenuated influenza virus to immunize the mucosa due to its mimicry of virus natural infection in the nasopharynx and activation of host inflammatory immune responses. Importantly, this transitory attenuated virus replication, known as viral shedding, increases Spn levels analogously to wild-type influenza virus infection and affects Spn-induced immunity as well as colonisation density, transmission and host susceptibility to invasive disease [281].

Similarly to Spn and wild-type influenza co-infection models, when mice were primarily colonised with Spn, LAIV is efficient in controlling infection and reducing mortality [76]. Additionally, in models where mice are first vaccinated, it has been shown an inflammatory state [274] presenting increased type I IFN levels [274], bacterial density and duration of Spn colonisation [282] as well as bacteria transmigration to the middle ear [274 283]. On the other hand, LAIV was not shown to increase Spn density in the lower respiratory tract [274] as the vaccine consists of temperature sensitive virus strains that replicates only in the upper airways. Interestingly, human models of co-infection with Spn and LAIV is also associated with increased Spn density [284] and disease. However, opposite to mice models, some studies indicate that LAIV in humans is protective against acute otitis media during influenza season [209,285,286].

1.5 Experimental Human Pneumococcal Challenge model for study of Spn and attenuated influenza virus co-infection

The Experimental Human Pneumococcal Colonisation (EHPC) [47] is an unique model of controlled infection that induces nasal Spn colonisation in healthy adults at a typical density and duration (1 to 3 weeks) in 50% of subjects following inoculation with different strains of Spn [47]. This model is the ideal biological system to study immune protection mechanisms as the onset, duration and termination of a colonisation episode are known and serial measurements of immune responses can be made with controlled rates of Spn acquisition and density.

In healthy adults, EHPC was used to determine that Spn challenge confers protection against recolonisation and development of invasive disease by inducing immunoglobulin production in the nasal mucosa [62] and blood [49] as well as pulmonary IL-17A$^+$ CD4$^+$ memory T-cells [287]. Furthermore, in the nasal mucosa, protection granted by EHPC is associated with pre-existing antibodies against PspA but not with antibodies against capsular polysaccharide [288]. In addition, establishment and maintenance of Spn colonisation after EHPC was associated with TGF-β1 and T regulatory cells [92].

The EHPC model has also been used as a new method for vaccine testing [51,52] and investigation of nasal microbiota and its interaction with Spn. Past studies have demonstrated that viral infections increase susceptibility to EHPC, eliciting higher levels of mucosal factor H and Spn density. This occurs due to higher Spn adherence caused by increased epithelial layer inflammation and factor H [46]. Moreover, different microbiota profiles were associated with Spn

colonisation of the nasopharynx using EHPC and different strains inoculated were demonstrated to cause disturbances in microorganism diversity [289].

1.6 Thesis Aims

Our main goals and specific aims for this thesis were to:

1. Determine the best methodology for investigation of immune responses in the nasopharynx by:
    a. Comparing phenotypes of cells collected by nasal curettage and nasal wash.
    b. Analysing cytokine levels using nasosorption and nasal wash.

2. Investigating if Spn colonisation, LAIV and TIV alter cell-mediated response in the nasal mucosa of human hosts by:
    a. Assessing recruitment of monocytes, neutrophils, T-cells and DCs.
    b. Measuring activation levels of neutrophils and T-cells.

3. Assess if Spn colonisation, LAIV and TIV alter cytokine responses in the nasal mucosa of human hosts by:
    a. Analysing induction of cytokines related to pro- and anti-inflammatory responses.
    b. Investigating levels of elicited cytokines that regulate cell growth and adaptive immunity.

4. Determine the effects of Spn colonisation, LAIV and TIV on immunity against influenza antigens by:
    a. Establishing levels of induced T-cell subsets producing cytokines after stimulation with influenza, including $CD4^+$, $CD8^+$, T-regs, TCR-$\gamma\delta$ and TRM T-cells.
    b. Comparing antibody levels to influenza (IgA and IgG) in serum, nasal wash and lung.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Funding and study approvals

Funding for Antecedent LAIV as well as Concurrent LAIV studies were received by the Bill and Melinda Gates Foundation, the UK Medical Research Council, the Liverpool School of Tropical Medicine, the Royal Liverpool, Broadgreen University Hospitals NHS Trust (United Kingdom) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Brazil).

Ethical approvals are shown in Table 2 and were obtained from the National Health Service Research Ethics Committees (REC), and Royal Liverpool and Broadgreen University Hospitals Trust. The clinical trial authorisation was granted by the Medicines and Healthcare products Regulatory Agency (MHRA). Volunteer appointments took place in the Clinical Research Unit at the Royal Liverpool University Hospital.

Table 2 Ethical and clinical trial approvals for Concurrent LAIV and Antecedent LAIV studies.

Registry	Approval
REC	14/NW/1460
EudraCT	2014-004634-26
ISRCTN Registry	ISRCTN16993271

2.2 Recruitment and ethics/ consent

The recruitment for the Antecedent LAIV study cohort took place in the period between October 2015 and April 2016 while the Concurrent LAIV study cohort was recruited between October 2016 and April 2017. Volunteers gave written and informed consent and recruited if they were healthy, non-smokers and aged 18-50.

Subjects were unable to participate if they fell under any of the following exclusion criteria during screen:

- Influenza or Spn vaccination or clinically confirmed disease in the preceding 2 years;
- Close contact with “high-risk” individuals (children under 5, immunosuppressed or elderly);
- Current febrile illness;
- Use of antibiotics;
- Immune-modulating medication;
- Pregnancy.

2.3 Study design

In both the Antecedent LAIV and Concurrent LAIV studies subjects were randomised using a permuted-block algorithm (1:1 in blocks of 10) held in sealed envelopes and distributed in two groups based on vaccination status: TIV (Control) or LAIV.

Volunteers participating in the Antecedent LAIV study were vaccinated at day -3 and inoculated at day 0 relative to Spn inoculation serotype 6B (Figure 1). On the other hand, subjects in the Concurrent LAIV study were inoculated with Spn at day 0 and vaccinated at day +3 post-inoculation. Vaccinations were done in the TIV control group with TIV flu jab (2015/2016 Fluarix Tetra, GlaxoSmithKline, UK) and the LAIV group with LAIV nasal spray (Fluenz Tetra and FluMist Tetra, AstraZeneca, UK).

In the Antecedent LAIV study, 222 participants consented, 162 were screened, 137 vaccinated (n=68 with LAIV, n=69 with TIV) and 130 were inoculated. Volunteers were excluded from analysis based on screen failure (n=5), stopping follow-up appointments (n=7), vaccination error (n=8), natural colonisation by Spn at baseline (n=1) and use of antibiotics (n=1). In addition, demographics were similar between groups (Table 4).

Furthermore, in the Concurrent LAIV study, 316 participants consented, of which 206 were screened, 198 were inoculated and 194 were vaccinated (n=97 with LAIV, n=97 with TIV). Volunteers were excluded from analysis based on screen failure (n=8), stopping follow-up appointments (n=4), vaccination error (n=13), naturally colonisation by Spn at baseline (n=13) and naturally acquired Spn colonisation during the study (n=4). Moreover, demographics were also similar between groups in this study (Table 5).

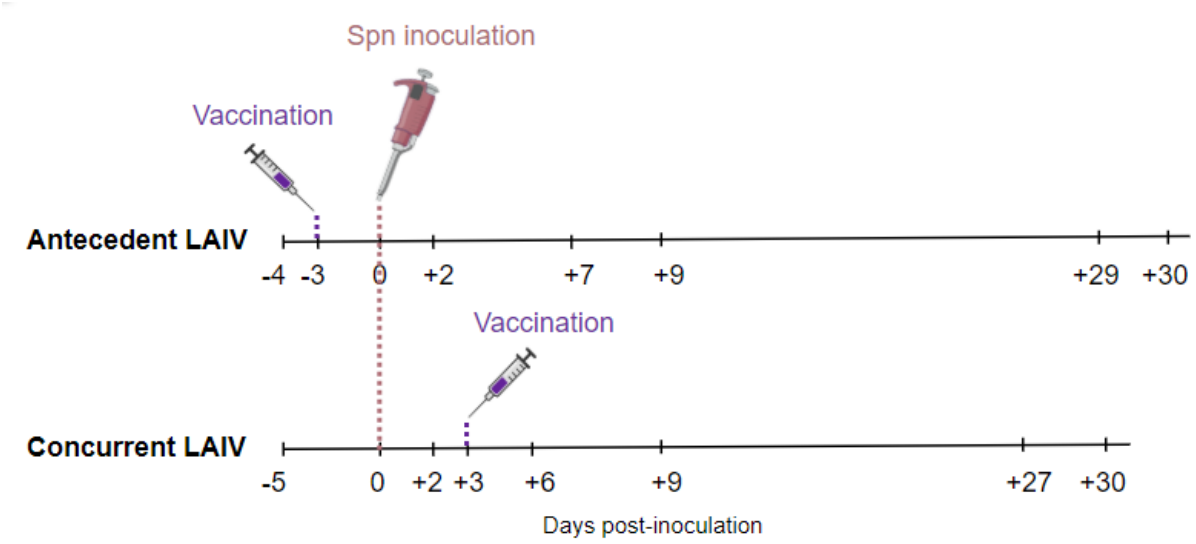

Figure 1. Design for Antecedent LAIV and Concurrent LAIV studies. The diagram represents nasopharyngeal inoculation with Spn serotype 6B as a red dotted line, and vaccination as a dotted purple line. In the Antecedent LAIV study, participants were screened on day -4, vaccinated on day -3, inoculated on day 0, and samples were collected on days +2, +7, +9, +29 and +30 relative to inoculation. In the Concurrent LAIV study, participants were screened at day -5, inoculated at day 0, vaccinated at day +3, and provided samples during visits on day +2, +6, +9, +27 and +30 post-inoculation.

Table 3. Demographics of volunteers enrolled in the Antecedent LAIV and Concurrent LAIV studies.

Antecedent LAIV			
	LAIV	TIV	Overall
Median age (range)	20.0 (18.0 - 34.0)	20.0 (18.0 - 48.0)	20.0 (18.0 - 48.0)
Female (%)	65.5	51.6	58.1
Median dose (range) – CFU/nostril	74,500 (51,000 - 88,000)	77,250 (51,000 - 88,000)	76,333 (51,000 - 88,000)
Time from vaccination to inoculation – days±SD	3.0 ± 0.1	3.0 ± 0.1	3.0 ± 0.1
Concurrent LAIV			
	LAIV	TIV	Overall
Median age (range)	20.0 (17.0 - 46.0)	20.0 (18.0 - 32.0)	20.0 (17.0 - 46.0)
Female (%)	53.4	56.7	55.2
Median dose (range) - CFU/nostril	82,167 (60,667 - 93,000)	81,083 (60,667 - 93,000)	82,167 (60,667 - 93,000)
Time from vaccination to inoculation – days ± SD	3.0 ± 0.2	3.0 ± 0.1	3.0 ± 0.1

2.4 Study schedules

During the studies, volunteers were sampled for nasal wash (NW), nasal cells, nasosorption (NS), serum and bronchoalveolar lavage (BAL) in specific timepoints after screening (Table 5 and 6). All sampling, processing and analysis were made while blinded to vaccination group and colonisation status, assuring unbiased analysis.

Table 4. Visit schedule for sample collections in the Antecedent LAIV study.

Sample collected	Screen (D-14 to D-4)	Vaccination (D-3)	Spn Inoculation (D0)	D2	D7	D9	D14	D22	D29	D30 to D197
Nasal wash	X			X	X	X	X	X	X	
Nasal cells	X			X	X	X			X	
Nasosorption	X		X	X	X	X			X	
Serum	X				X				X	
BAL										X

Table 5. Visit schedule for sample collections in the Concurrent LAIV study.

Sample collected	Screen (D-14 to D-1)	Spn Inoculation (D0)	D2	Vaccination (D3)	D6	D9	D14	D21	D27	D28 to D197
Nasal wash	X		X		X	X	X	X	X	
Nasal cells	X		X		X	X			X	
Nasosorption	X	X	X		X	X				
Serum	X				X				X	
BAL										X

2.5 Clinical procedures

2.5.1 Clinical consumables

Procedure	Consumables
Vaccination	LAIV (Fluenz Tetra and FluMist Tetra, AstraZeneca, UK); TIV (2015/2016 Fluarix Tetra, GlaxoSmithKline, UK)
Nasal cells collection	Rhinoprobe (ASL Rhino-Pro©, Arlington Scientific); PBS+Ca+Mg
Nasosorption collection	Nasosorption strip (Nasosorption™, Hunt Developments)
BAL collection	Benzodiazapine antagonist (flumazenil) Benzodiazepine (midazolam) Sphygmomanometer Instilagel (lidocaine gel) 2% and 4% lidocaine (Xylocaine) Nasal speculae Cannula (18-20G)

2.5.2 Volunteers symptoms log and evaluation of procedures

Volunteers from the Antecedent study (n=148) documented local and general symptoms for a period of 7 days since baseline (day -4). Severity ratings ranged from 1 (less severe) to 7 (more severe). Symptoms evaluated were sneezing, runny or itchy nose, congestion, throat symptoms, cough, eye or ear symptoms, headache, as well as additional symptoms added by the participants.

For evaluation of the tolerance to the sample collection procedures, volunteers were asked to use a 5-point modified Likert scale. A range of 1 to 5 was used to collect individual opinions about how painful and how uncomfortable the procedure was, along with if it made their eyes water.

2.5.3 Experimental Human Pneumococcal Carriage (EHPC) model

Volunteers (n=130 in Antecedent and n=198 in Concurrent study) received 100µl of Spn inoculum prepared in advance (see section 2.6.3) inside each nostril and remained seated for up to 15 minutes without blowing their nose or sniffing up. Each subject received an emergency pack including: Amoxicillin 9x500mg, antibiotic information sheet, thermometer and inoculation information sheet.

2.5.4 Vaccination

Participants were vaccinated (n=137 in Antecedent and n=194 in Concurrent study) with either nasal LAIV (Fluenz Tetra or FluMist Tetra, AstraZeneca, UK, used interchangeably due to acquisition shortages) paired with intramuscular placebo (0.5 mL of normal saline) or with intramuscular TIV (2015/2016 Fluarix Tetra, GlaxoSmithKline, UK) paired with nasal placebo (0.2 mL normal saline) as control. In the Antecedent study, 65 volunteers received LAIV and 64, TIV, whereas in the Concurrent study 97 subjects were vaccinated with LAIV and 97 with TIV.

2.5.5 Nasal wash collection

The NW method used in the thesis was previously described by Gritzfeld et al. [47] and based on the methodology published by Naclerio et al. [45]. For collection of NW, participants received 5 mL of saline in one nostril, rapidly expelling the liquid for collection in a foil bowl [45]. This procedure was repeated until 20 mL was used in one nostril. The liquid collected was stored at room temperature for further processing described in Section 2.6.5. Importantly, NW was performed after nasosorption but before nasal curettage in order to keep samples uncontaminated.

2.5.6 Nasal cells collection

The nasal inferior turbinates were visualised with a light with the participant being seated with the head tilted posteriorly. A curette (ASL Rhino-Pro©, Arlington Scientific) was used to scrape a small collection of cells from the nasal mucosa. Two scrapes per nostril were taken and placed in a 15 mL Falcon tube

placed on ice containing PBS+Ca+Mg (Section 2.5.1) for processing, described in Section 2.6.6. Importantly, nasal scrape was performed before NW and nasosorption since the intervention could contaminate other collected samples.

For this procedure to be done consistently, all clinical staff responsible for nasal cell collection was trained using 3D models of human noses as well as volunteers. The results of collected epithelial, immune, neutrophils, T-cells and monocytes were compared between the clinical staff. In addition, quality checks of the results were performed continuously throughout the studies and additional training was given if staff did not show the accuracy necessary for trustworthy analysis.

2.5.7 Nasosorption collection

The nasosorption collection technique used in this thesis was based on the methodology previously published by Thwaites et al. [290]. An adsorptive matrix strip (Nasosorption™, Hunt Developments) was held parallel to the volunteers' nasal septum with the absorptive part inside one of the nostrils for nasal fluid collection. The nostril was pressed so that the strip held its place for 2 minutes. After that, the strip was deposited in its original tube and froze immediately in a -80^{o}C freezer for further analysis. Importantly, nasosorption was the first sample collected from the nose of volunteers, before NW and nasal curettage, in order to preserve the nasal lining from contamination.

2.5.8 Serum collection

Serum was sampled from volunteers by collecting 5 mL of blood and storing in a tube with anticoagulant EDTA. Immediately after collection, the sample was placed in a -80^{o}C freezer for future experiments.

2.5.9 Bronchoalveolar lavage collection

The collection of BAL cells was conducted at the clinical research unit (CRU) of the Royal Liverpool Hospital. To assure safety during the procedure, monitoring equipment was available (three lead ECG, pulse oximeter, spygnomanometer and anaesthetic support, Section 2.5.1).

First, oxygen was delivered via nasal cannula at up to 4L/min and topical anaesthesia with lidocaine was achieved in the nasal passages (using Instilagel) and the oral mucosa (using Xylocaine). After topical anaesthesia at the larynx

was completed using 4% lidocaine, further mucosal anaesthesia was achieved by using 2 mL aliquots of 2% lignocaine.

Following, the bronchoscope was inserted in the nostril and positioned. Further, hand suction was performed using 50 mL saline syringe and repeated 3 times, using a maximum volume of 200mL. The sample was kept in ice and the bronchoscope was slowly withdrawn and processed as described in Section 2.6.8. For an additional following of volunteers' health, participants were followed-up at 1-5 days post-procedure with a clinical examination.

2.5.10 Safety monitoring

Every volunteer that enrolled in the studies was required to send a text message to the clinical team every day for a week. If the text was not received, the volunteer was contacted personally to ensure their safety.

Antibiotics were used if the volunteer was colonised with Spn at the end of the study or in the event they were unwell. In case of serious disease, a direct admission to the Infectious Disease ward at the Royal Liverpool University Hospital was available.

2.6 Laboratory procedures

2.6.1 Laboratory consumables

Procedure	Consumables
Bacterial stock preparation	- Blood agar plates PB0122A (Oxoid, Basingstoke, UK) - Vegitone media (Oxoid, Basingstoke, UK) - Glycerol - PBS (Thermo Fisher Scientific, Waltham, MA) - 6 and 12-well plates (Thermo Fisher Scientific, Waltham, MA) - Spectrophotometer (FLUOstar Omega plate reader)
Experimental Human Spn Inoculation	- Blood agar plates PB0122A (Oxoid, Basingstoke, UK) - 96-well Nunc Maxisorp plates (Thermo Fisher Scientific, Waltham, MA)
Nasal cells staining	- 70 µm filter (Thermo Fisher Scientific, Waltham, MA) - Cytometer (LSRII, BD Bioscience, UK) - Antibodies for Concurrent LAIV study: Epcam-PE; HLADR-PE.Cy7; CD66b-FITC (Biolegend, San Diego, CA); CD3-APC.Cy7; CD14-Percp.Cy5.5 (BD Biosciences, San Jose, California, USA); CD45-PACOrange (Thermo Fisher Scientific, Waltham, MA). - Antibodies for Antecedent LAIV study: Epcam-PE; HLADR-PE.Cy7; CD16-APC; CD66b-FITC, CD19-BC650, CD8-BV785 (Biolegend, San Diego, CA); CD3-APC.Cy7; CD14-Percp.Cy5.5 (BD Biosciences, San

	Jose, California, USA); CD45-PACOrange (Thermo Fisher Scientific, Waltham, MA). - Anti-Rat Ig, κ/Negative Control Compensation Particles Set (BD Biosciences, San Jose, CA). - Anti-Mouse Ig, κ/Negative Control Compensation Particles Set (BD Biosciences, San Jose, CA).
Luminex human cytokine 30-plex	- Human cytokine magnetic 30-plex panel (Thermo Fisher Scientific, Waltham, MA) - Hu Cytokine Mag 30-plex antibody bead solution (Thermo Fisher Scientific, Waltham, MA) - Hu Cytokine Mag 30-plex Biotinylated Ab (Thermo Fisher Scientific, Waltham, MA) - Hu 14-Plex standard (Thermo Fisher Scientific, Waltham, MA) - Hu 16-Plex standard (Thermo Fisher Scientific, Waltham, MA) - Wash Solution Concentrate (Thermo Fisher Scientific, Waltham, MA) - Incubation buffer (Thermo Fisher Scientific, Waltham, MA) - Biotin Diluent (Thermo Fisher Scientific, Waltham, MA) - Streptavidin RPE diluent (Thermo Fisher Scientific, Waltham, MA) - Streptavidin RPE concentrate (Thermo Fisher Scientific, Waltham, MA) - Assay diluent (Thermo Fisher Scientific, Waltham, MA) - Luminex MAGPIX calibration/verification kits. Calibration Kit MPX-CAL-K25 and Performance Verification Kit MPX-PVER-K25 (Thermo Fisher Scientific, Waltham, MA)

	- Luminex MAGPIX + xPonent software for Luminex (Thermo Fisher Scientific, Waltham, MA) - 96-well Nunc Maxisorp plates (Thermo Fisher Scientific, Waltham, MA) - Magnetic 96-well plate separator (Thermo Fisher Scientific, Waltham, MA) - Luminex Flat bottom 96-plate and black cover (Thermo Fisher Scientific, Waltham, MA)
BAL processing	- 6-, 12-, 24-, 48- and 96-well plates (Thermo Fisher Scientific, Waltham, MA) - Complete medium: RPMI, 10% of heat-inactivated fetal bovine serum (FBS, Thermo Fisher Scientific, Waltham, MA), PSN antibiotic mixture (Penicillin-Streptomycin-Neomycin). - FBS-DMSO: 90% heat-inactivated fetal bovine serum (FBS) and 10% Dimethyl sulfoxide (DMSO), (Thermo Fisher Scientific, Waltham, MA)
BAL intracellular staining	- PBS (Thermo Fisher Scientific, Waltham, MA) - Cytometer (LSRII, BD Bioscience, UK and Becton Dickinson, UK) - TIV (2015/2016 Fluarix Tetra, GlaxoSmithKline, UK) - Antibodies: CD3-APCH7 (clone SK7), TCR-γδ–PECy7 (clone 11F2), IL-10–BV786 (clone JES3-9D7), IL-17A–BV510 (clone N49-653), (BD Biosciences, San Jose, California, USA), CD4–PerCP5.5 (clone SK3), CD8–AF700 (clone SK1), CD69–BV650 (clone FNSO), CD25-PE.TxsRed (clone M-A251), CD103–BV605 (clone Ber-ACT8), CD49a-APC (clone TS2/7), FOXP3-FITC (clone 259D), IFN-γ-PE (clone 4S.B3), TNF-α–BV711 (cloneMAb11) (Biolegend, San Diego, CA).

	- Anti-Rat Ig, κ/Negative Control Compensation Particles Set (BD Biosciences, San Jose, CA). - Anti-Mouse Ig, κ/Negative Control Compensation Particles Set (BD Biosciences, San Jose, CA). - ArC Amine Reactive Compensation Bead Kit (Invitrogen Corporation, Carlsbad, CA).
IgG and IgA ELISA	- 96-well Nunc Maxisorp plates (Thermo Fisher Scientific, Waltham, MA) - TIV (2015/2016 Fluarix Tetra, GlaxoSmithKline, UK) - PBS (Sigma Aldrich, Gillingham, UK) with 0.005% Tween 20 (Sigma, PP1379, Deisenhofen, Germany) - 1% bovine serum albumin (BSA) in PBS - 0.1% bovine serum albumin (BSA) in PBS - Detection antibodies: anti-human-IgG (Sigma, A3188, Deisenhofen, Germany) and anti-human-IgA (Sigma, I1261, Deisenhofen, Germany) - Streptavidin−Alkaline Phosphatase (Bio-rad, STAR6B, Hercules, CA) - p-Nitrophenyl Phosphate (Sigma-Aldrich, Poole, U.K.) - FLUOstar Omega ELISA microplate reader (BMG Labtech) - Omega Analysis (BMG Labtech).

2.6.2 Reagent preparations

2.6.2.1 For Experimental Human Spn Inoculation

Reagent	Consumables and procedures
STGG medium[291]	Consumables: - Oxoid tryptone-soya broth (CM 129) 3.0mL - glucose 0.5g - Oxoid skim milk powder (CM L31) 2.0g - glycerol 10.0mL; double distilled water 100.00mL.
	Procedure: 1 mL amounts were dispensed into bijoux's and autoclaved at 15lb for no more than 10 min. The tubes were stored at 4-6°C.

2.6.2.2 For nasal wash processing

Reagent	Consumables and procedures
STGG medium[291]	Consumables: - Oxoid tryptone-soya broth (CM 129) 3.0mL - glucose 0.5g - Oxoid skim milk powder (CM L31) 2.0g - glycerol 10.0mL; double distilled water 100.00mL.
	Procedure: 1 mL amounts were dispensed into bijoux's and autoclaved at 15lb for no more than 10 min. The tubes were stored at 4-6°C.

2.6.2.3 For nasal cells processing and staining

Reagent	Consumables and procedures
PBS+Ca+Mg	Consumables: - phosphate-buffered saline (PBS, Thermo Fisher Scientific, Waltham, MA) - heat-inactivated fetal bovine serum (FBS, Thermo Fisher Scientific, Waltham, MA) - ethylenediaminetetraacetic acid (EDTA, Thermo Fisher Scientific, Waltham, MA)
	Procedure: Phosphate-buffered saline (PBS) + 0.5% heat-inactivated fetal bovine serum (FBS) and 2.5 mM ethylenediaminetetraacetic acid (EDTA)
Violet viability dye working solution	Consumables: - LIVE/DEAD Fixable Dead Cell Stain kit (Invitrogen, UK) - PBS+Ca+Mg
	Procedure: 1µL of Violet Live/Dead staining in 500µL PBS+Ca+Mg.

2.6.2.4 For Luminex human cytokine 30-plex

Reagent	Consumables and procedures
Wash buffer	Consumables: Wash Solution Concentrate (Thermo Fisher Scientific, Waltham, MA)
	Procedure: 15 mL of Wash Solution Concentrate (20X) to 285 mL of ddH20.
Detector antibody mix	Consumables: Biotin Diluent and Biotinylated Antibody (Thermo Fisher Scientific, Waltham, MA)
	Procedure: 25µL of Biotin Diluent and 2.5µL of 10x Biotinylated Antibody per well
Substrate mix	Consumables: RPE-Diluent and Streptavidin-RPE (Thermo Fisher Scientific, Waltham, MA)
	Procedure: 25µL of RPE-Diluent and 2.5µL of 10x Streptavidin-RPE per well

2.6.2.5 For staining of non-adherent cells from BAL tissue

Reagent	Consumables and procedures
Complete Medium	- RPMI (Thermo Fisher Scientific, Waltham, MA) - FBS (10%, heat inactivated, Thermo Fisher Scientific, Waltham, MA) - PSN antibiotic mixture (Penicillin-Streptomycin-Neomycin)
TIV for stimulation	TIV (2015/2016 Fluarix Tetra, GlaxoSmithKline, UK)
Violet Viability dye working solution	Consumables: - Violet Viability dye (LIVE/DEAD Fixable Dead Cell Stain kit, Invitrogen, UK) - PBS (Thermo Fisher Scientific, Waltham, MA)
	Procedure: 1µL of Live/Dead diluted in 300µL PBS
BD Golgiplug	BD Golgiplug (BD Biosciences, San Jose, California, USA)
Fix/Perm working solution	Consumables: Foxp3/Transcription Factor Staining Buffer Set (eBioscience, 00-5523)
	Procedure: 300µL of FOXP3 Fix/Perm Concentrate in 900µL of FOXP3 Diluent.
FIX/Perm wash buffer	Consumables: Foxp3/Transcription Factor Staining Buffer Set (eBioscience, 00-5523)
	Procedure: 90µL of 10x Permeabilisation buffer (Foxp3/Transcription Factor Staining Buffer Set, eBioscience, 00-5523) in 810µL of ddH2O

2.6.3 Bacterial stock preparation

For the preparation of the bacterial stock used in the Experimental Human Spn Inoculation, first a “parent” stock was prepared, quantified, aliquoted and frozen for future use. To assure no animal products besides Spn was inoculated into volunteers, the stock was grown in Vegitone Infusion broth (Oxoid, Basingstoke, UK) as well as all processing involving the bacteria was performed with sterile fumehood, incubator and pippetes.

The first stock was prepared by streaking Spn of serotype 6B in a blood agar plate (Oxoid, Basingstoke, UK), which was incubated overnight at 37°C and 5% CO_2. After that, each half of the plate was swabbed and mixed separately with 12 mL of Vegitone. The cell cultures were incubated in plates (Thermo Fisher Scientific, Waltham, MA) at 37°C until detection of turbidity in the liquid was achieved or at least for a period of 2 hours.

Subsequently, 40 mL of Vegitone was added to each culture, the OD was quantified in a Spectrophotometer and adjusted to 0.15 OD at 620nm using the Bacterial stock quantification method (see section 2.6.3.1) for further incubation. During incubation at 37°C and 5% CO_2, the culture’s OD were measured hourly until an OD between 0.30-0.35 - early-mid log phase - was achieved. In one culture, 10% sterile glycerol was added, and 1 mL aliquots were prepared and frozen in a -80°C freezer while the other culture was centrifuged at 3345xg for 15 minutes. Afterwards, the supernatant was removed, and the pellet was resuspended with 22.5 mL of Vegitone.

Aliquots of 1 mL were prepared and stored at -80°C. Later, 10% sterile glycerol was added to the culture and after at least 48 hours, three aliquots were quantified to ensure the accuracy in the stocks’ CFU.

2.6.3.1 Bacterial stock quantification

To determine the CFU/mL in the final aliquots of bacterial stock, we diluted it by adding 20µL of stock to 180µL of sterile saline. After that, a dilution of 1:10 was prepared in a 96-well plate (Thermo Fisher Scientific, Waltham, MA) and three 10µL drop of the 6 first dilutions were added to a blood agar plate divided in 6 parts (Figure 2). The plate was left to dry and then incubated for 9 to 16 hours at 37°C and 5% CO_2.

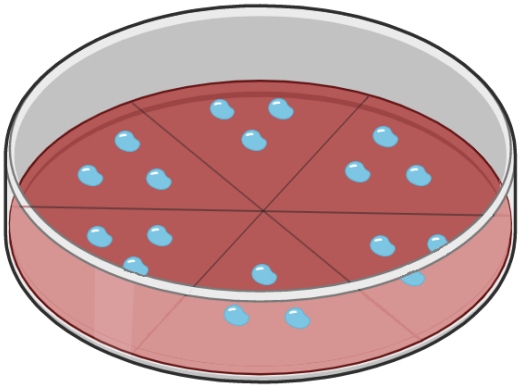

Figure 2. Agar blood plate layout for quantification. Division of agar blood plate into 6 sections represented by black lines and three 10µL drops of diluted bacterial stock per session represented by blue drops.

In the next day, the visible colonies in each plate division were counted by dividing the number of colonies by three, taking the average number, multiplying by the dilution factor and further dividing by the volume of 10mL. Finally, the number was multiplied by 1000 to obtain a result in CFU/mL as described in the equation below with *x* being the number of colonies:

$$\left(\frac{\frac{x}{3} \times 10^{x}}{10\mu L}\right) \times 1000$$

2.6.4 Experimental Human Spn Inoculation

For the inoculation of volunteers, after quantification of stable and accurate CFU results from the bacterial stock, an aliquot was thawed from the -80ºC freezer and centrifuged at 17000xg for 3 minutes. The supernatant was removed, the pellet was resuspended with 1 mL of saline and centrifuged again with the same specifications. One more time, the supernatant was removed, and 1 mL of saline was added to the sample.

Using the original concentration of stock, the aliquots were diluted 1:10 according to the desired dose of 80,000CFU/100µl by using the Bacterial stock quantification procedure (see section 2.6.3.1).

2.6.5 Nasal Wash Processing

Following NW collection (see section 2.5.4), the sample obtained from the volunteer was immediately centrifuged at 3345xg for 10 min at room temperature. Aliquots of 1 mL were taken from the supernatant and stored in a -80ºC freezer for further analysis.

After that, the pellet was resuspended with 100µL of STGG and quantified for investigation of respiratory pathogens, using the Bacterial stock quantification procedure (see section 2.6.3.1).

2.6.6 Nasal cells processing

Upon collection of nasal cells described in Section 2.5.5, the sample was dislodged from the rhinoprobes by washing the cells with PBS+Ca+Mg (Section 2.6.2.3). Subsequently, the cells were centrifuged at 440xg for 5min at 4^{o}C and the supernatant was removed so the cells could be stained (see section 2.6.6.1).

2.6.6.1 Nasal cells staining

After processing, the nasal cells from volunteers were resuspended in 50µL of Violet viability dye working solution (Section 2.6.2.3) and incubated for 15 minutes in ice. Afterwards, the antibody mixture was added accordingly (Section 2.6.6.1.1) and incubated for 15 minutes in ice.

Then, the stained cells were washed with 3.5 mL of PBS+Ca+Mg (Section 2.6.2.3) at 440g for 5mins and 4^{o}C, resuspended, filtered over a pre-wetted 70µm filter and placed into a tube for further cytometry analysis. The contents were centrifuged again and resuspended in 200µL of PBS+Ca+Mg in the Antecedent LAIV and of cell fix (BD Biosciences) in the Concurrent LAIV study for acquisition on a flow cytometer (LSRII, BD Bioscience, UK and Becton Dickinson, UK).

2.6.6.1.1 Antibodies for nasal cell staining

In order to investigate cells populations in the human nasopharynx and cell-mediated immune responses to LAIV and Spn colonisation, nasal cells were stained for monocytes, neutrophils, lymphocytes and cell activation markers.

During the Antecedent LAIV study (Table 6), cells were dyed with the extracellular antibodies CD45 (for identification of neutrophils and immune cells), Epcam (the epithelial cellular adhesion molecule was used as a marker for

epithelial cells), CD14 (to distinct monocytes), CD3 (for recognition of T-cell population), CD16 and CD66b (for measurement of neutrophil recruitment) as well as the marker HLA-DR was analysed, an MHC class II cell surface receptor expressed by human leukocytes and commonly used to identify an activation signal [292,293].

Likewise, in the Concurrent LAIV study (Table 6), volunteers' cells were stained with CD45, Epcam, CD14, CD19, CD3, CD66b and HLADR, with additional BDCA-1, BDCA-2, CD4 and CD8, markers for a deeper immunophenotyping of subsets of dendritic and T-cells present in the sample. The results were analysed using FlowJo X (Treestar Oregon, USA) and the gating strategy is shown in Figures 3 and 4 for the Antecedent and Concurrent LAIV studies, respectively. Samples with less than 500 immune cells (15% of all samples measured) or 250 epithelial cells were excluded from further analysis.

Flow cytometry compensation was set individually for each of the fluorochromes in the panel in order to adjust for spillover (physical overlap) between fluorochrome channels and acquire trustworthy results. Two sets of single-stained compensation samples were prepared using Anti-mouse and anti-rat BD positive and BD negative compensation beads accordingly (Biosciences, San Jose, CA, Section 2.6.1). Compensation staining for the Violet fluorescent dye was performed separately using the ArC Amine Reactive Compensation Bead Kit (Biosciences, San Jose, CA, Section 6.2.1).

Table 6. Surface antibody markers applied to nasal cells in each study as well as volume used for each sample.

Antecedent LAIV study	
Antibody	Volume per test (µL)
CD3-APC.Cy7	3
CD16-APC	5
CD14-PerCP.Cy5.5	5
CD66b-FITC	5
CD45-PacOrange	5
EpCAM-PE	5
HLA-DR-PE.Cy7	5

Concurrent LAIV study	
Antibody	Volume per test (µL)
CD66b-FITC	1
EpCAM-PE	1,5
HLA-DR-PE.cy7	1,5
CD8-BV785	1,5
CD45-PacOrange	3
CD3-APC.Cy7	3
CD19-BV650	3
CD4-BV605	3
CD14-PerCP.Cy5.5	5
BDCA-1-BV711	5
BDCA-2-APC	5

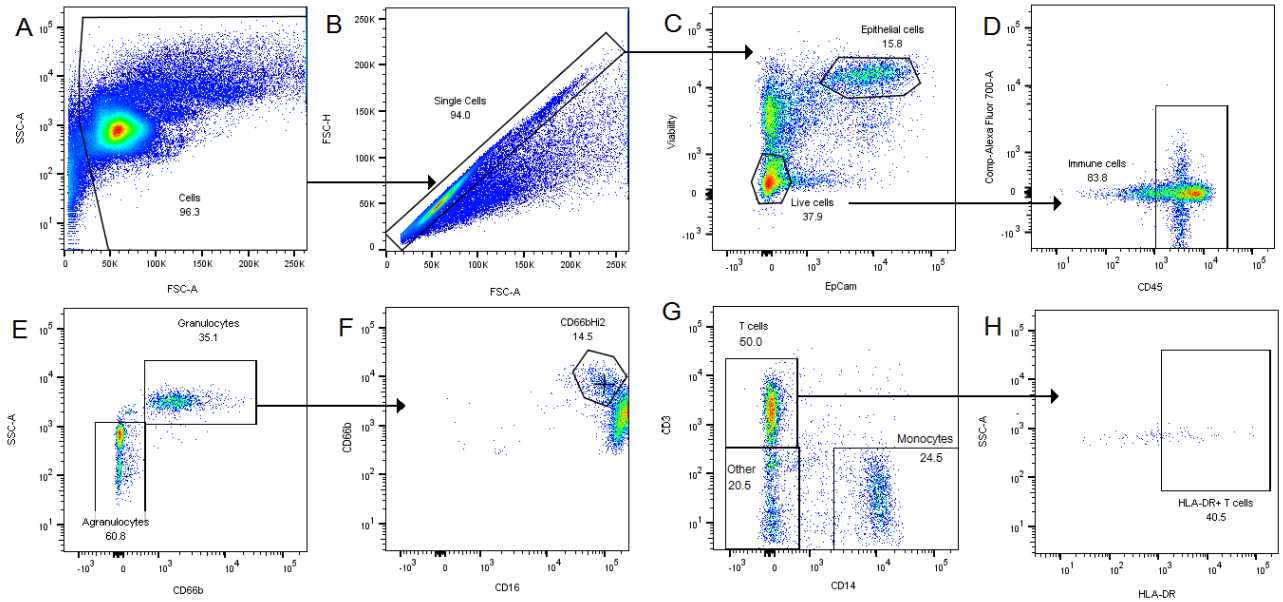

Figure 3. Representative flow plots of nasal ells from the Antecedent LAIV study analysis by flow cytometry. The gating tree was set as follows. A: FSC-A/FSC/SSC-A (represents the distribution of cells in the light scatter based on size and intracellular composition, respectively) to B: FSC-H/FSC-A (to distinct single cells) to C: Viability/Epcam (Live gate represents the fraction of viable cells within the sample analysed and Epcam was used for identification of epithelial cells) to D: AlexaFluor/CD45 positive (represents the immune cells) to E: SSC-A/CD66b (identifies non-neutrophils and neutrophils) to F: CD66b/CD16 (from the analysed neutrophils, CD66bHi were gated) or G: CD3/CD14 (from the analysed aneutrophils, T-cells, monocytes and other cells populations were identified) to H: SSC-A/HLA-DR (from analysed T-cells, HLA-DR^{+} cells were gated)

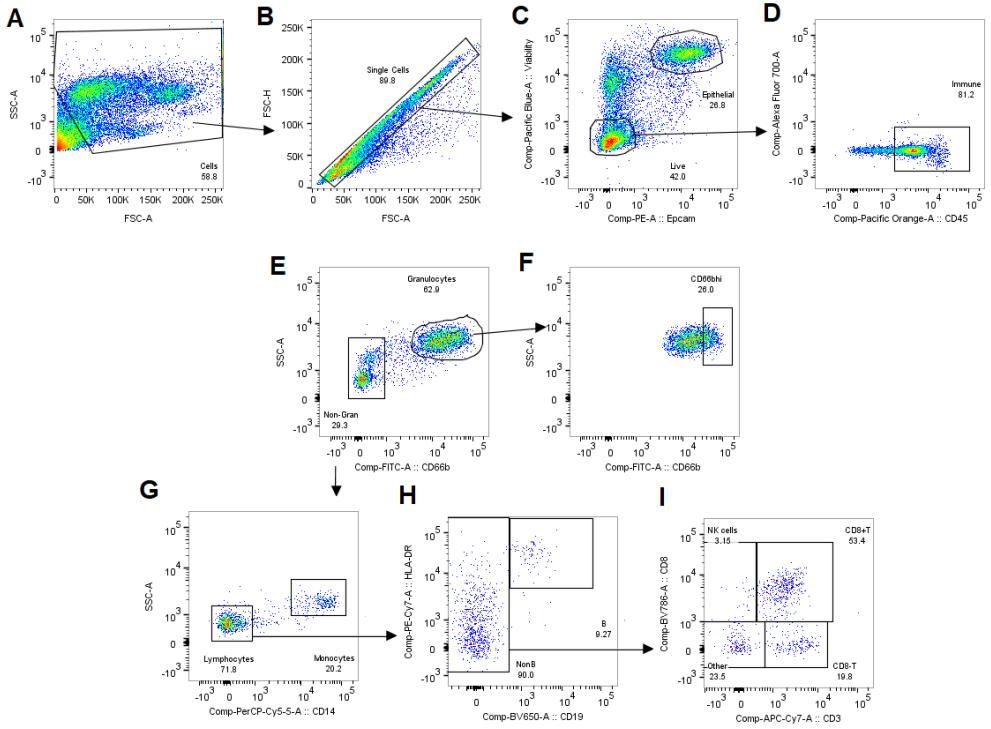

Figure 4. Representative flow plots of nasal T-cells from the Concurrent LAIV study analysed by flow cytometry. The gating tree was set as follows. A: FSC-A/FSC/SSC-A (represents the distribution of cells in the light scatter based on size and intracellular composition, respectively) to B: FSC-H/FSC-A (to distinct single cells) to C: Viability/Epcam (Live gate represents the fraction of viable cells within the sample analysed and Epcam was used for identification of epithelial cells) to D: AlexaFluor/CD45 positive (represents the immune cells) to E: SSC-A/CD66b (identifies non-neutrophils and neutrophils) to F: SSC-A/CD66b (from the analysed neutrophils, CD66bHi were gated) or G: SSC-A/CD14 (from the analysed non-neutrophils, T-cells, monocytes and other cells populations were identified) to H: HLA-DR/CD19 (identifies B-cells) to I: CD8/CD3 (from non-B-cells, identifies NK cells, $CD8^+$ T-cells and $CD8^-$ T-cells).

2.6.7 Luminex Human Cytokine 30-plex of nasal fluid

Nasal fluid was collected, processed and frozen from a set of volunteers (see section 2.5.6). After thawing, the nasosorption strip (NS) was washed with 100µL of Assay Diluent (Thermo Fisher Scientific, Waltham, MA. Section 2.6.1) and centrifuged for 10 min at 16000xg in order to elute the sample. Subsequently, 30µL of the liquid was placed on a plate followed by addition of 30µL of Assay diluent and 30µL of incubation buffer to each well.

Afterwards, 75µl of the mixture was transferred to the Luminex plate. Furthermore, 60µL of standard was combined with 30µL of incubation buffer and 75µL of this solution was also moved to the Luminex plate (all Thermo Fisher Scientific, Section 2.6.1). At this time, 12.50µL of Antibody Bead was added into the wells of the Luminex plate filled with samples or standards. The duplicate samples were acquired by transferring 43.75µl from a well to the well beside it. The plate was incubated overnight at 4ºC under agitation on an orbital shaker (500 rpm agitation).

On the next day, the plate was washed twice with 200µL Wash Solution and 25µL Biotinylated Detector Antibody was added to each well (Thermo Fisher Scientific). Following incubation for 1 hour, the plate was washed twice one more time and 25µL Streptavidin-RPE solution was added to each assay well (Thermo Fisher Scientific). The samples were incubated for 30 minutes at room temperature on an orbital plate shaker.

Moreover, the wells were washed three times and 105µL 1X Wash Solution to each assay well and placed on an orbital plate shaker for 2–3 minutes prior to analysis. The plate was then acquired using a 30-plex magnetic human Luminex cytokine kit ((Thermo Fisher Scientific). Results were analysed on a

LX200 with xPonent 3.1 software following manufacturer's instructions. Cytokines with a CV>50% for a given sample were excluded from further analysis.

2.6.8 BAL Processing

2.6.8.1 Isolation of non-adherent cells from BAL tissue

The BAL sample collected from a set of volunteers (see Section 2.5.8) was passed through a sterile medical cotton gauze into 50 mL Falcon tubes in ice in order to remove viscous portions of the lavage. Next, 10 mL of sample from the filtered sample was separated and centrifuged at 400xg for 10min at 4ºC. Its supernatant was removed, and the pellet resuspended with 2 to 3 mL of complete medium (Section 2.6.1). After that, the centrifugation process was carried on again and the pellet was resuspended in FBS-DMSO (Section 2.6.1) to obtain a concentration of $2x10^5$ cells/mL Finally, the BAL cells were stored in a -80ºC freezer overnight then transferred to liquid nitrogen for storage and further analysis.

In order to isolate BAL lymphocytes for antibody staining, on the next day, the aliquot was resuspended and washed with 1 mL of complete medium at a time until clean. The sample was topped up with media to complete 50 mL and centrifuged at 400xg for 10 minutes in 4ºC. The pellet was then reconstituted with complete medium and plated (Thermo Fisher Scientific) to obtain a final concentration of $5x10^5$ macrophages per well. Following, the plates were incubated at 37°C for 2 to 3 hours to promote adherence of macrophages. Finally, the non-adherent cells were removed from the plate supernatant after rinsing and washing with 3 mL of complete medium.

2.6.8.2 Intracellular staining of non-adherent cells from BAL tissue

Non-adherent BAL cells obtained from volunteers (see section 2.6.8.1) were counted and incubated at $1x10^6$ cells/mL in complete medium at 37ºC. Samples were stimulated with influenza antigens (TIV) concentrated at 1.2μg/mL (see Section 2.6.2.5) or left unstimulated as negative control and incubated for 2 hours. Then, 1000x diluted BD Golgiplug (see Section 2.6.2.5) was added and cells were incubated for an additional 16 hours as previously described.

After 16 hours, the cells were washed with 3 mL of PBS, resuspended and stained with Violet Viability dye working solution (see Section 2.6.2.5). After 15 minutes, cells were stained with the surface markers CD3-APCH7 (clone SK7), TCR-γδ–PECy7 (clone 11F2) from BD Biosciences (San Jose, California, USA), CD4–PerCP5.5 (clone SK3), CD8–AF700 (clone SK1), CD69–BV650 (clone FNSO), CD25-PE.TxsRed (clone M-A251), CD103–BV605 (clone Ber-ACT8), CD49a-APC (clone TS2/7) from Biolegend (San Diego, CA) according to Table 7 and incubated for 15 minutes. Cells were fixed and permeabilised using the Foxp3/Transcription Factor Staining Buffer Set as per manufacturer's instructions described in Section 2.6.2.5. Cells were stained with intracellular markers FOXP3-FITC (clone 259D), IFN-γ-PE (clone 4S.B3), TNF-α–BV711 (cloneMAb11) Biolegend (San Diego, CA) and IL-10–BV786 (clone JES3-9D7) IL-17A–BV510 (clone N49-653) from BD Biosciences (San Jose, California, USA). (Table 7). For investigation of TRM T-cell responses to influenza, we used the extracellular markers CD69, CD103 and CD49a. In this thesis, TRM was ultimately defined as $CD4^+$ $CD69^+$ cells, following scientific consensus [294], as over one third of $CD4^+$ $CD69^+$ cells did not express the additional resident memory

markers CD103 and CD49a. In addition, to assess the frequency of regulatory T-cells (T-regs) in the lung, we measured the frequency of CD25hi FOXP3^{+} T-regs among CD4^{+} T-cells using intracellular staining.

After 30 minutes, samples were washed with 3 mL of PBS and resuspended in 200µL of PBS for acquisition on a BD LSR flow cytometer (Becton Dickinson, UK). Flow cytometry data was analysed using FlowJo cell analysis software version 10 (FlowJo, LLC, Ashland, Ore) and the gating strategy as shown on Figure 5 and 6. For participants displaying less than 500 immune cells for a given sample, the participant was excluded from further analysis. Similarly, to staining nasal cells, flow cytometry compensation was done individually for each of the fluorochromes in the panel as described on section 2.6.6.1.1.

Table 7 Surface antibody markers applied to BAL cells as well as volume used for each sample.

Extracellular staining	
Antibody	Volume (µL)
CD49a-APC	3
CD69 – BV650	3
CD3-APCH7	3
TCR-γδ – PECy7	3
CD25-PE.TxsRed	2
CD103 – BV605	1.5
CD4 – PerCP5.5	0.5
CD8 – AF700	0.5

Intracellular staining	
Antibody	Volume (µL)
FOXP3-FITC	5
IL-10 – BV786	3
IFN-γ - PE	3
IL17A – BV510	3
TNF-α – BV711	3

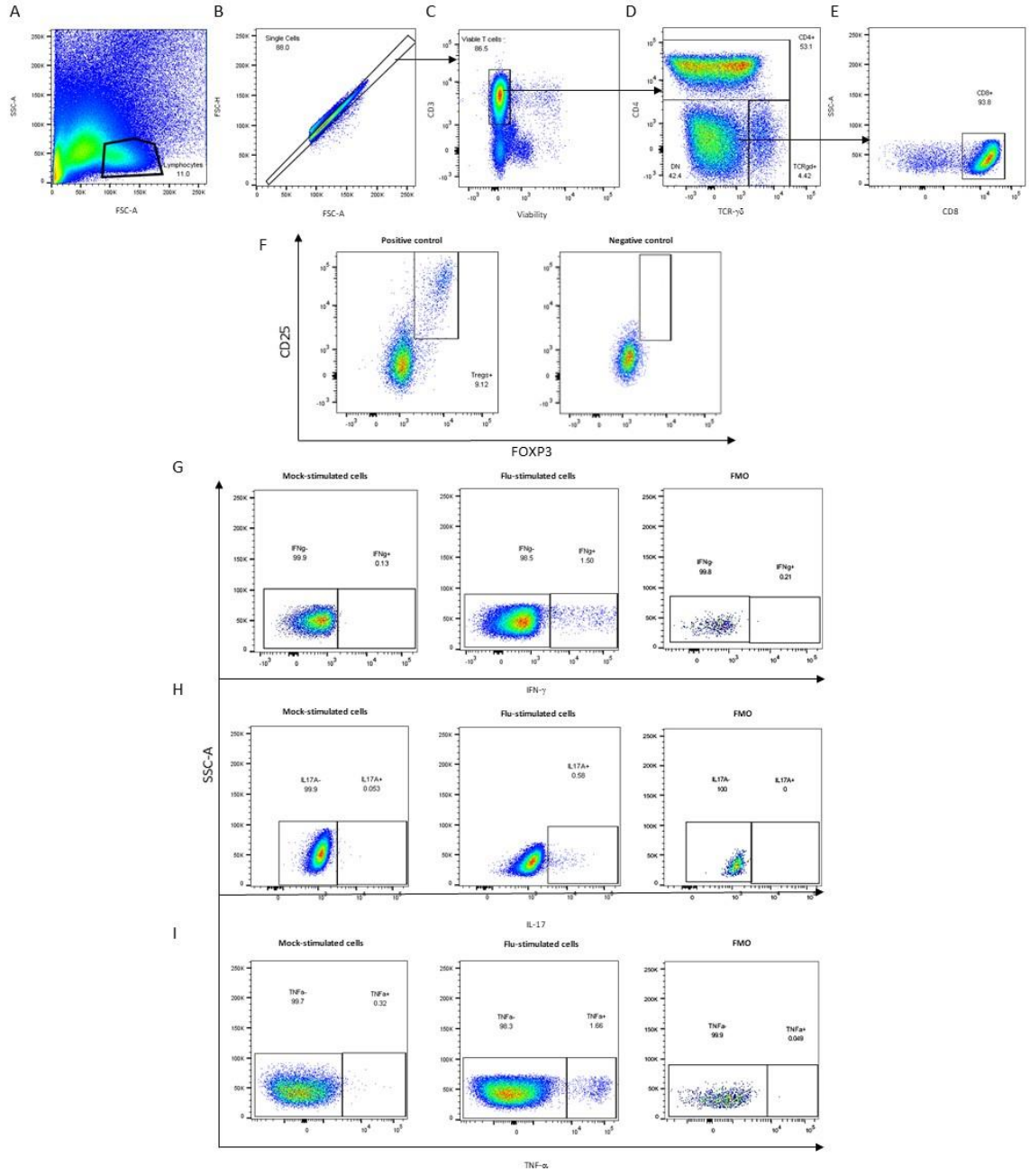

Figure 5. Representative flow plots of BAL T-cells and cytokine production analysed by flow cytometry using intracellular staining. To assess the frequency of cells in human BAL, we employed the following gating strategy. (A) FSC-A/SSC-A to (B) FSC-H/FSC-A (in order to exclude doublets) to (C) CD3/Viability to (D) CD4/TCR-γδ to (E) SSC-A/CD8. (F) The markers CD25/FOXP3 ($CD25^{hi}$ and $FOXP3^{+}$) were used to assess the frequency of T-regulatory cells in human BAL after restimulation with influenza antigens. A negative control ($CD25^{-}$ and $FOXP3^{-}$) was used to validate flow cytometric data. To assess cellular production of (G) IFN-γ, (H) IL-17A, (I) TNF-α, cells were stained by intracellular staining after overnight mock or influenza-stimulation. Fluorescence Minus One (FMO) controls were used to verify flow cytometric data

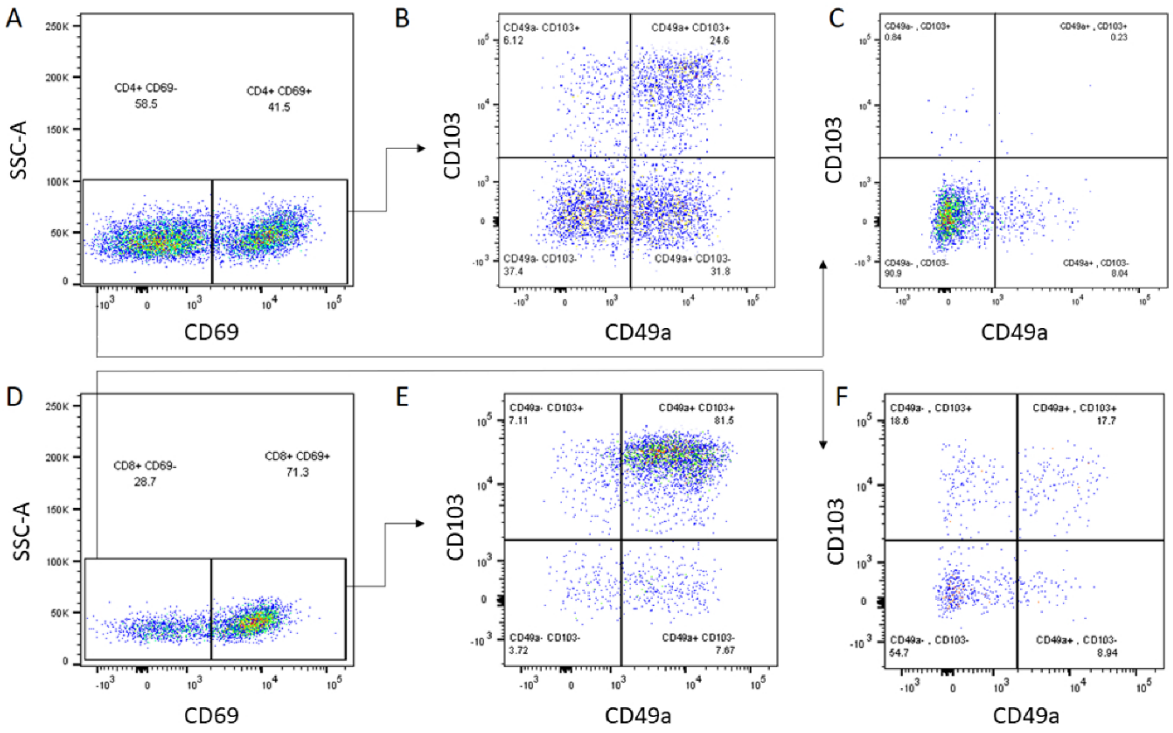

Figure 6. Representative plots of TRM T-cells identified by flow cytometry. The markers CD69, CD103 and CD49a were used to assess the frequency of TRM cells in human BAL. (A) $CD4^+$ T-cells gated into CD69 negative and positive cells. CD103 and CD49 marker expression are shown for (B) $CD4^+$ $CD69^+$ T-cells and (C) $CD4^+$ $CD69^-$ T-cells. (D) $CD8^+$ T-cells gated into CD69 negative and positive cells. CD103 and CD49 marker expression are shown for (E) $CD8^+$ $CD69^+$ T-cells and (F) $CD8^+$ $CD69^-$ T-cells.

2.6.9 ELISA procedures

2.6.9.1 IgG and IgA ELISA on serum, nasal wash and BAL

ELISA was used to quantify levels of IgG and IgA antibodies to influenza in the serum, nasal wash and BAL supernatant from volunteers participating in the Antecedent LAIV study. Pooled sera of 7 TIV vaccinated volunteers was heat-inactivated (at 56^{o}C for 30 min) and used as standard in both total IgA and IgG to influenza ELISA. Antibody levels were expressed in arbitrary units. For IgG standard preparation, a dilution of 1:4000 was applied, while for IgA was diluted 1:40.

Briefly, 96-well plates (Thermo Fisher Scientific) were coated with 100µL of 0.2 µg/mL TIV in PBS overnight at room temperature. During the experiment, each wash consists of washing plates three time with PBS with 0.005% Tween (Sigma, see Section 2.6.1). After incubation overnight, plates were washed following blocking with 100µL of PBS with 1% BSA for 1 hour in room temperature. Then, plates were washed, and samples were added in duplicate and incubated for 2 hours at room temperature.

For detection of IgG and IgA, a 1:5000 and 1:4000 dilution of anti-human-IgG and anti-human-IgA antibodies respectively(Sigma, see Section 2.6.1), was made using 0.1% BSA and 100µL added to each well after washing and incubated at room temperature for 2 hours. For IgA ELISA, 100µL of 1:2000 dilution of Streptavidin−Alkaline Phosphatase using 0.1% BSA was added to each well and incubated at room temperature for 1 hour.

Then, for both IgA and IgG to influenza, plates were washed and 100µL of p-Nitrophenyl Phosphate (Sigma) was added to the wells. Plates were incubated

at room temperature for a fixed period (IgA: 30 min and IgG: 90 minutes). The optical density of each well was measured at 405nm using a FLUOstar Omega ELISA microplate reader, the average blank corrected value was calculated for each sample and the data analysed using Omega Analysis.

2.7 Statistical analysis

All sampling, processing and analysis were performed while blinded to vaccination and colonisation group to achieve unbiased results. Non-parametric tests were used for statistical analysis since number of samples were insufficient for a normal distribution of results. Statistics were calculated in GraphPad prism version 5.0, 6.0 and 7.0 for Windows (GraphPad Software, California USA) and R Statistical Software (R Foundation for Statistical Computing) with packages gplots, shiny, mass, vegan and RcolorBrewer. Differences were considered statistically significant if $p \leq 0.05$. Benjamini-Hochberg multiple correction was performed in R for 30-plex cytokine data.

CHAPTER THREE

DEVELOPMENT OF METHODOLOGY FOR NASAL MUCOSA ANALYSIS

3.1 Introduction

The human oro/nasopharynx is the major site of Spn colonisation [295] and LAIV attenuated virus replication [153,296]. In order to establish reliable and reproducible methodologies for nasal cell immunophenotyping and cytokine analysis of the mucosal immune responses, we assessed and compared 3 minimally-invasive micro-sampling techniques (nasal wash, nasal curettage and nasosorption).

Currently, the most used method for collecting cells from within the nasopharynx is a NW procedure. The technique is well tolerated, however, luminal cell populations can vary significantly from intra-mucosal populations [297,298]. Cell collection using nasal curettes is an alternative method that has previously been used to collect epithelial cells for culture, as well as for gene expression analysis [299,300]. Herein, we sampled the nasal mucosa using nasal curettes and studied the composition of nasal cells using flow cytometry. Nasal immune cell yields and viability were compared between nasal curettes and nasal washes.

Cytokines and other soluble immune mediators are also commonly measured in NW samples. However, an absorptive matrix to collect nasal fluid (NS) has been tested in neonates, and has the potential to be better tolerated and more widely applicable [301]. This technique has recently been used to investigate nasal responses to grass pollen, LPS and rhinovirus [302–304].

Immunological findings in murine, or other animal models, often fail to translate to humans, which indicate the need for more accurate non-invasive techniques to measure immunological response in the nose. Furthermore, an improved method of sampling the nasal mucosa would certainly lead to a greater

understanding of the cellular components in the human nasopharynx. In this chapter, we aimed to compare cells and cytokines detection between different techniques.

3.2 Methods

3.2.1 Volunteers recruitment

In total 240 samples were collected from 139 healthy individuals (requirements described in Section 2.2) from the Antecedent Study (Section 2.3) to investigate techniques for studying immune responses at the mucosal level.

3.2.2 Collection of nasal samples

All participants were sampled for NW (Section 2.5.4) using 20 mL of sterile saline and underwent nasal curettage and NS collection.

In order to collect nasal cells by curettage, volunteers had their inferior turbinates scraped using a rhinoprobe (BRAND ASL Rhino-Pro©, Arlington Scientific) in each nostril, as described in Section 2.5.5. The sample was placed in 8 mL of PBS +Ca+Mg and placed on ice for further analysis.

For NS collection, an adsorptive matrix strip (Nasosorption™, Hunt Developments) was inserted into the nostril and placed against the nasal lining of the inferior turbinate for 2 minutes and then placed in its transport tube (Section 2.5.6).

3.2.3 Flow cytometry analysis

As described in Section 2.6.6.1, cells were dislodged from the curette and, were spun down (440xg for 5 minutes) and resuspended in PBS+Ca+Mg containing LIVE/DEAD Fixable Aqua Dead Cell Stain (ThermoFisher). After 15 minutes incubation on ice, a cocktail of conjugated antibodies against key cell surface markers such as Epcam-PE, HLADR-PECy7, CD16-APC, CD66b-FITC (Biolegend), CD3-APCCy7, CD14-PercpCy5.5 (BD Biosciences) and CD45-PACOrange (ThermoFisher) was added to the cells. Following a further 15 minutes incubation on ice, cells were filtered over a 70µm filter (ThermoFisher). Next, the cells were spun down (440xg for 5 minutes), resuspended in PBS+Ca+Mg and acquired on a flow cytometer (LSRII, BD). NWs were similarly processed excluding the dislodging step as described in Section 2.6.5. Flow cytometry data were analysed using Flowjo V.10 (Treestar).

3.2.4 Cytokine detection

As described in Section 2.6.7, nasal lining fluid was extracted from nasosorption strips by centrifugation (1880xg for 10 minutes) and frozen at -80^{o}C until use. Supernatant from NW was collected by centrifugation at 1008xg for 3 minutes and was stored at -80^{o}C until use. The human magnetic 30-plex cytokine kit (ThermoFisher) was used to detect 30 cytokines simultaneously on a LX200 with xPonent3.1 software (Luminex) following manufacturer's instructions described in Section 2.6.7.

3.2.5 Scoring tolerability of nasal sampling procedures

Following NS, nasal curettage and NW, participants rated using a 5-point modified Likert scale (Section 2.5.2) how ‘painful’ and how ‘uncomfortable’ each procedure was, and how much it made their ‘eyes water’. 39 participants also completed a symptoms log for 7 days documenting both local and general symptoms with severity ratings from 1 to 7 (Section 2.5.2).

3.2.6 Multi-dimensional scaling and heat map generation

Multi-dimensional scaling and heat map representations were generated using statistical software R. Flow cytometry data (epithelial cell yield, immunophenotyping and activation) was log-transformed and a distance-matrix was calculated. The Kruskal stress was calculated using the ‘MASS’ package on the R software. Heat maps of log-transformed cytokine data were generated using the ‘gplots’ package.

3.2.7 Statistical analysis

As described in Section 2.7, non-parametric two-tailed tests were used throughout using Prism 5 (Graphpad). If 2 groups were compared, a Mann-Whitney test was used. If multiple groups were compared, a Kruskal-Wallis test was used, followed by a Dunn’s post-test. A Spearman test was used to measure correlations between 2 continuous variables. Analysis of similarity (ANOSIM) testing was performed using the ‘vegan’ package in R.

3.3 Results

Nasal curettage yields robust and reproducible data

To verify the repeatability of nasal curettage, we initially collected samples from the left and right nostril of 3 healthy volunteers and performed flow cytometry to identify cellular composition (Figure 7).

Samples from both nostrils were processed independently and frequencies of neutrophils, monocytes and T-cells were compared for each of the 3 volunteers. Cellular samples collected from the 2 nostrils were similar for each of the 3 volunteers, compared to samples collected from the other 2 volunteers. These results demonstrate the repeatability of nasal curettage as well as the presence of inter-individual variation in immune cells in the nose.

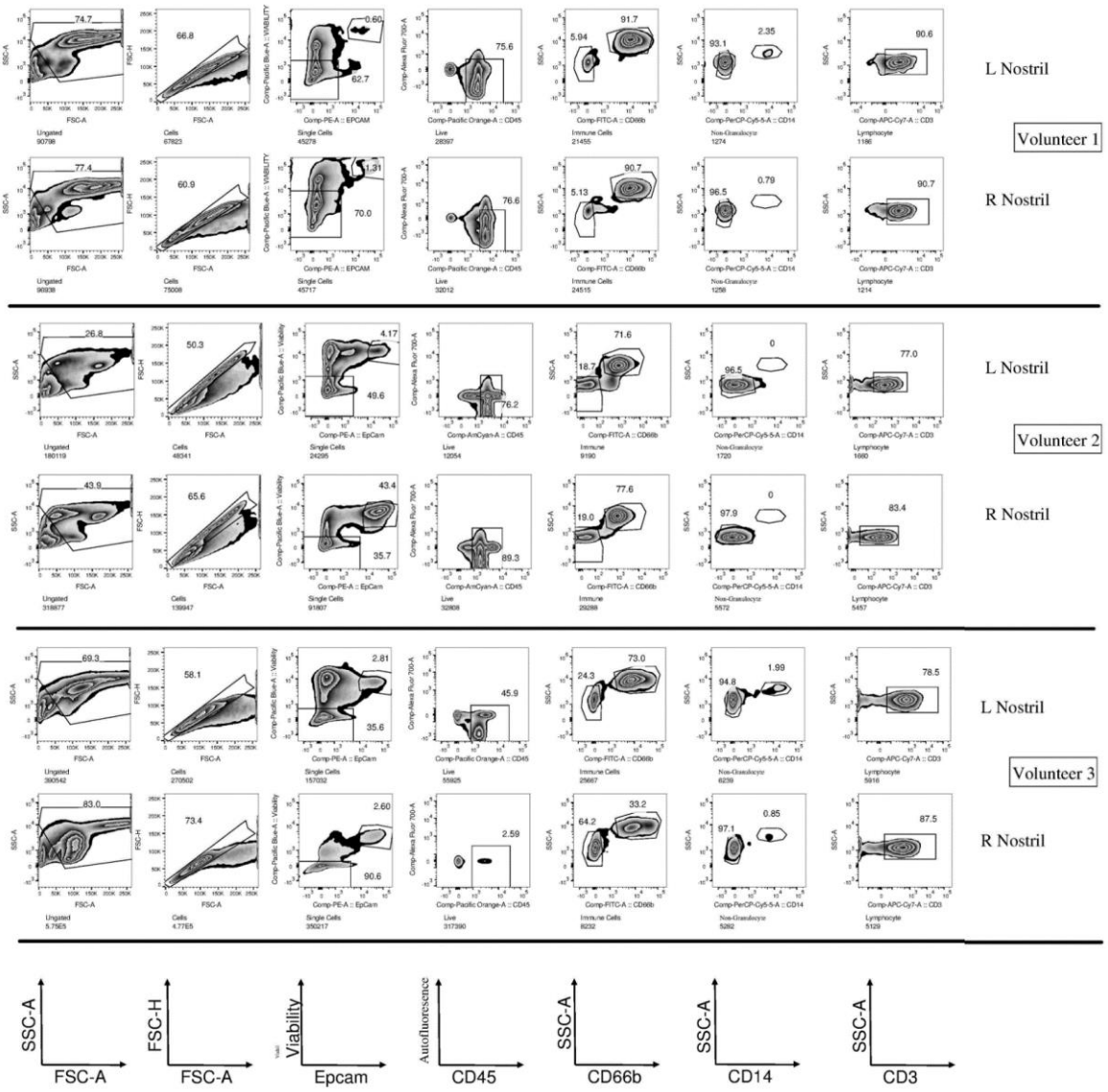

Figure 7. Repeatability of nasal curettage sampling. Nasal cells were collected from the left (L) and right (R) nostril of three volunteers, processed independently and their composition was assessed by flow cytometry. After excluding debris and doublets, epithelial cells were identified by Epcam expression. A viability dye and CD45 were used to identify live immune cells. Among those cells, side scatter, CD66b, CD14 and CD3 were used to identify neutrophils, monocytes and T-cells respectively.

To verify that nasal curette sampling yields stable data, cells were collected from healthy volunteers (n=117) over a 5-month period (Figure 8). The percentage of neutrophils and T-cells among immune cells was stable during this period (Figure 8A). Moreover, for a subset of volunteers, up to 4 nasal samples were collected during a 33 day period. The levels of both neutrophils and T-cells correlated on an intra-individual level between repeated sampling (Figure 8B and 8C). These results demonstrate that despite variation between individuals, the immunological profile in the nose is stable in the absence of disease or immune intervention such as vaccination.

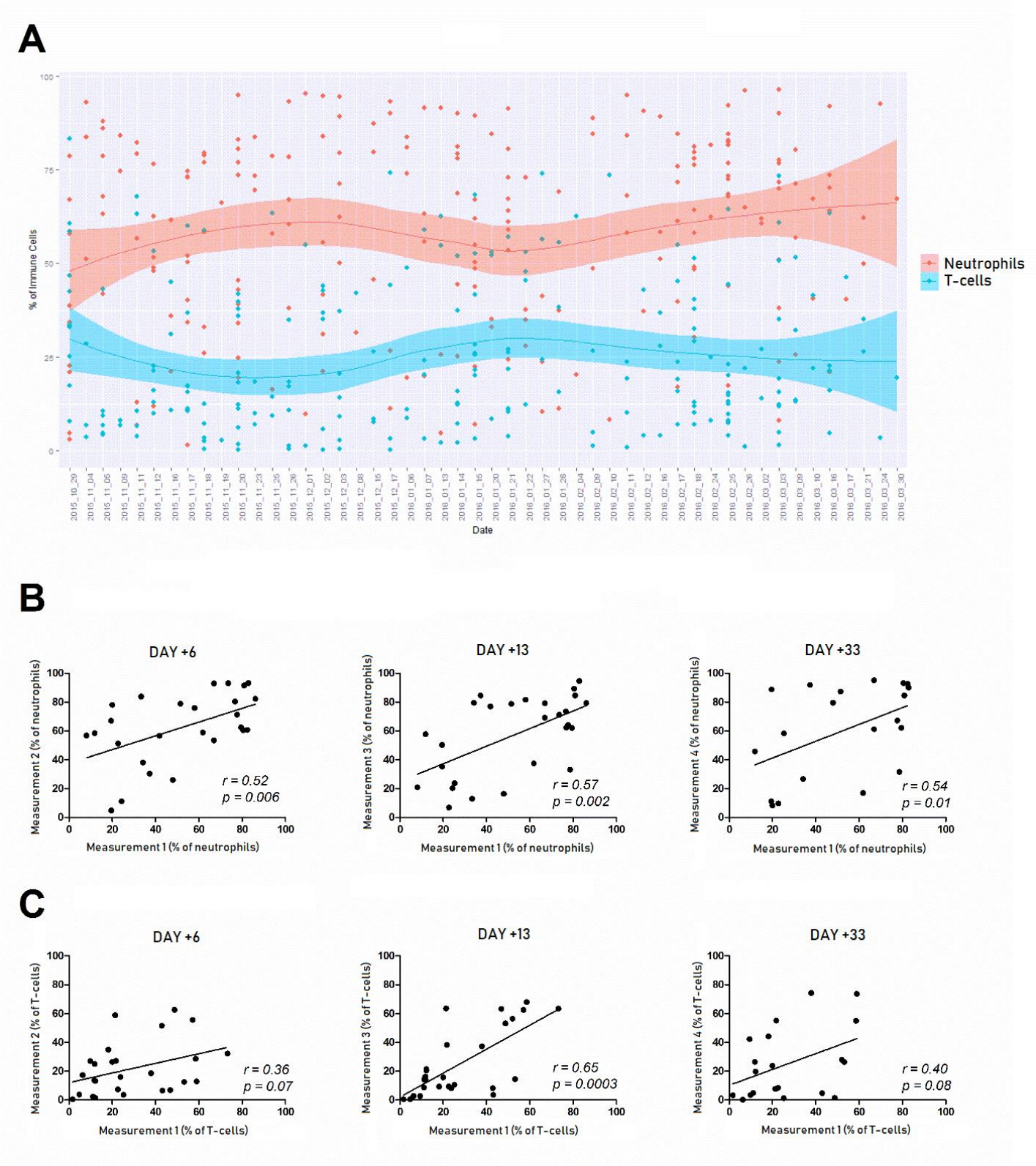

Figure 8. Nasal curettage yields reproducible and consistent results over time. (A) The percentage of neutrophils (red) and T-cells (blue) in 218 nasal cell samples collected over a 5-month period (n=117 volunteers, sampled up to 5 times). Circles represent individual samples and loess curves are depicted for both populations. (B, C) Correlations for individuals in 4 repeated measurements over a 33-day period for (B) neutrophils and (C) T-cells

Nasal curettage and nasal wash yield different cell populations

We then compared the yield and composition of nasal cells collected using curettes to those collected using a NW (Figure 9A and 9B). Nasal curettage yielded a median of 4367 (interquartile range, IQR: 1511–10348) immune cells and 1407 (IQR: 570–3194) epithelial cells, respectively. The number of immune cells obtained was similar between NW and nasal curette. In contrast, there were a median 22.7-fold increased numbers of epithelial cells collected by nasal curette (Figure 9A, $p<0.05$). Figure 9B shows the composition of the collected immune cells. NW immune cells consisted almost exclusively of neutrophils (median 96%, IQR: 93–97%). On the other hand, nasal curette samples contained predominantly neutrophils (median 64%, IQR: 39–79%, $p<0.0001$ compared to NW), but also consisted of a larger fraction of T-cells than NW (median 16%, IQR: 9–38%, $p<0.0001$ when compared to NW).

A median of 2591 (IQR: 691–7666) neutrophils and 633 (IQR: 210–1740) T-cells were acquired per sample. Nasal curette samples also contained more HLA-DR$^+$ cells, which are likely to consist of B-cells and DCs (median 1.7%, IQR 0.9–3.2%, $p<0.001$ compared to NW). Of all immune cells collected by curettage and NW, 81.7% and 96.4% could be characterised, respectively.

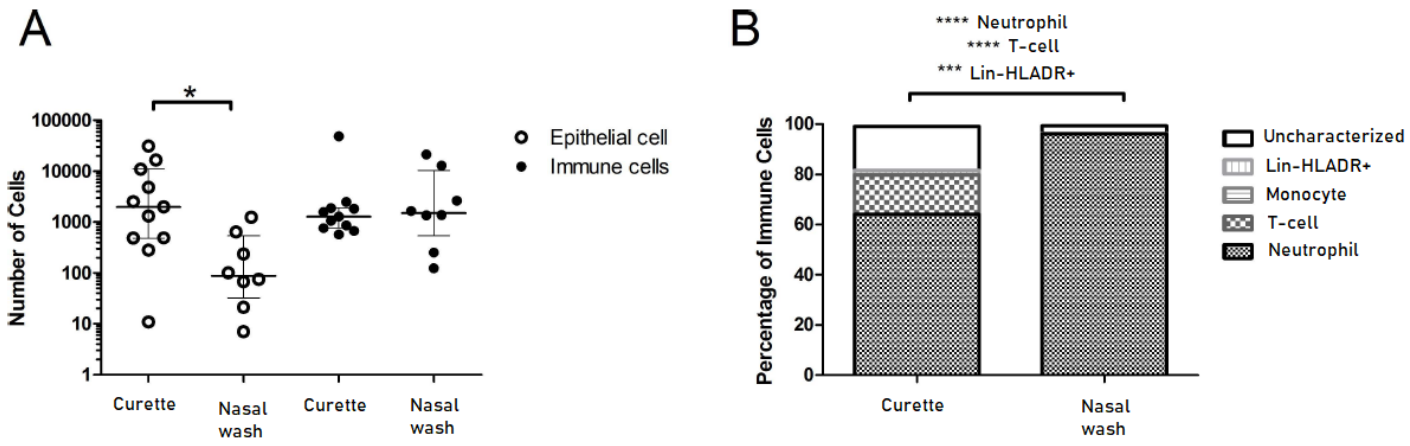

Figure 9. Comparison of samples collected by nasal wash and nasal curette. (A) Immune (black) and epithelial (grey) cell yields were compared between nasal wash pellets and nasal curette samples. Individuals samples and median with interquartile range are shown. (B) Median levels of neutrophils (blue), T-cells (red), monocytes (black) and lineage- HLA-DR+ (grey) among immune cells in nasal curette (n=139 individuals) and nasal wash (n=8) samples. **p<0.01, ***p<0.001, ****p<0.0001 Mann-Whitney test.

Cytokine detection from nasal lining fluid using nasosorption devices

To investigate cytokines in the nose, we used NW and NS to collect nasal lining fluid. The median volume of nasal lining fluid returned using this technique was 42.5µL (IQR: 29.25–71.25µL, n=41). We compared cytokine levels in nasal lining fluid and NW supernatant for 30 cytokines by Luminex (Figure 10A).

Levels of different cytokines varied considerably, with median levels of IL-1RA at 212,000pg/mL and GM-CSF at 2pg/mL in nasal lining fluid. Relative cytokine abundancy correlated well between NS and NW, as cytokines that were abundant in NS were also highly present in NW. Of interest, T-cell cytokines (IL-10, IL-17, IFN-γ, TNF-α, IL-4, IL-5, IL-2) were only present at low levels (Figure 10B), which correlates with the absence of T-cells in the lumen. Growth factors as EGF, HGF and VEGF were expressed at moderately high levels, reflecting the homeostatic nature of mucosal surfaces.

Levels of cytokines were higher in NS compared to NW (median 4.7x, IQR: 3.1–8.0x). However, some cytokines had a ratio between NS and NW that differed substantially from this: IL-1RA and IL-5 were respectively 51.5x and 45.2x higher in NS than in NW. In contrast, MIG, RANTES and IP-10 were found at similar levels in NS and in NW (ratio of 1.5x, 1.0x and 0.9x, respectively) (Figure 10B).

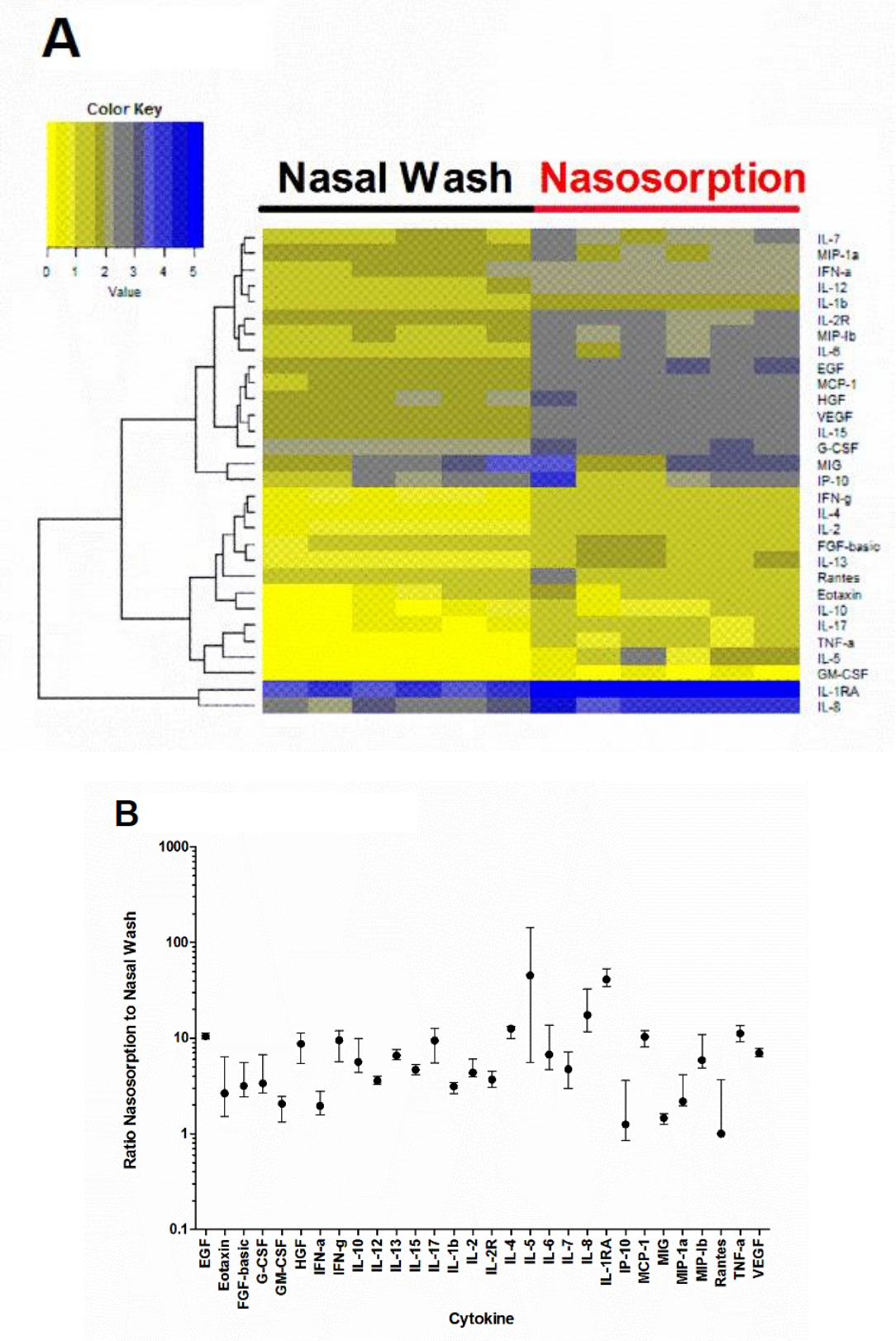

Figure 10. Comparison of cytokine levels in samples collected by NW and using NS strips. (A) A heat map depicts log-transformed cytokine concentrations, with yellow and blue indicating low and high levels, respectively. Each of the columns corresponds to 1 sample (n=6 NW and NS) and each of the rows to 1 cytokine. A distance tree shows similarity between cytokines. (B) The ratio of cytokine concentrations measured in paired NS and NW (median and IQR are shown, n=6).

Methods of nasal micro-sampling are well tolerated by volunteers and do not lead to symptoms

Using the 5-point modified Likert scale, 20 participants gave ratings for nasal curettage (88 ratings) and NS (60 ratings) with regards to pain, discomfort and lacrimation (Table 2).

For nasal curettage and NS (Figure 11), the proportion of responses that reported any degree of pain (any score > 1) were 73% and 10%, respectively. Additionally, the proportion of responses that reported any degree of discomfort (score > 1) were 86% and 47%, respectively. Moreover, the proportion of responses that reported any degree of lacrimation (score > 1) were, 84% and 28%, respectively.

For NS the maximum rating was moderate for levels of discomfort, pain or causing lacrimation. A small proportion of responses rated nasal curettage as very painful (2%), uncomfortable (8%) or causing lacrimation (6%).

Finally, we assessed whether these sampling methods led to increased general and nasal symptoms over a longer period in 39 healthy volunteers (Table 3). All participants had NW procedures and 20 of those participants had nasal curettage and NS to investigate whether these additional sampling methods affect nasal symptoms. Daily symptom logs for nasal and general symptoms were completed by all volunteers. Age and sex distribution were similar in each group. The median ratings for overall nasal symptoms were 1 (range 1–4) and 1 (range 1–5) in the group with and without additional nasal sampling, respectively.

In the symptoms log, a score of 1 represented ‘none to occasional symptoms’ and 5 represented ‘moderately bothersome’. Comparison of area under the curve between the 2 groups showed no significant difference in either overall nasal symptoms or general symptoms. In addition, we compared nasal

and general symptoms between participants with and without additional nasal sampling on day 1 (before additional sampling) and day 3 (after additional sampling). There were no significant differences between nasal or general symptoms with or without additional nasal sampling at these time points.

Table 8. Levels of pain, discomfort and lacrimation associated with nasal curettage and NS.

Volunteer responses (%, n)		
PAIN RATING		
Pain rating	Nasal curettage	Nasosorption
1 - Not at all	27% (24)	90% (54)
2 – Slightly	43% (38)	10% (6)
3 – Moderately	27% (24)	0% (0)
4 – Very	3% (2)	0% (0)
5 – Extremely	0% (0)	0% (0)
DISCOMFORT RATING		
Discomfort rating	Nasal curettage	Nasosorption
1 - Not at all	13% (12)	53% (32)
2 – Slightly	47% (42)	40% (24)
3 – Moderately	32% (28)	7% (4)
4 – Very	8% (7)	0% (0)
5 – Extremely	0% (0)	0% (0)
LACRIMATION RATING		
Lacrimation rating	Nasal curettage	Nasosorption
1 - Not at all	17% (14)	71% (4)
2 – Slightly	60% (53)	22% (13)
3 – Moderately	18% (16)	7% (4)
4 – Very	3% (3)	0% (0)
5 – Extremely	2% (2)	0% (0)

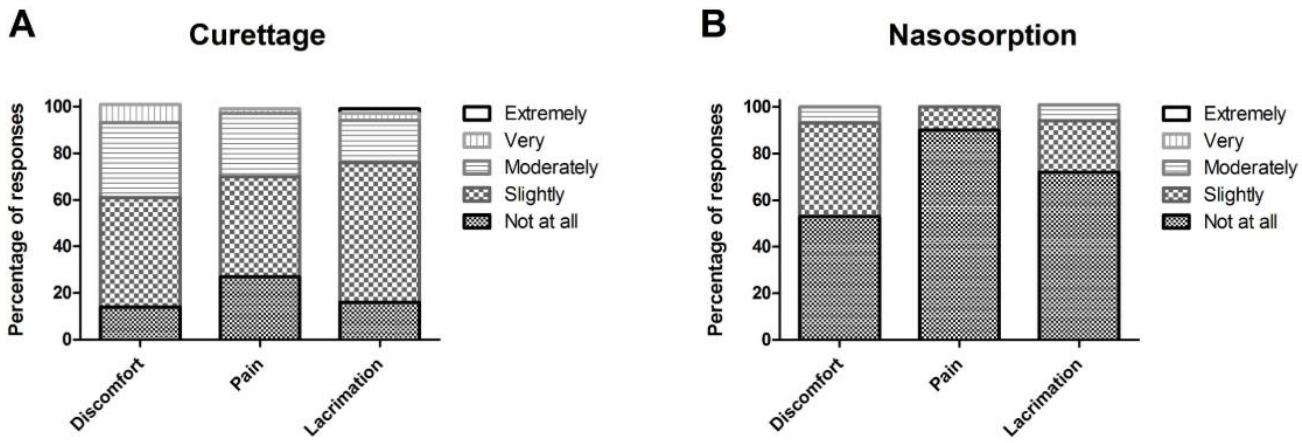

Figure 11.Tolerability of novel nasal sampling methods. The percentage of volunteers rating (A) nasal curettage and (B) nasosorption on discomfort, pain and lacrimation.

3.4 Discussion

Here we described 2 novel non-invasive nasal mucosal micro-sampling techniques and their use for measuring immunological parameters in the nasal mucosa: 1) using nasal curettes to collect cells from the inferior turbinate and; 2) absorptive matrices to collect nasal lining fluid (NS). Both techniques were well tolerated and yielded reproducible and robust data. We demonstrated differences in immune populations and activation state in nasal mucosa compared to nasopharyngeal lumen in healthy adults. We also found superior cytokine detection with NS compared to NW. This was the first time that nasal cells collected in this way were analysed by flow cytometry, demonstrating the presence of immune cells in these samples.

NW yielded almost exclusively neutrophils, indicating differences in immune cells were collected by NW and nasal curette. The lack of T-cells in NWs reflects earlier findings showing that neutrophils and monocytes readily enter the lumen in the gut, while T-cells are mostly associated with the sub-epithelial layer [47,305,306].

We also assessed the use of NS devices to collect nasal lining fluid. Importantly, the nasal lining fluid contained cytokines in concentrations that were increased compared to concentrated NW. Moreover, a positive correlation between cytokine levels that were detected in both nasal lining fluid and those from NW was observed. However, the ratio between cytokine concentrations in nasal lining fluid and NW was not similar for all cytokines assessed, which could be due to the existence of cytokines in different nasopharyngeal compartments. As NWs sample the entire nasopharynx in contrast to a localised sample coming from the NS strip this might lead to different returned cytokine levels.

Alternatively, these differences could arise from cytokines binding affinities to the NS paper. NW is currently the most commonly used method to obtain samples from the nasopharynx, however, it has limited application in multiple clinical scenarios such as sampling of children as NW consists of holding in and expelling saline through the nose. Here we have demonstrated that NS has greater sensitivity than the traditional NW and is extremely well tolerated by participants. Nevertheless, there are potential issues with sample collection using NS devices, such as poor return volume, which was corrected in future samplings by adding diluent buffer to the samples afterwards.

In conclusion, non-invasive mucosal sampling yields nasal cells and nasal lining fluid that can be used to study both cellular and soluble immune responses at the mucosal surface. Such samplings are well-tolerated and do not lead to a change in nasal symptoms being ideal for research. These techniques were used throughout the 2 LAIV studies and can be widely implemented in order to provide researchers with an effective tool to study immunological responses in the respiratory tract.

CHAPTER FOUR

EFFECT OF SPN AND LAIV ON RECRUITMENT OF IMMUNE CELLS TO NASAL MUCOSA

4.1 Introduction

The mucosal tissue is described to have an epithelial cell layer that interacts directly with the environment, making it the body's first line of defence against infectious agents. Mucosal surfaces have a high tolerance to antigens that are not dangerous, coming from ingested food or commensal microorganisms, inhibiting unnecessary local and systemic immune responses in order to maintain homeostasis and normal physiological functions [307].

The mucosa form the largest mammalian lymphoid organ system. As part of this complex barrier, the nasal mucosae are protected by specialised innate and adaptive immune mechanisms can recruit a large number of immune cells such as monocytes, neutrophils, DCs and T-cells, which can be resident cells or migratory cells that travel through the mucosa-associated lymphoid tissue (MALT) [308,309].

Nasopharyngeal Spn colonisation is the primary reservoir for bacterial transmission [310] and a prerequisite of invasive disease [311]. However, it is common for humans to be naturally colonised by Spn asymptomatically many times during lifetime for weeks or even months at a time [312]. Therefore, a successful control of the Spn load by immune cells in the nasal mucosa is the key for preventing severe illness [313] as uncontrolled Spn colonisation and increased bacterial load has been associated with transmission within households [310], elevated risk of Spn pneumonia [266] and mortality [314].

Moreover, it is known that during Spn and wild-type influenza virus co-infection, the host ability to control bacterial growth is affected [75,270,315–317]. The presence of the virus induces additional adaptive $CD8^+$ T-cell responses in the nasal mucosa to achieve virus clearance and support innate host defences [188].

Consequently, adaptive and innate responses synergize to culminate in an inflammatory cytokine and chemokine storm [271].

It is important to notice that influenza attenuated viruses contained in the LAIV can impact on Spn density in children [318]. Furthermore, LAIV in 2 to 4 year old increased prevalence of Spn when compared to other microbiota in the nose [318]. In adults, asymptomatic viral infection has been shown to predispose to experimental colonisation by facilitating Spn adherence to the epithelium [46]. Murine models also confirms that LAIV increases susceptibility to and duration of Spn colonisation with similar mechanisms to the wild-type influenza virus [282]. However, as most of the observed interactions between Spn, attenuated influenza virus and host derive from mice and children models, these findings may not accurately correspond to the human adult counterpart. Therefore, it becomes important to confirm this observation using samples from healthy adults.

4.2 Methods

4.2.1 Volunteer recruitment, vaccination and inoculation

216 healthy adult volunteers were recruited to the Antecedent and Concurrent LAIV study (Section 2.2) and received vaccination (LAIV or TIV as control) and intranasal inoculation with Spn6B (EHPC). In the Antecedent study, vaccine was administered 3 days before inoculation, while in the Concurrent study, subjects received the vaccine 3 days after the inoculation (Section 2.3).

4.2.2 Nasal cells collection, processing and staining

Before and after vaccination and inoculation, 106 volunteers recruited to the Antecedent study and 110 to the Concurrent study had their inferior turbinates scraped with a rhinoprobe (Section 2.5.5) on predetermined timepoints.

Nasal cells were washed (Section 2.6.6) and stained with extracellular antibodies (Section 2.6.6.1). In the Antecedent study, nasal cells were stained with against CD45, Epcam, CD14, CD3, CD16, CD66b and HLADR cell surface markers , whereas in the Concurrent study 2 additional antibodies for DCs (anti-BDCA-1 and anti-BDCA-2) were included in the flow cytometry panel.

Volunteers were stratified into 4 groups for analysis: TIV Spn-, vaccinated with TIV and negative for Spn colonisation (Antecedent n=25, Concurrent n=33); TIV Spn+, vaccinated with TIV and Spn colonised (Antecedent n=18, Concurrent n=25); LAIV Spn-, vaccinated with LAIV and not colonised (Antecedent n=26, Concurrent n=30); LAIV Spn+, vaccinated with LAIV and Spn colonised (Antecedent n=22, Concurrent n=22).

4.2.3 Flow cytometry and statistical analysis

To account for the differences in the length of sample collected by curettage and accurately compare the number of immune cells recruited to the nasal mucosa, the analysis of specific cell numbers was made using the number of the specific cell population compared to the epithelial cell numbers contained in the collected sample. The recruitment of immune cells was analysed in each group by comparing the median number of immune cells to epithelial cell ratio at each timepoint compared to baseline (Antecedent study: day -4; Concurrent study: day -5 from Spn inoculation) by Wilcoxon test. Comparisons of median results between groups were done by Mann-Whitney test.

Stained nasal cells were analysed in a flow cytometer using FlowJo X (Treestar) and gating strategy is shown in Section 2.7. Statistical analysis was done using Prism 7 (Graphpad) software.

4.3 Results

4.3.1 Antecedent LAIV

In the Antecedent study, 106 volunteers were vaccinated with either TIV (n=51) or LAIV (n=55) and inoculated with Spn 3 days later. Subjects were sampled for nasal cells on days -4 (baseline), +2, +7, +9, +29 relative to Spn inoculation. For analysis, volunteers were stratified into the groups: TIV Spn-, TIV Spn+, LAIV Spn- and LAIV Spn+. In addition, for analysis regarding only Spn colonisation status, volunteers were divided into the groups: Spn- (not Spn colonised, n=60) and Spn+ (Spn colonised, n=46).

Induction of monocyte recruitment to the nose by Spn colonisation is impaired by antecedent LAIV vaccination

Here we compared the recruitment of monocytes to the nasopharynx using the median of monocytes to epithelial cell ratio. When assessing all volunteers colonised by Spn independent of vaccine, Spn-induced monocyte recruitment was significant at day +2 (Spn- vs. Spn+, median 2.1-fold increase, p=0.014, Figure 12B). Importantly, when stratified by vaccination, colonised volunteers showed a significant induction in recruitment at day +2 when compared to baseline, but only when vaccinated with TIV (TIV Spn+: median 2.3-fold increase, p=0.038). Further, at day +9, a median 3.4-fold increase in monocyte levels was still observed in TIV Spn+ (p=0.002). The significant induction shown in this group continued to be demonstrated as far as day +29, with 3.1-fold increase (p=0.030, Figure 12A). However, in colonised subjects, LAIV given before inoculation reduced the Spn-induced recruitment of monocytes.

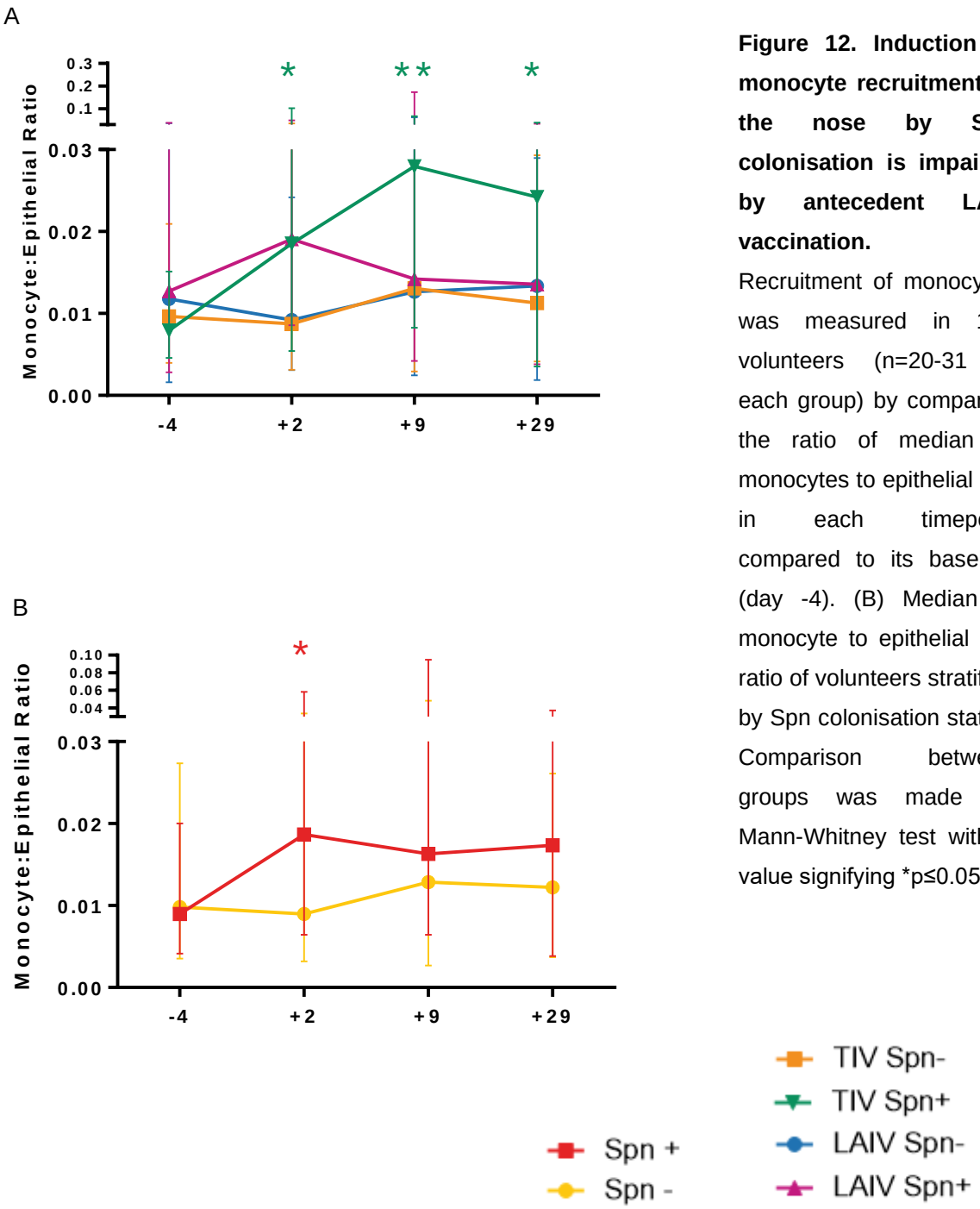

Figure 12. Induction of monocyte recruitment to the nose by Spn colonisation is impaired by antecedent LAIV vaccination. (A) Recruitment of monocytes was measured in 106 volunteers (n=20-31 in each group) by comparing the ratio of median of monocytes to epithelial cell in each timepoint compared to its baseline (day -4). (B) Median of monocyte to epithelial cell ratio of volunteers stratified by Spn colonisation status. Comparison between groups was made by Mann-Whitney test with p value signifying *p≤0.05.

Monocyte recruitment is associated with density of Spn only in TIV vaccinated.

In order to investigate if the monocyte infiltration observed was associated with the increase in Spn density in the nasal mucosa, we performed correlation tests by using the maximum Spn density and maximum ratio of monocytes in the nasal mucosa in a subset of 45 colonised volunteers of the Antecedent LAIV-EHPC study (TIV n=22, LAIV n=23). We demonstrated that the peak in monocyte numbers correlates with Spn density in the TIV- ($p=0.016$, $R^2=0.64$, Figure 13A), but not in the LAIV-vaccinated group ($p=0.70$, $R^2=-0.03$, Figure 13B).

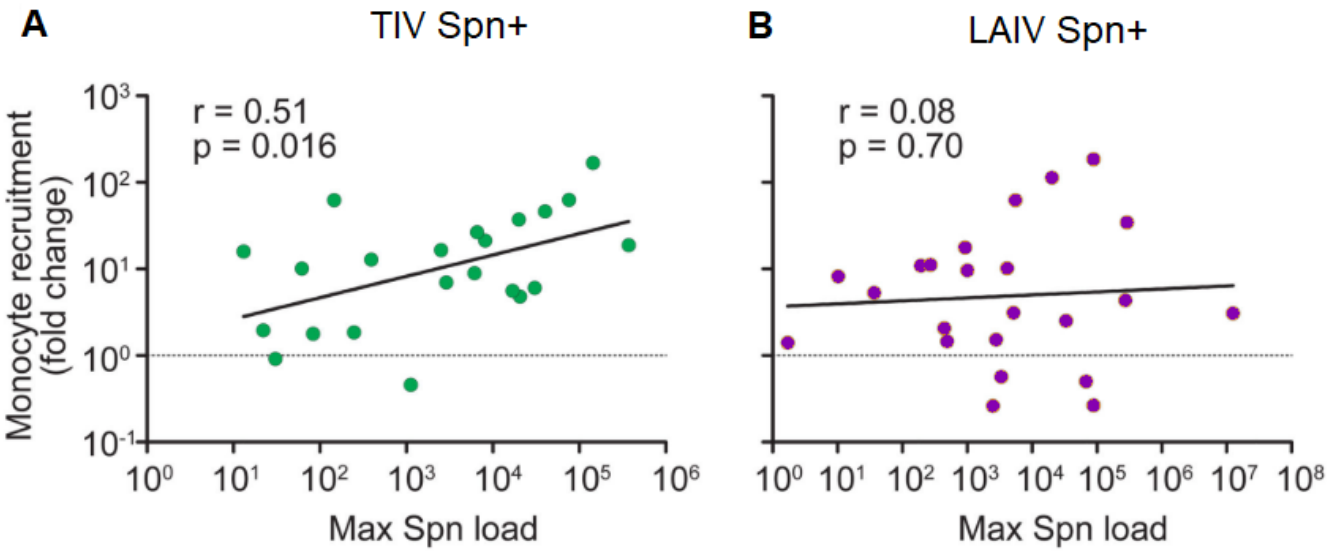

Figure 13. Monocyte recruitment is associated with density of Spn only in TIV vaccinated group. Levels of maximum Spn load are shown for the (A) TIV Spn+ (n=22) and (B) LAIV Spn+ group (n=23) and correlated with the maximum monocyte recruitment (fold-change to baseline). Individual subjects and Spearman correlation analysis are shown

Spn-induced activation of neutrophils in the nose is impaired by antecedent LAIV.

To assess the levels of neutrophils we analysed CD66b, a marker for active neutrophils [319,320], in 106 volunteers divided in the groups. In addition, we measured the average median fluorescence intensity of CD66b from neutrophil (MFI of CD66b) to test the intensity of this marker.

We observed that neither Spn colonisation nor influenza vaccination significantly alters the number of active neutrophils recruited to the nasal mucosa (Figure 14A, 14B). However, in TIV Spn-, the number of active neutrophils were slightly higher at day +2 when compared to baseline (Day +2 with median MFI of 8534,8 and IQR 6495-14560 vs Day -4 with median 6846,6 and IQR 5260-11101, p=0.002, Figure 14C). Such a response was impaired in the LAIV group. When compared by Spn colonisation status, no significant differences in neutrophil recruitment between the groups were observed (Figure 14D).

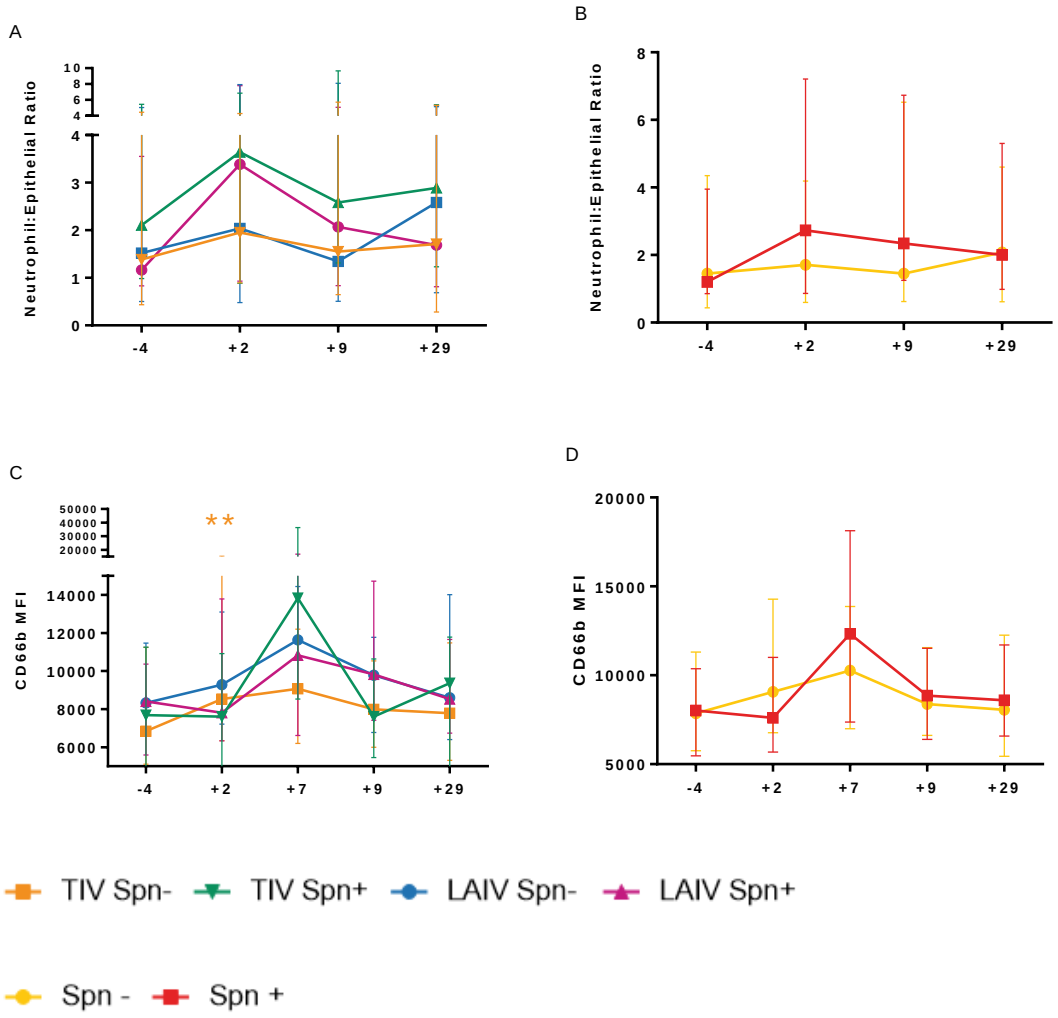

Figure 14. Spn-induced activation of active neutrophils in the nose is impaired by antecedent LAIV. Recruitment of neutrophils was measured in 106 volunteers with groups stratified by vaccination status (TIV or LAIV) and Spn colonisation (Spn-, non-colonised, and Spn+, colonised): TIV Spn- (n=31), TIV Spn+ (n=20), LAIV Spn- (n=29) and LAIV Spn+ (n=26). We compared (A) the median of neutrophils to epithelial cell ratio in each timepoint to baseline (day -4). (B) Median of neutrophils to epithelial cell ratio in volunteers stratified by Spn colonisation status (Spn- and Spn+). (C) Activation of neutrophils was assessed by comparing the median of MFI of CD66b in each timepoint to baseline. (D) Median of MFI of CD66b was compared between groups stratified by Spn colonisation. Comparisons within groups were performed by using Wilcoxon test and between groups by Mann Whitney test with **p<0.01

.

Antecedent LAIV induces activation of T-cells in the human nose.

We examined T-cell recruitment to the nasal mucosa, comparing 106 volunteers divided in groups considering vaccine and Spn colonisation status. Additionally, activation of T-cells was determined by analysing T-cell populations positive for the marker HLA-DR.

Similar to neutrophils, neither the influenza vaccines nor Spn colonisation induced significant recruitment of $CD3^+$ T-cells to the nose (Figure 15A and 15B). Nevertheless, antecedent LAIV induced T-cell activation independent of Spn colonisation status. LAIV administration before the bacterial challenge resulted to a peak of T cell activation 10 days (LAIV Spn+, day +7 median 1.3-fold increase, p=0.039) and 13 days after vaccination (LAIV Spn+, day +9, median 1.2-fold increase, p=0.042), whereas non-colonised subjects demonstrated a similar peak after 7 days (LAIV Spn-, median 1.3-fold increase, p=0.007) with another median 1.3-fold increase 29 days post-inoculation (LAIV Spn-, p=0.046, Figure 16).

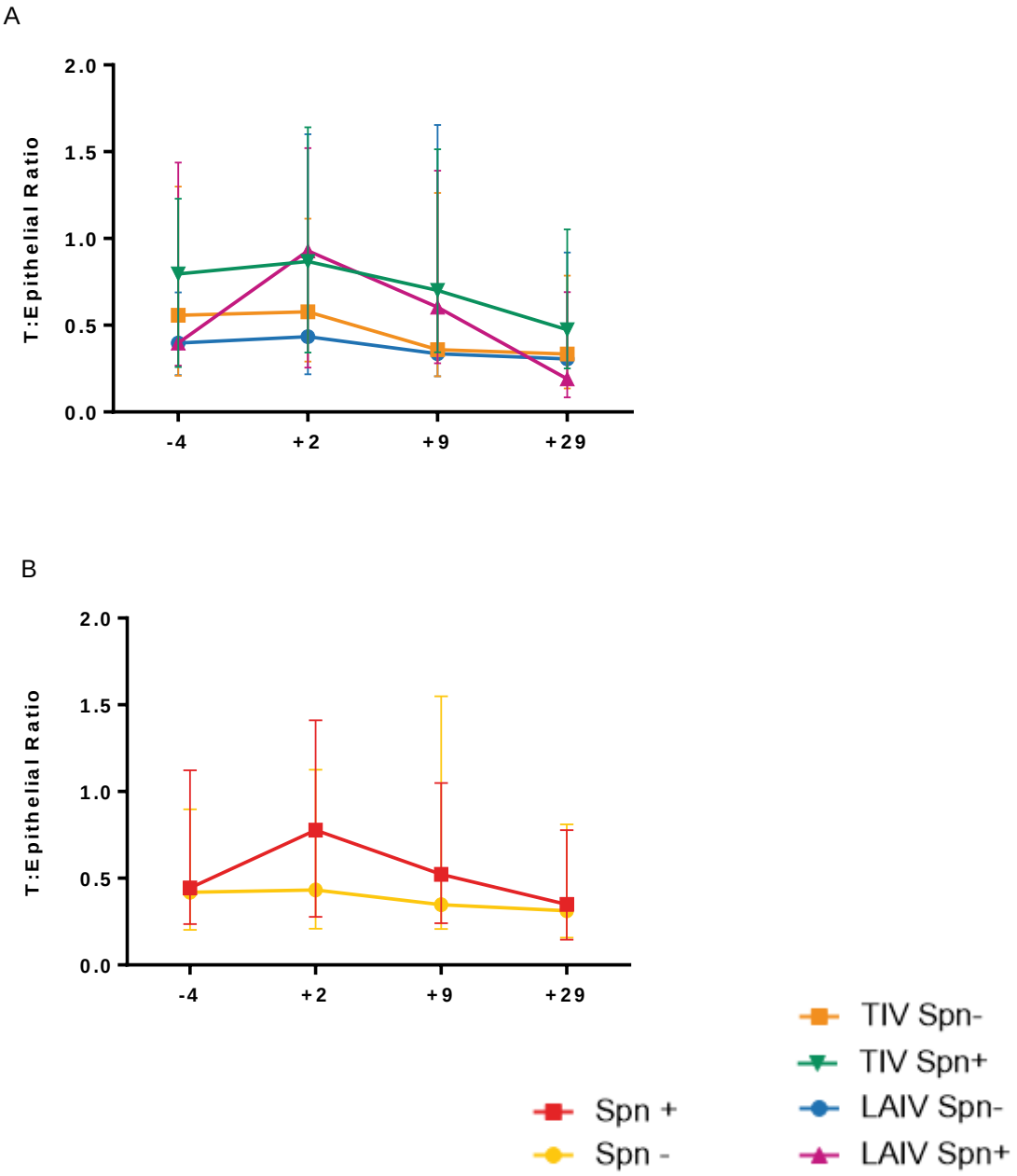

Figure 15. Antecedent influenza vaccines and Spn colonisation do not affect T-cell recruitment to the human nasal lumen. (A) Nasal recruitment of T-cells was measured in 106 volunteers divided into groups: TIV Spn- (n=31), TIV Spn+ (n=20), LAIV Spn- (n=29) and LAIV Spn+ (n=26). We compared the (A) Median of T-cells to epithelial cell ratio in each timepoint to baseline (day -4) within groups using Wilcoxon test. (B) Median of T-cells to epithelial cell ratio in volunteers stratified by Spn colonisation status using Mann Whitney test for comparison between groups

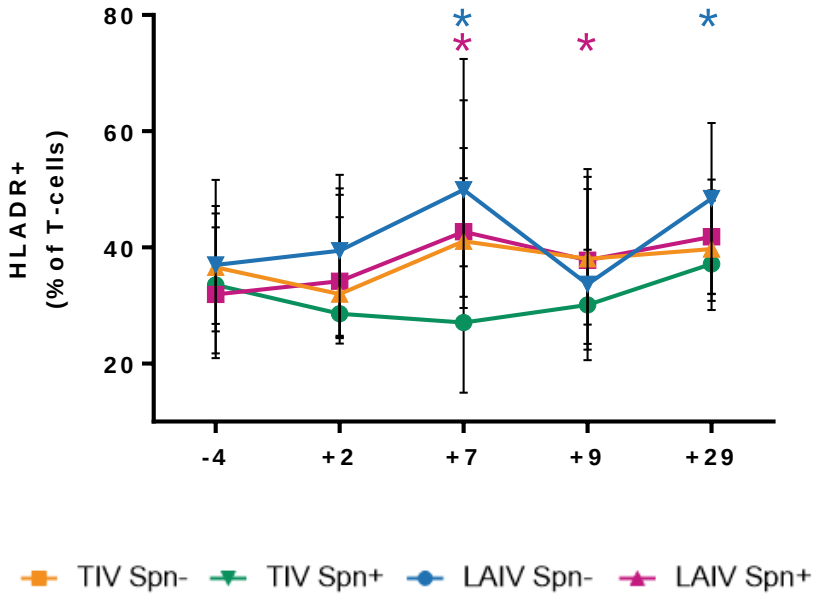

Figure 16. Antecedent LAIV, but not TIV, induces activation of T-cells in the human nose. Activation of T-cells was measured by comparing median percentage of HLA-DR+ T-cells in 106 volunteers (n=20-31) using Wilcoxon test for each timepoint to baseline (day -4) with *p≤0.05.

4.3.2 Concurrent LAIV

During the Concurrent LAIV-EHPC study, 194 volunteers were inoculated with Spn and vaccinated with either TIV (n=97) or LAIV (n=97) 3 days later. Nasal cells were sampled by curettage on days -5 (baseline), +2 (no vaccine), +6, +9, +27 relative to Spn inoculation. To perform the analysis, subjects were stratified into the groups: TIV Spn-, TIV Spn+, LAIV Spn- and LAIV Spn+.

Spn colonisation of the nasopharynx induce neutrophil activation.

For assessment of monocyte recruitment in nasal mucosa, we compared the median of monocytes to epithelial cell ratio in each timepoint to baseline using extracellular staining and flow cytometry analysis. Contrarily to the Antecedent study, no nasal recruitment of monocytes was observed after influenza vaccination or Spn colonisation (Figure 17A and 17B).

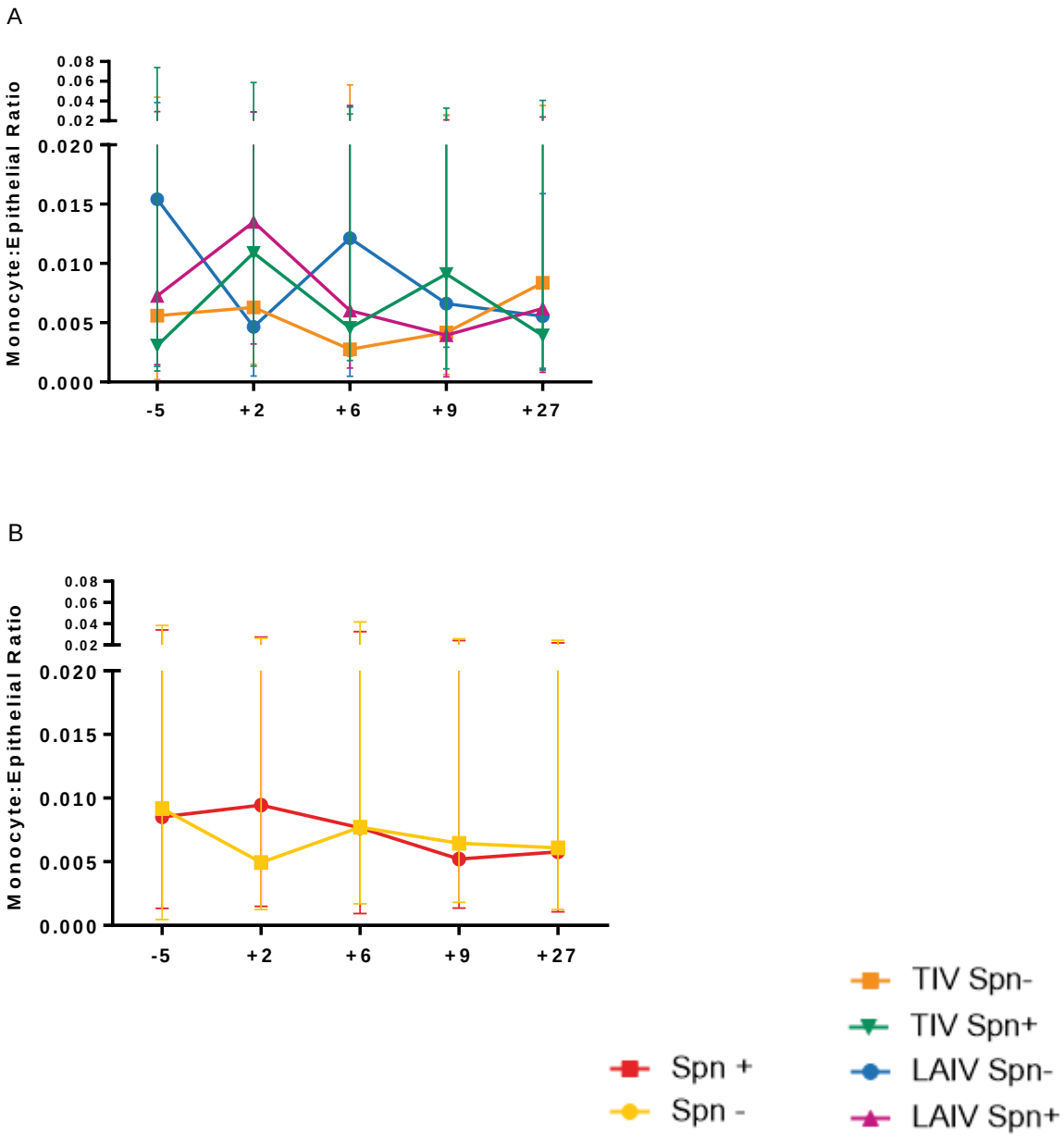

Figure 17. Induction of monocyte recruitment to the nose by Spn colonisation is affected by influenza vaccination. (A) Recruitment of monocytes was measured in 99 volunteers (n=24-27) by comparing the ratio of median monocytes to epithelial cell in each timepoint compared to its baseline (day -5). (B) Median of monocyte to epithelial cell ratio of volunteers stratified by Spn colonisation status (Spn- and Spn+). Comparison within groups was made by Wilcoxon test and between groups was made by Mann-Whitney test.

Neutrophil levels were assessed in the same way by using the ratio of neutrophils to epithelial cells ratio in each timepoint. The concurrent influenza vaccines did not affect significantly the number of neutrophils at the nasal lumen (Figure 18A). However, in non-colonised (Spn-) group, levels of neutrophils were reduced by median 2.5-fold 27 days post Spn challenge when compared to baseline (p=0.046, Figure 18B).

In response to Spn colonisation, LAIV Spn+ had a significant increase in neutrophil activation 2 days post-inoculation and before influenza vaccination (median 1.4-fold increase, p=0.048, Figure 18C). Although, the same pattern of neutrophil activation was observed in TIV Spn+, this increase did not differ significantly from the baseline. As these 2 groups received the influenza vaccine at Day 3 post pneumococcal inoculation, they can be pooled together up to Day 2. Therefore, when volunteers were stratified by colonisation status, it was shown that indeed Spn induced neutrophils activation 2 days after inoculation (Spn+, median 1.66-fold increase, p<0.0001, Figure 18D). The induction was sustained 6 days post-inoculation (median 1.4-fold increase, p=0.013), increasing further after 9 days (median 1.8-fold increase, p=0.022).

Notably, differences in neutrophils activation were already significant at baseline (day -5), with TIV Spn+ being a median 1.5 and 1.6 times higher when compared to TIV Spn- (p=0.040) and LAIV vaccinated groups, respectively (vs LAIV Spn-, p=0.022, Figure 18C).

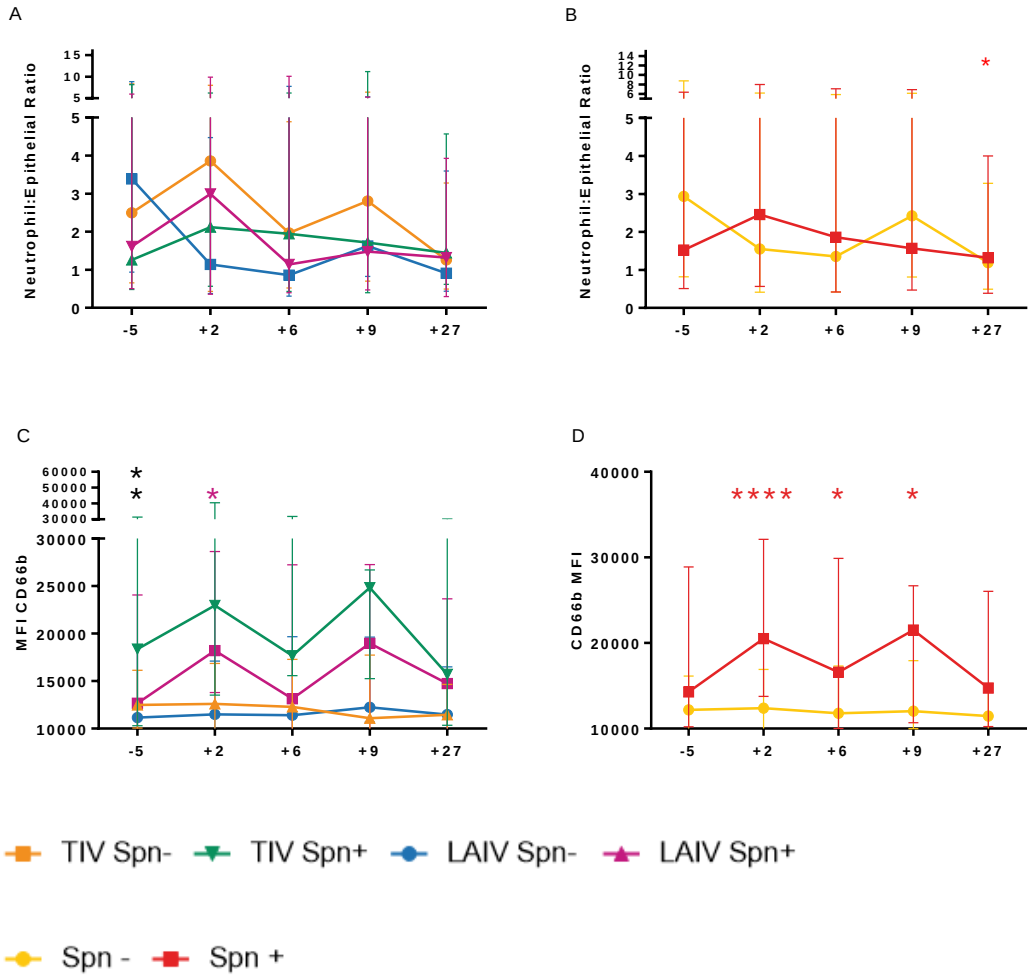

Figure 18. Spn colonisation of the nasopharynx induces neutrophil activation. (A) Recruitment of neutrophils was measured in 99 volunteers (n=24-27) by comparing the median of neutrophils to epithelial cell ratio in each timepoint to baseline (day -5) within groups using Wilcoxon test. (B) Median of neutrophils to epithelial cell ratio was measured in volunteers stratified by Spn colonisation status (Spn- and Spn+). The groups were compared in each timepoint by Mann Whitney test. (C) Activation of neutrophils was measured by comparing the median MFI of CD66b in each timepoint to baseline using Wilcoxon test and between groups using Mann Whitney test, with *p≤0.05. (D) MFI of CD66b stratified by Spn colonisation using Mann Whitney test with ****p<0.0001 and *p≤0.05.

LAIV in Spn colonised alters T-cell subsets induction and T-cell activation.

We investigated $CD3^+$ T-cell recruitment to the nose, as well as activation of T- cells using the marker HLA-DR in 99 volunteers divided in groups: TIV Spn- (n=24), TIV Spn+ (n=27), LAIV Spn- (n=24) and LAIV Spn+ (n=24).

Interestingly, concurrent LAIV and Spn colonisation prevented a small median increase of 1.1-fold in T-cells levels 27 days post-inoculation which was significant in TIV Spn- (p=0.013, Figure 19A, Figure 19B). Interestingly, at this timepoint, the group TIV Spn- showed higher $CD4^+$ T-cells levels, presenting median 1.6-fold increase in levels (p=0.013, Figure 20A) as well as a decrease of $CD8^+$ T-cells with a small median 1.1-fold reduction (p=0.007, Figure 20B).

Importantly, LAIV Spn- showed a significant decrease in T-cell activation 2 days after Spn inoculation which was impaired in Spn colonised (median 1.4-fold decrease, p=0.025, Figure 21).

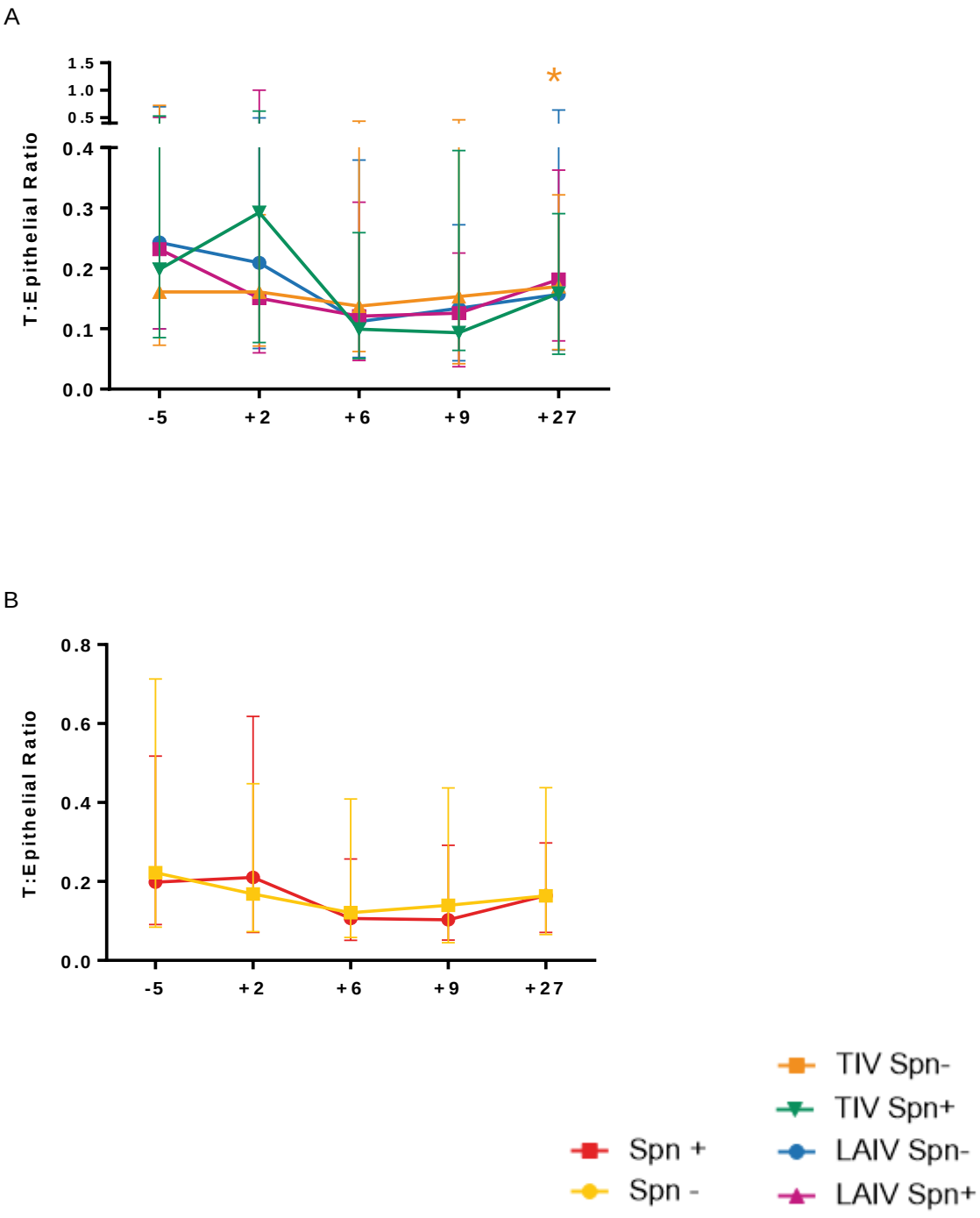

Figure 19. Late increase of T-cells in Spn non-colonised. (A) Nasal recruitment of T-cells was measured in volunteers (n=24-27) by comparing the median of T-cells to epithelial cell ratio in each timeline to baseline (-5) within groups using Wilcoxon test with *p≤0.05. (B) Median of T-cells to epithelial cell ratio in volunteers stratified by Spn colonisation status (Spn-, n=60, and Spn+, n=46) using Mann Whitney test for comparison between groups.

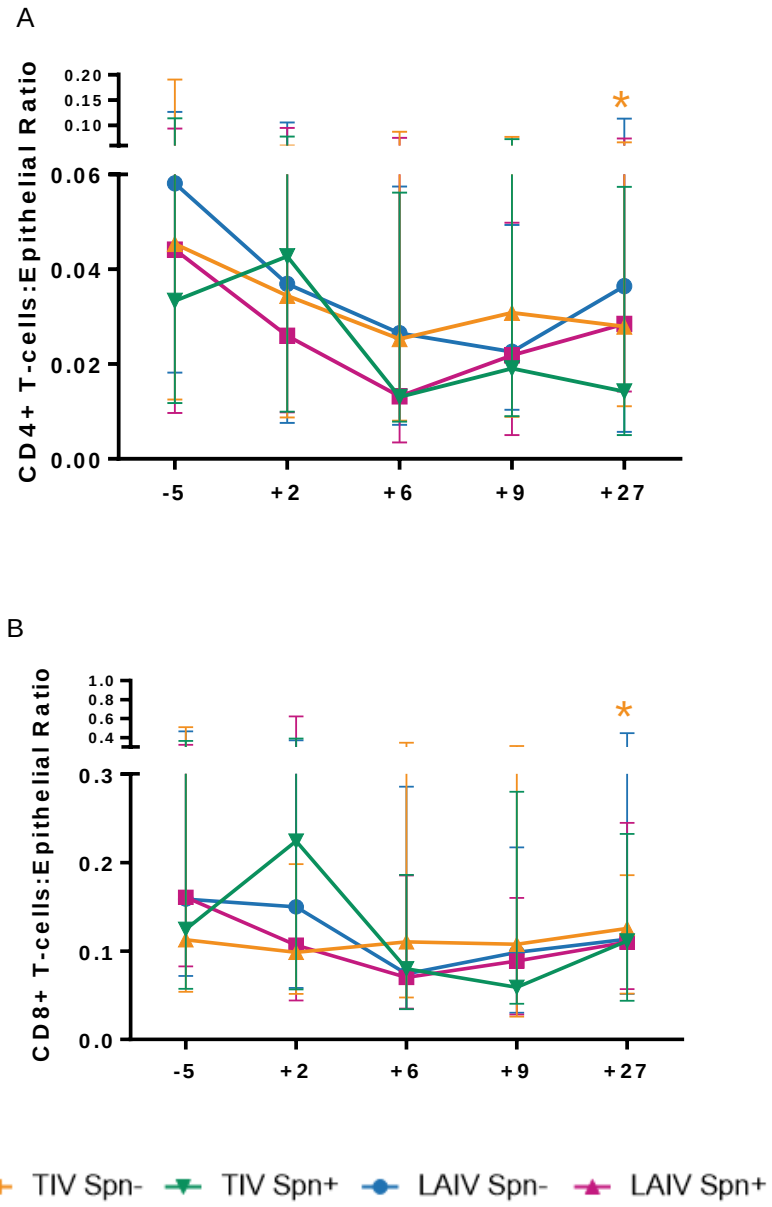

Figure 20. LAIV and Spn colonisation alters late CD4⁺ T-cells decrease and CD8⁺ increase. (A) Nasal recruitment of T-cells was measured in volunteers (n=24-27) by comparing the median of CD4⁺ T-cells to epithelial cell ratio in each timeline to baseline (-5). (B) Median of CD8⁺ T-cells to epithelial cell ratio. Comparisons were made within groups using Wilcoxon test with * p≤0.05.

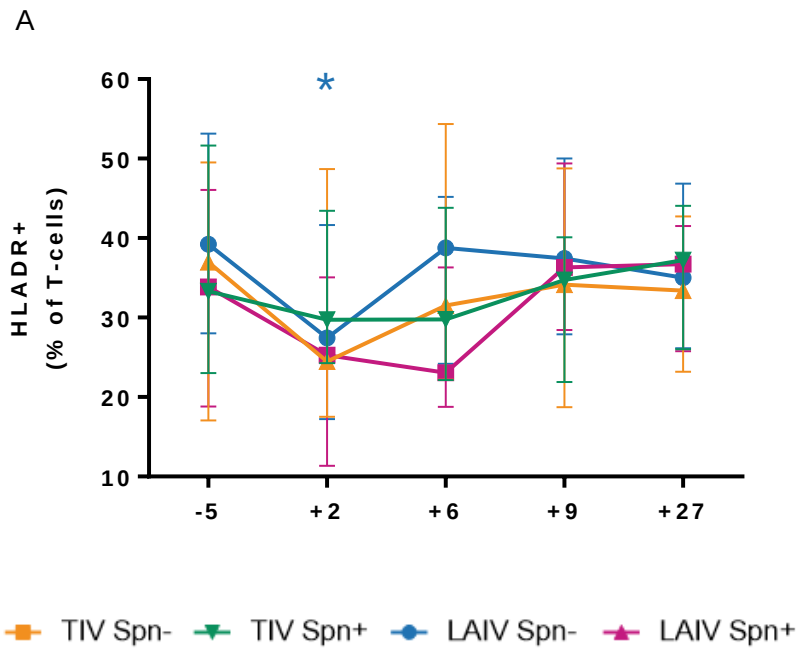

Figure 21. LAIV and Spn colonisation alters T-cell activation. (A) Activation of T-cells was measured by comparing median percentage of HLA-DR positive T-cells using Wilcoxon test for each timepoint to baseline (day -5) with *p≤0.05. Groups were stratified by vaccination and Spn colonisation status (n=24-27). (B) Fold-change of median percentage of HLA-DR positive T-cells using Wilcoxon test with *p≤0.05.

We also assessed the recruitment of DCs, using the markers BDCA1 (CD1c) for inflammatory DCs and BDCA-2 (CLEC4C) for plasmacytoid DCs. BDCA-1$^+$ DCs is a subset of cells that present dendritic morphology and can induce robust T-cell stimulation [321], whereas BDCA-2$^+$ DCs can affect the innate immune responses via TNF-α and IFN-γ production [322]. The median of BDCA-1$^+$ and BDCA-2$^+$ cells to epithelial cell ratio was measured in each timepoint and compared to baseline demonstrating no significant differences in cell recruitment (Figure 22A and 22B).

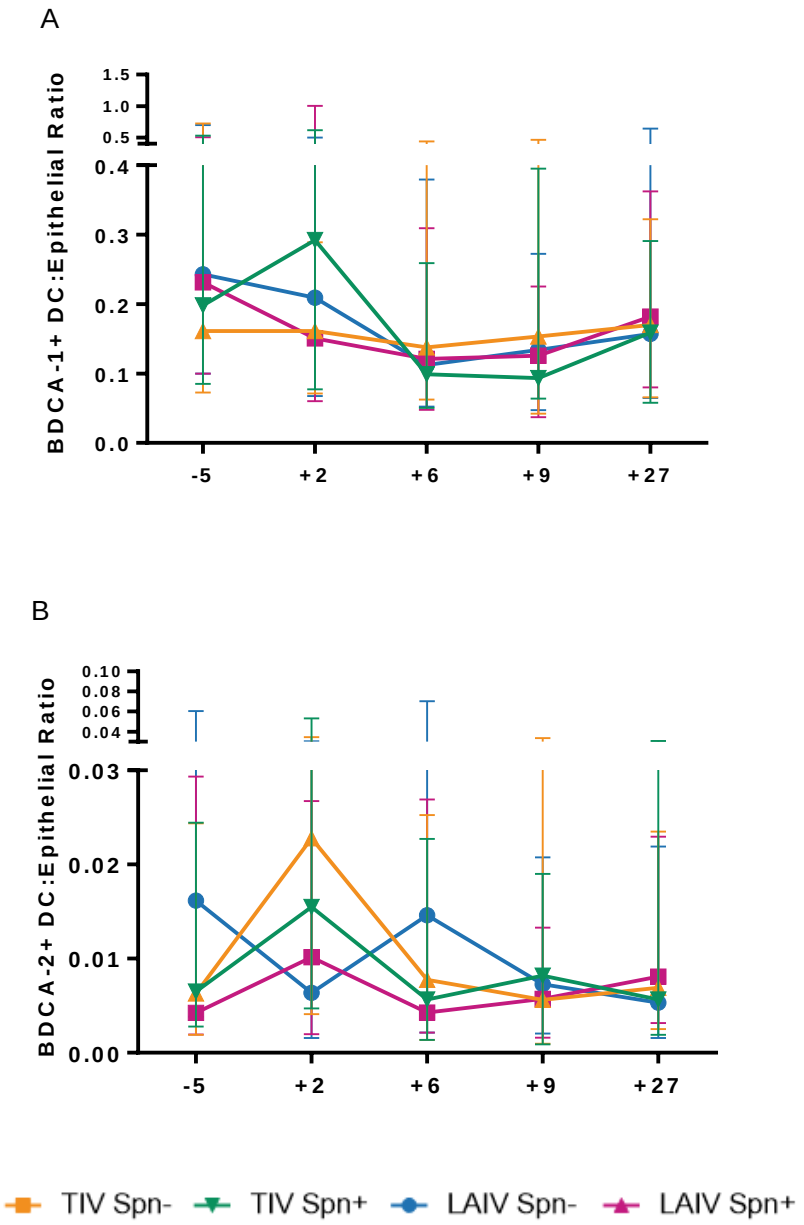

Figure 22. Concurrent influenza vaccines and Spn colonisation do not significantly affect BDCA-1$^+$ and BDCA-2$^+$ DC levels in the human nasal mucosa. (A) Nasal recruitment of DCs was measured in volunteers (n=24-27) by comparing the (A) median of BDCA1$^+$ DC. (B) median of BDCA-2$^+$ DC.

4.4 Discussion

The immunological mechanisms that control Spn colonisation and the impact of LAIV vaccination thereon have been studied extensively in mice [76,186,323,324] but remain poorly understood in humans. The Antecedent and Concurrent LAIV-EHPC studies allowed us to study the recruitment of immune cells to the human nasopharynx following Spn inoculation and influenza immunisation. We demonstrated that when LAIV proceeds Spn colonisation affects cellular responses, such monocytes recruitment in the nasal lumen, as well as activation of neutrophils and T-cells. Additionally, Spn colonisation induced neutrophil activation and impaired T-cells activation and abundance in the nasal mucosa.

The human nasopharynx, the site where pneumococcal colonisation occurs, is equipped with both epithelial and immune cells that mount responses to *S. pneumoniae* [306]. In mice, nasal mucosal immune responses to Spn are described by classic activation of macrophages and increase in regulatory T-cells (T-reg) levels [92]. During a colonisation episode, monocytes together with neutrophils are supposed to contribute to the control of Spn density, as demonstrated by the recruitment of these type of cells in mice deficient for macrophage [325,122,326]. In line with the data deriving from mice [327], in healthy adults monocytes were recruited to the nasal mucosa after Spn colonisation. Monocytes recruitment peaked at Day 9 post the bacterial challenge in the control group. On the other hand, primary infection with LAIV, which leads to a transient influenza virus replication in the nasal mucosa, impaired monocyte recruitment in those colonised with Spn. Moreover, only in the control group Spn density was associated with increased monocyte recruitment, which suggests that the

attenuated influenza viruses contained in the vaccine possibly disrupts host cytokine and chemokine signalling and subsequently impairs monocyte recruitment.

Our finding on the effect of LAIV in Spn colonised individuals corroborate with studies in which LAIV impact monocyte recruitment similarly to the wild-type influenza infection in mice, offering an explanation for the association of LAIV administration with increase in Spn acquisition and bacterial load found in human studies [318,328]. Monocytes seems to be key cells in controlling nasopharyngeal pneumococcal colonisation [94 329] and inhibition of their recruitment to the nasal mucosal has been associated with reduced bacterial clearance [108,330]. Although our results are robust, they may not be translated accurately to young children, as monocyte recruitment to the nasal mucosa is observed during influenza infection [331]. Interestingly, although concurrent LAIV was shown to increase levels of monocytes, recruitment of these immune cells was not significant. A possible explanation for this discrepancy between the two studies is that baseline levels of resident macrophages were approximately the double in the Antecedent LAIV study when compared to the Concurrent LAIV study. Further, in the presence of Spn or when undergo apoptosis, resident macrophages signal for the recruitment of blood circulating monocytes, which could further explain the increased concentration of monocytes in volunteers who already displayed high levels at baseline. In addition, reduced Spn density and colonisation periods were reported in the Concurrent study when compared to the Antecedent [50] which can also affect monocyte recruitment.

Unlike monocytes, high levels of neutrophils can be found in healthy individuals [327]. During Spn colonisation in murine models, Th17 cells secrete IL-

17A contributing to neutrophil recruitment to the nasal mucosa [94]. In the first 24 hours of Spn colonisation, neutrophils perform essential roles in controlling the infection mainly through serine proteases [332] as well as degranulation, production of reactive oxygen and nitrogen species, and pro-inflammatory cytokines [92,327].

Additionally, co-infection studies with antecedent wild-type influenza virus and Spn in mice demonstrate impairment in recruitment of neutrophils due to reduction in KC and chemokine MCP-1 expression, critical for infection control [186], as well as increased expression of anti-inflammatory cytokine IL-10, commonly produced by $CD8^+$ T-regs after viral clearance [92,127,188]. In contrast to this mechanism described in murine models of antecedent influenza co-infection, we did not observe neutrophil recruitment to the nose following Spn colonisation. This underlies the importance of confirmation of data with the attenuated virus and human data to elucidate the impact of LAIV.

However, when Spn colonised the nasal mucosa prior to influenza vaccination, it induced activation of neutrophils during 9 days after inoculation, indicating that the co-infection elicits additional immune mechanisms for control of both pathogens. The results corroborate neutrophil studies in mice which have demonstrated that Spn infection within 3 days of influenza infection induce clearance whereas co-infection within 6 to 10 days conferred inefficient bacterial control and colonisation of the nasopharynx [192]. Importantly, although neutrophils are present in the nasal lumen of children of 9 to 11 years old [333], the results may not be transferable to younger ages considering studies in which Spn-induced neutrophils are not detected in the nasal mucosa of naive mice [94]. Additionally, the depletion of these immune cells did not increase susceptibility to pneumococcal invasive disease in a mouse model [334].

When the innate immunity is not enough to allow for effective viral clearance, adaptive responses are also induced in the nasopharynx. Murine models show that in the human upper airways, viral pathogens are recognised through pattern recognition receptors (PRRs) such as TLRs and intracellular viral sensors that in turn induce adaptive responses [196], eliciting secretory antibodies and T-cells with mucosal homing properties, especially influenza-specific $CD8^+$ T-cells, common in natural influenza infections [187]. Here, we demonstrate that in human adults the presence of the attenuated influenza viruses did not affect T-cell recruitment, although it induced T-cell activation. Increased activation indicates that the attenuated virus is presented to the host as a natural infection by producing intracellular influenza antigens eliciting T-cell responses similar to the wild-type influenza [335].

In conclusion, the results presented in this chapter address important questions about the immune responses that control and clear Spn and how LAIV can alter these immune mechanisms, using for the first time an experimental human challenge model of co-infection with LAIV and Spn (EHPC).

CHAPTER FIVE

EFFECT OF SPN AND LAIV ON THE NASOPHARYNX CYTOKINE PRODUCTION

5.1 Introduction

A stable and asymptomatic Spn colonisation is not uncommon in children and adults, and provide benefits to the host as it develops cellular and adaptive immunity in the nasopharynx and systemically [49,62] . The immunising asymptomatic colonisation is maintained by a fine balance between pro- and anti-inflammatory cytokines [92].

However, influenza virus has the ability to interact with and modify the host nasal environment [196], and can leave individuals susceptible to uncontrolled Spn colonisation [262]. First shown in a ferret model by McCullers [273], wild-type influenza virus causes deregulation between immune tolerance and inflammatory responses, which can lead to damage in nasal epithelium [190] and, in turn, facilitate Spn adhesion to cells and impairment of cellular protection against the bacteria [76,92,274]. Spn replicated continuously in the nose, increasing density and bacterial infiltration to the lungs as well as inducing more cytokines, chemokines and antimicrobial peptides [132,188,270]. These elicited cytokines are shown to have a wide range of effects during co-infection in mice, acting on both innate and adaptive processes of the immune system [76,92,274]. They can affect recruitment of immune cells, including monocytes, neutrophils and T-cells, as well as their activation [92,132,186,188,262,270,271].

Furthermore, influenza vaccination with live attenuated virus – although an effective strategy to prevent infection in children [213] – also influence host immune responses to Spn, affecting Spn acquisition and density, transmission and susceptibility to invasive disease [281] due to its mimicry of wild-type influenza natural replication in the nasopharynx, known as viral shedding. Similarly to studies with wild-type influenza, LAIV vaccination in mice colonised with Spn also

induced deregulated cytokine responses in the nasopharynx [274], which highlights the necessity of validation of these findings in human. Herein, we used a controlled human LAIV-Spn co-infection model to investigate the cytokine responses elicited to Spn colonisation and how these responses are altered by either a primary or a simultaneous influenza viral infection.

5.2 Material and methods

5.2.1 Volunteer recruitment, vaccination and inoculation

75 adult volunteers were recruited for the Antecedent and Concurrent studies (as described in Section 2.2) and received influenza vaccination (LAIV or TIV as control) and were inoculated with live Spn using EHPC. Individuals were vaccinated 3 days previous to Spn inoculation in the Antecedent study and 3 days after in the Concurrent study (Section 2.3 and 2.5.4).

5.2.2 Nasosorption collection

An adsorptive matrix strip was used in the nasal septum of volunteers for collection of nasal fluid and stored at -80^{o}C (Section 2.5.7).

5.2.3 Luminex and statistical analysis

As described in Section 2.6.7 nasal fluid was washed and prepared for Luminex Human Cytokine 30-plex per manufacturer's instructions. Results obtained were processed and analysed by a LX200 and xPonent 3.1 software. Statistical analysis was made in Prism 6 and 7 (Graphpad) and R software (Section 2.7).

5.3 Results

In the Antecedent study, 75 volunteers were vaccinated with LAIV (n=38) or TIV as control (n=37) and 3 days later inoculated intranasally with live Spn. Volunteers were stratified into groups: TIV Spn- (n=19), TIV Spn+ (n=18), LAIV Spn- (=19) and LAIV Spn+ (n=19). NS was collected at baseline (day -4), day +0, +2, +7, +9 and +29 relative to Spn inoculation. Levels of 30 cytokines were measured in the nasal lining fluid by Luminex.

In the Concurrent study, 56 volunteers were inoculated with live Spn, following vaccination with LAIV (n=28) or TIV (n=28) 3 days later. NS was collected at baseline (day -5) and at days +6, +9 and +27 relative to inoculation (or 3, 6 and 24 days after vaccination). Levels of 30 cytokines were measured in the nasal lining fluid by Luminex, however, only 6 volunteers became colonised in this subset of volunteers. Therefore, stratification for analysis was done regarding vaccination status with groups: TIV (n=28) and LAIV (n=28).

LAIV induces pro-inflammatory cytokines in nasopharynx, especially in Spn colonised.

Firstly, we investigated the cytokine profile elicited by concurrent influenza vaccines in the human nasal mucosa in volunteers challenged by Spn but not colonised.

Three days after vaccination (day +6), volunteers in the control group induced a median 1.4-fold decrease in levels of pro-inflammatory cytokine IL-1β (p=0.033, Figure 23A) and 6 days after (day +9) a median 2-fold decrease in GM-CSF (p=0.049, Figure 23B).

In contrast to TIV, LAIV vaccination elicited increase of IL-1β levels 3 days after vaccination (day +6) (median 1.2-fold increase) (p=0.049, Figure 24A). Moreover, we observed that LAIV induced a high pro-inflammatory cytokine response in the nose, with median 1.6-fold increase of IFN-α and MIP-1α (p=0.013, Figure 24B and Figure 24C), median 1.9-fold increase of MIP-1β levels (p=0.009, Figure 24D) and median 2.5-fold increase of TNF-α levels (p=0.011, Figure 24E).

Six days after vaccination (day +9), LAIV group maintained a similar to day +6 induction of IL-1β, MIP-1α, TNF-α and MIP-1β levels (IL.1β: median 1.3-fold increase, p=0.021, Figure 24A; MIP-1α: median 1.5-fold increase, p=0.048, Figure 24C; TNF-α: median 1.5-fold increase, p=0.026, Figure 24E; MIP.1β: median 1.5-fold increase, p=0.035, Figure 24D) but not for IFN-α. Additional pro-inflammatory cytokine response was elicited with increases in levels of MCP-1, IL-6 and IL-8 (MCP-1: median 1.8-fold increase, p=0.010, Figure 25A; IL-6: median 1.9-fold increase, p=0.035, Figure 25B; IL-8: median 1.3-fold increase, p=0.041, Figure 25C) with IL-8 sustained until day +29 (median 1.5-fold increase,

p=0.035, Figure 25C). Moreover, at day 24 after influenza vaccination (day +29), TNF-α levels were sustained at levels similar to those observed at day +6 and +9 (median 2.3-fold increase, p=0.018, Figure 25E) post LAIV.

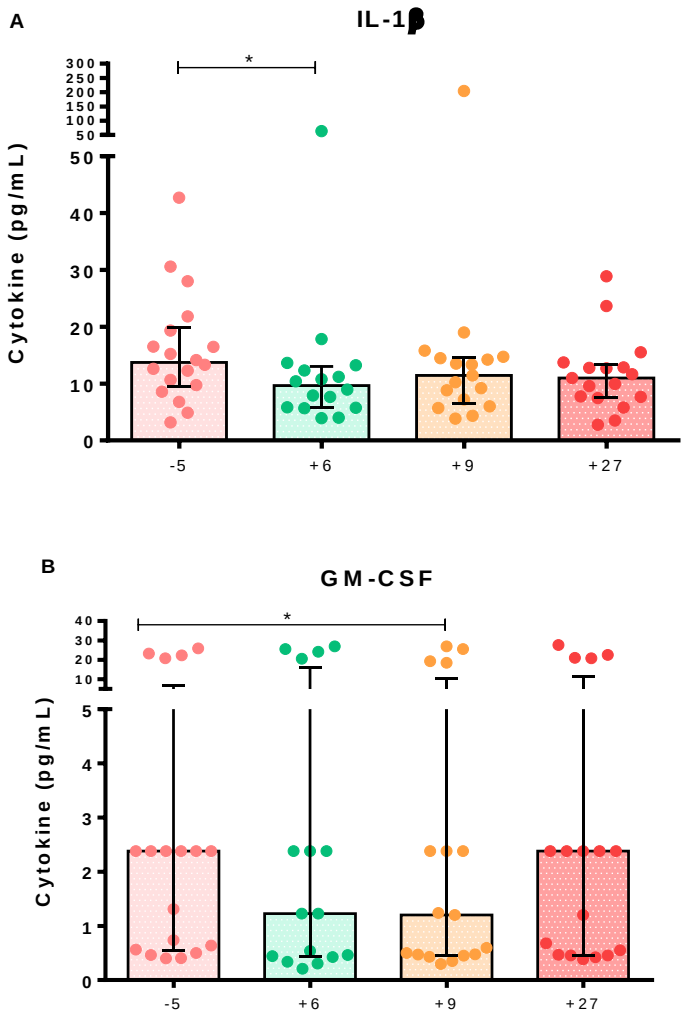

Figure 23. TIV vaccinated group exhibits decrease in pro-inflammatory cytokines IL-1β and GM-CSF in the nasopharynx. Levels of (A) IL-1β and (B) GM-CSF measured in nasal lining of volunteers vaccinated with TIV (n=28) were compared at each timepoint to baseline (day -5) by Wilcoxon test with *p≤0.05. Medians and interquartile range of each timepoint are shown.

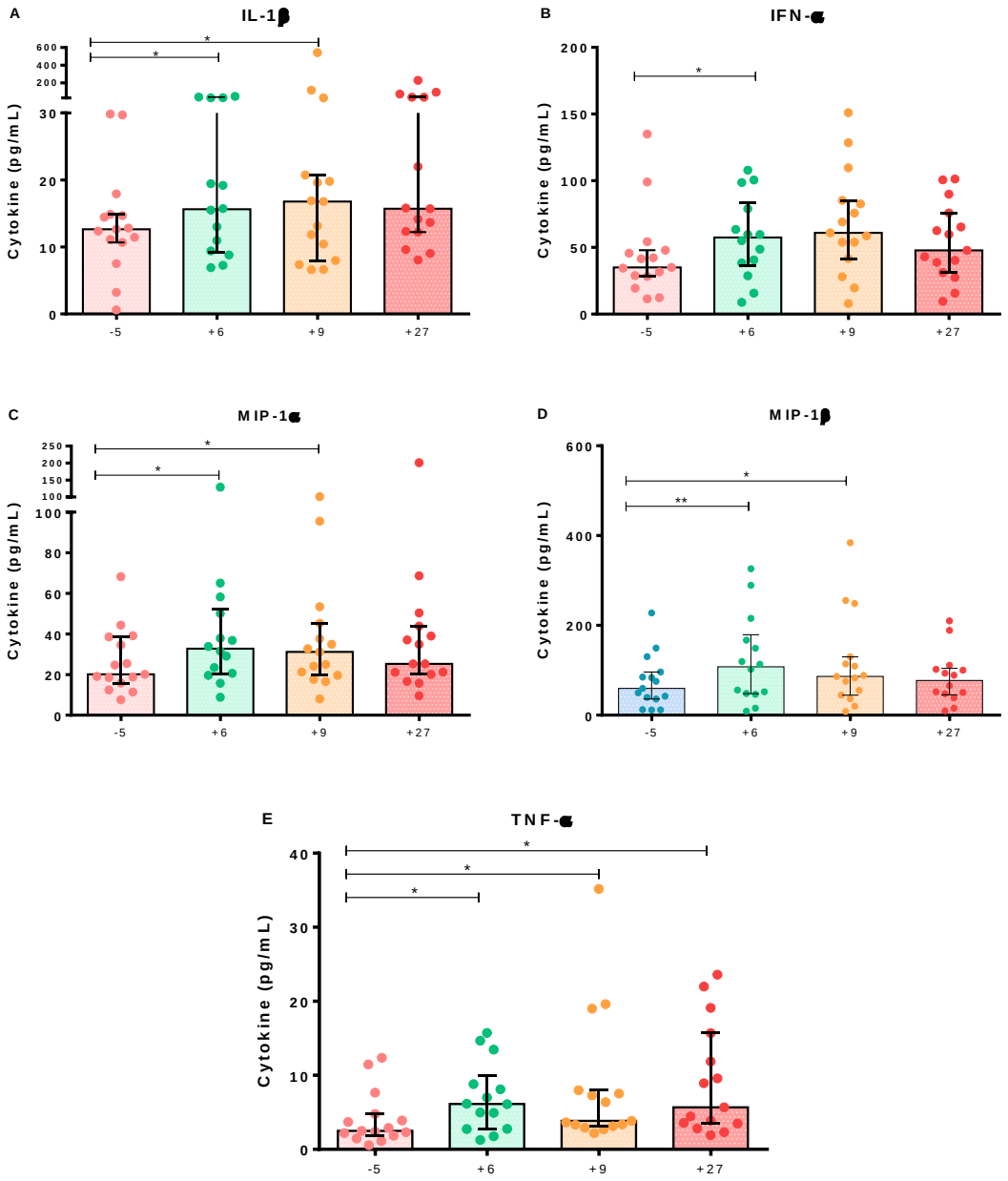

Figure 24. LAIV vaccinated group exhibits increase in pro-inflammatory cytokines in the nasopharynx. Levels of (A) IL-1β, (B) IFN-α, (C) MIP-1α, (D) MIP-1β and (E) TNF-α measured in nasal lining of volunteers vaccinated with LAIV (n=28) were compared at each timepoint to baseline (day -5) by Wilcoxon test with *p≤0.05 and **p<0.01. Medians and interquartile range of each timepoint are shown.

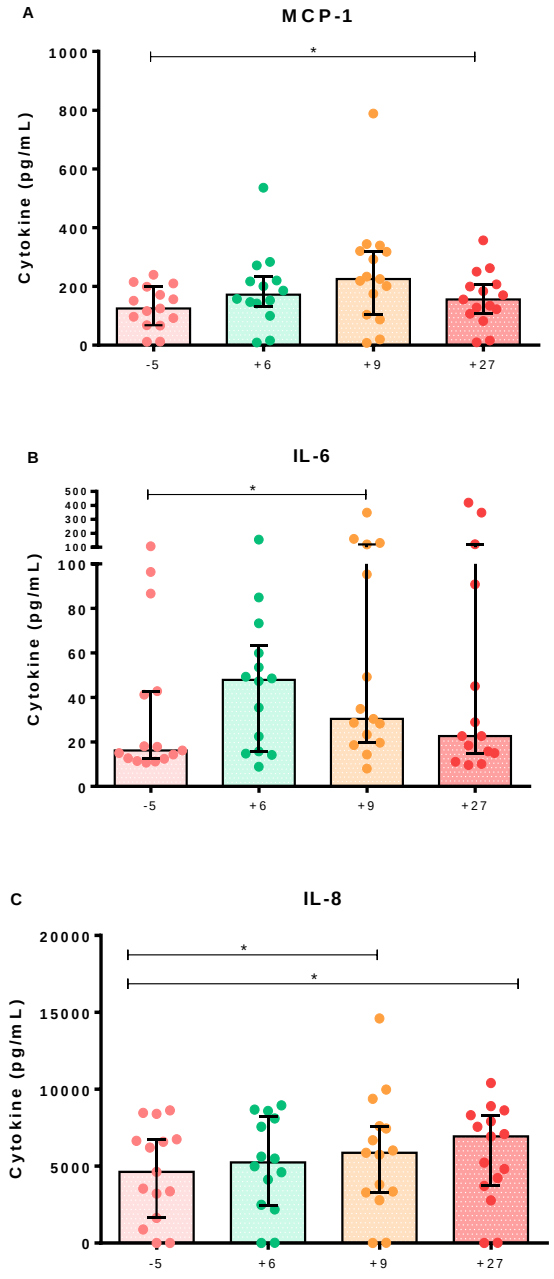

Figure 25. LAIV vaccinated group exhibits increase in pro-inflammatory cytokines in the nasopharynx. Levels of (A) MCP-1, (B) IL-6 and (C) IL-8 measured in nasal lining of volunteers vaccinated with LAIV (n=28) were compared at each timepoint to baseline (day -5) by Wilcoxon test with *p≤0.05. Medians and interquartile range of each timepoint are shown.

Next, we examined the cytokine profile elicited in a second set of volunteers by antecedent influenza vaccines followed by Spn inoculation in the nose, with groups stratified by vaccination and colonisation status.

Notably, antecedent LAIV in absence of Spn colonisation (day +0) induced cytokines IL-6 and MIG, as LAIV Spn- presented median 1.4- and 2.5-fold increase of IL-6 and MIG levels, respectively (p=0.021 and p=0.003, Figure 26A and 26B, respectively). Moreover, LAIV induced MCP-1 and TNF-α secretion in the nasopharynx, but only MCP-1 levels were maintained up to Day 9 post inoculation in the LAIV Spn- (MCP-1, median 1.3-fold increase, p=0.029; TNF-α, median 1.9-fold increase, p=0.030, Figure 27A and 27B).

Additionally, in LAIV Spn-, Spn challenge also induced the secretion of the pro-inflammatory cytokines MIP-1α, MIP-1β, IL-1β and RANTES 2 days after challenge (MIP-1α, median 1.3-fold increase, p=0.024; MIP-1β, median 1.4-fold increase, p=0.036; IL-1β, median 1.7-fold increase, p=0.004; RANTES, median 2.9-fold increase, p=0.026, Figure 28A, 28B, 28C and 28D respectively).

Seven days after inoculation, volunteers continued to express Spn-induced IL-1β although no other cytokine increase was demonstrated (median 1.3-fold increase, p=0.020, Figure 28C). On the other hand, 9 days post-inoculation another peak of pro-inflammatory cytokine levels was observed in this group with induction of MIG, MCP-1, IL-1β and RANTES (MIG, median 1.7-fold increase, p=0.030; MCP-1, median 1.4-fold increase, p=0.010, IL-1β, median 1.5-fold increase, p=0.044; RANTES, median 2.5-fold increase, p=0.018. Figures 26B, 27A; 28C and 28D respectively).

The inflammation profile observed at day +0 in LAIV Spn+ was similar to the non-colonised group for cytokines IL-6 and MIG, presenting a median 1.6-

and 2.2-fold increase, respectively (p=0.012 and p=0.023, Figure 29A and 29B, respectively). Furthermore, LAIV with the influence of Spn, induced the production of cytokines IFN-α, MIP-1α, MIP-1β and IP-10 (median 1.1-, 1.2-, 1.3- and 3.6-fold increase, respectively. IFN-α, p=0.009; MIP-1α, p=0.023; MIP-1β, p=0.035; IP-10, p<0.001, Figure 30A, 30B, 30C and 30D respectively). Interestingly, LAIV Spn+ LAIV-induced IL-6 cytokine exhibited increased levels 5 days after LAIV administration (median 1.8-fold increase, p<0.001, Figure 29A).

Also 5 days after LAIV, in volunteers of the LAIV Spn+ group, Spn colonisation induced a median 1.4-fold increase in IL-1β (p=0.003, Figure 31). These volunteers also demonstrated higher levels of MIP-1α and MIP-1β (Figure 30B and 30C), however this induction started before Spn inoculation (MIP-1α, median 1.4-fold increase, p=0.038; MIP-1β, median 1.6-fold increase, p=0.017). Additionally, likewise responses at day +0, 2 days after Spn challenge, LAIV-vaccinated and colonised were still the only group to induce IFN-α (median 1.2-fold increase, p=0.001, Figure 30A) and even higher levels of IP-10 (median 4.8-fold increase, Figure 30D).

Opposite to non-colonised, LAIV Spn+ did not show great cytokine level increases after 2 days since colonisation. In this group, we also observed only a moderate increase in IFN-α induction 7 and 9 days post-inoculation (day +7, median 1.1-fold increase, p=0.028, day +9, median 1.1-fold increase, p=0.018, Figure 30A).

Interestingly, the TIV Spn- exhibited an induction of MIG before (day +0), 7 and 9 days after Spn challenge, which was not observed in the first set of volunteers vaccinated with concurrent TIV (day +7, median 1.4-fold increase,

p=0.010; day +9, median 1.9-fold increase, p=0.026. Figure 32A) as well as IP-10 at day +9 (median 1.4-fold increase, p=0.026, Figure 32B).

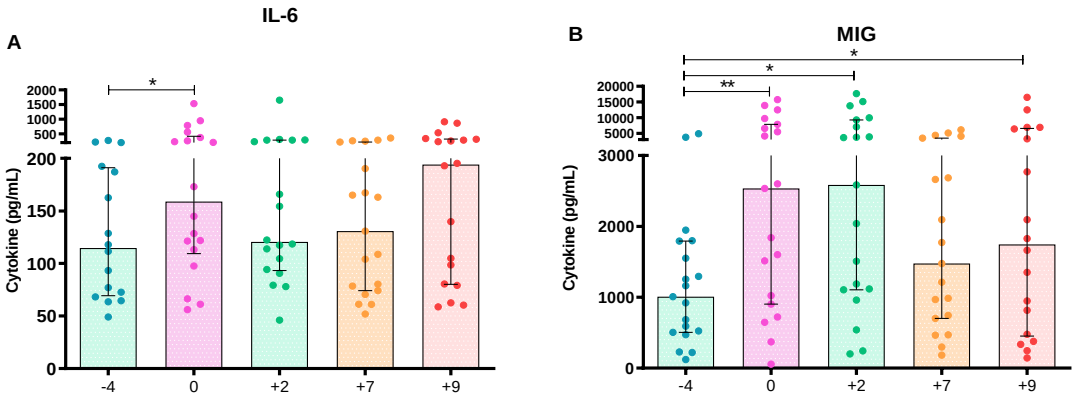

Figure 26. Antecedent LAIV induces pro-inflammatory cytokines IL-6 and MIG in nasopharynx of non-colonised volunteers. Levels of (A) IL-6 and (B) MIG were measured in nasal lining of volunteers vaccinated with antecedent LAIV and not colonised by Spn (LAIV Spn-, n=18-19). Results were compared at each timepoint to baseline (day -4) by Wilcoxon test with *p≤0.05 and **p<0.01 and medians and interquartile range of each timepoint are shown.

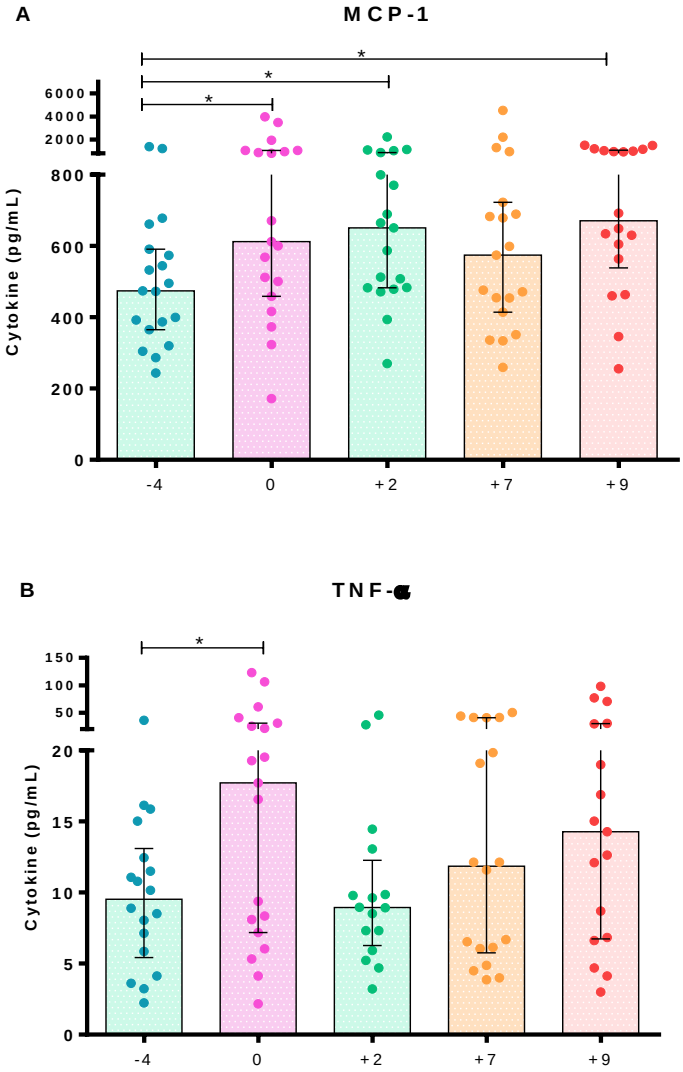

Figure 27. Antecedent LAIV induces pro-inflammatory cytokines MCP-1 and TNF-α in nasopharynx of non-colonised volunteers. Levels of (A) MCP-1 and (B) TNF-α were measured in nasal lining of antecedent LAIV-vaccinated and non-colonised volunteers (LAIV Spn-, n=18-19) and compared at each timepoint to baseline (day -4) by Wilcoxon test with *p≤0.05. Medians and interquartile range of each timepoint are shown.

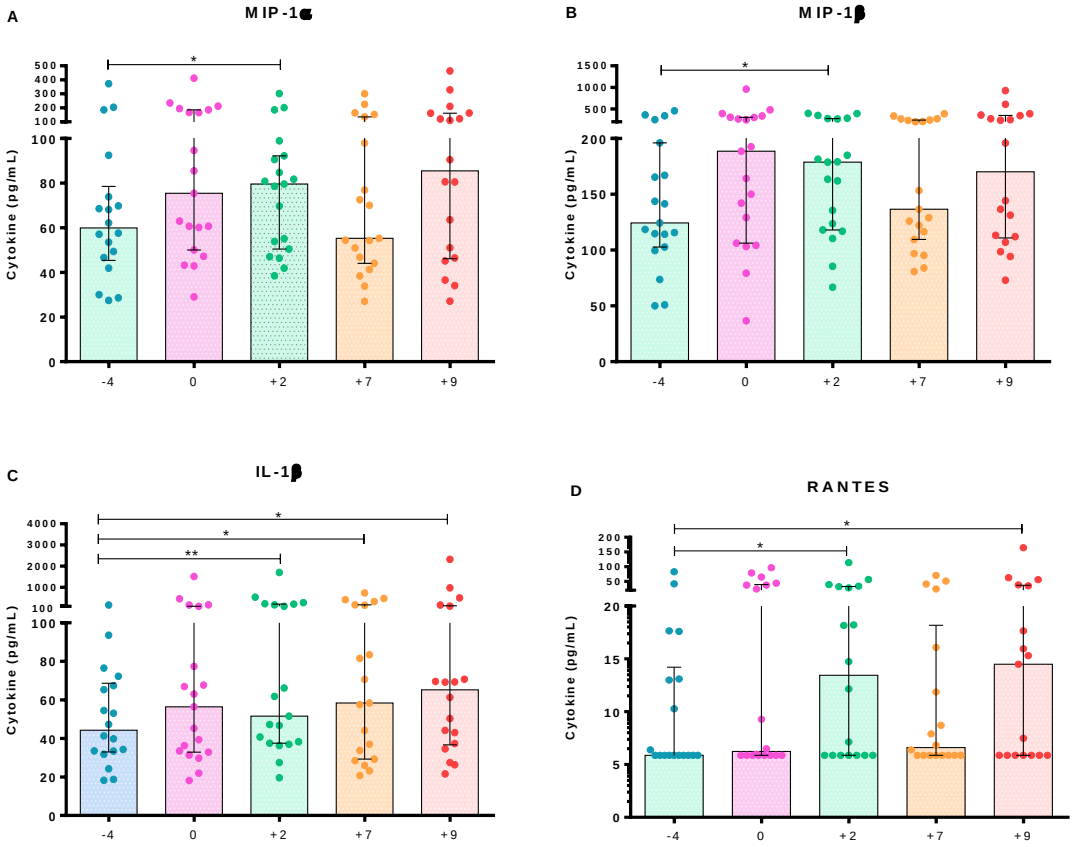

Figure 28. Antecedent LAIV induces additional pro-inflammatory cytokines in nasopharynx of non-colonised volunteers. Levels of (A) MIP-1α, (B) MIP-1β, (C) IL-1β and (D) RANTES were measured in nasal lining of volunteers vaccinated with antecedent LAIV and not colonised by Spn (LAIV Spn-, n=18-19). Results were compared at each timepoint to baseline (day -4) by Wilcoxon test with *p≤0.05 and **p<0.01 and medians and interquartile range of each timepoint are shown.

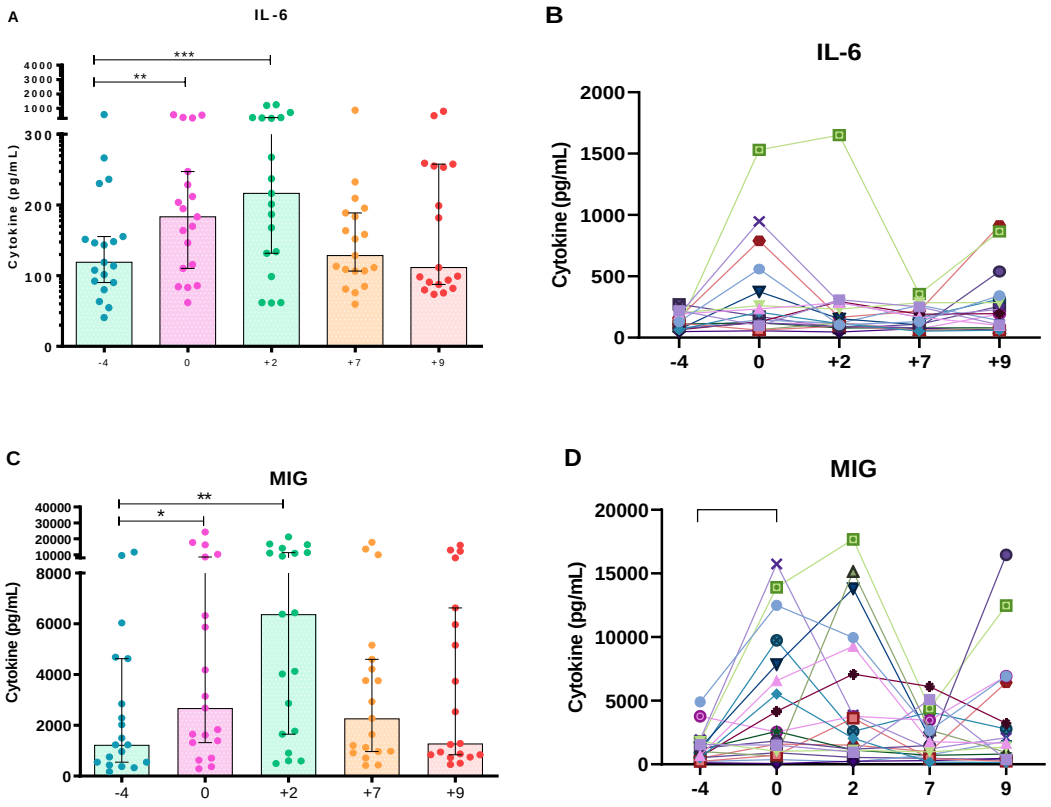

Figure 29. Antecedent LAIV induces pro-inflammatory cytokines IL-6 and MIG in nasopharynx of Spn colonised volunteers. Levels of (A, B) IL-6 and (C, D) MIG were measured in nasal lining of volunteers vaccinated with antecedent LAIV and colonised by Spn (LAIV Spn+, n=18-19). Results were compared at each timepoint to baseline (day -4) by Wilcoxon test with *p≤0.05, **p<0.01 and ***p<0.001. In A and C, medians and interquartile range of each timepoint are shown. In B and D each line represents one volunteers and each point the result of a single volunteers in a specific timepoint.

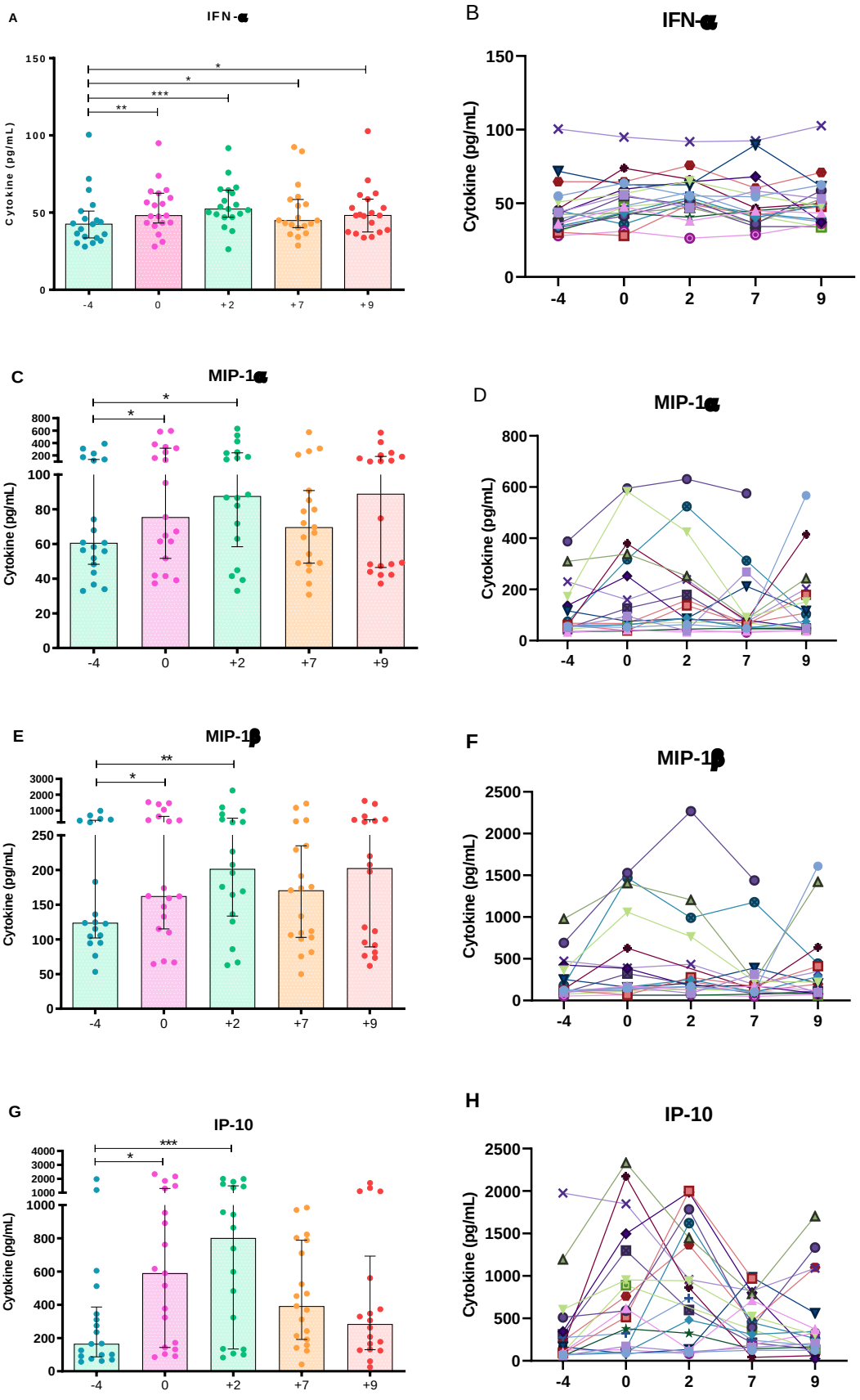
A
IFN-α
Cytokine (pg/mL)
150
100
50
0
*
*
***
**
-4
0
+2
+7
+9
B
IFN-α
Cytokine (pg/mL)
150
100
50
0
-4
0
2
7
9
C
MIP-1α
Cytokine (pg/mL)
800
600
400
200
100
80
60
40
20
0
*
*
-4
0
+2
+7
+9
D
MIP-1α
Cytokine (pg/mL)
800
600
400
200
0
-4
0
2
7
9
E
MIP-1β
Cytokine (pg/mL)
3000
2000
1000
250
200
150
100
50
0
**
*
-4
0
+2
+7
+9
F
MIP-1β
Cytokine (pg/mL)
2500
2000
1500
1000
500
0
-4
0
2
7
9
G
IP-10
Cytokine (pg/mL)
4000
3000
2000
1000
1000
800
600
400
200
0
***
*
-4
0
+2
+7
+9
H
IP-10
Cytokine (pg/mL)
2500
2000
1500
1000
500
0
-4
0
2
7
9

Figure 30. Antecedent LAIV induces additional pro-inflammatory cytokines in nasopharynx of Spn colonised volunteers. Levels of (A, B) IFN-α, (C, D) MIP-1α, (E, F) MIP-1β and (G, H) IP-10 were measured in nasal lining of volunteers vaccinated with antecedent LAIV and colonised by Spn (n=18-19) and compared at each timepoint to baseline (day -4) by Wilcoxon test with *p≤0.05, **p<0.01 and ***p<0.001. In A, C, E and G medians and interquartile range of each timepoint are shown. In B, D, F and H each line represents one volunteers and each point the result of a single volunteers in a specific timepoint.

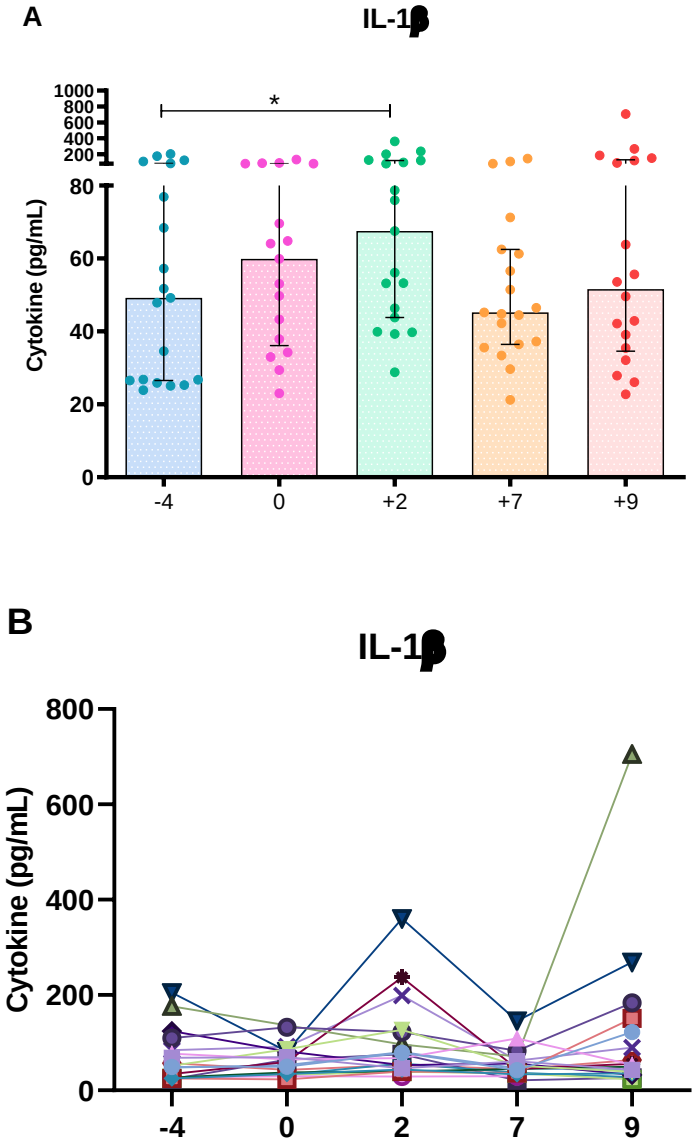

Figure 31. Antecedent LAIV induces pro-inflammatory cytokine IL-1β in nasopharynx of Spn colonised volunteers. Levels of IL-1β was measured in nasal lining of volunteers vaccinated with antecedent LAIV and colonised by Spn (LAIV Spn+, n=18-19). Results were compared at each timepoint to baseline (day -4) by Wilcoxon test with *p≤0.05 and **p<0.01. (A) Medians and interquartile range of each timepoint are shown. (B) Each line represents one volunteer and each point the result of a single volunteers in a specific timepoint.

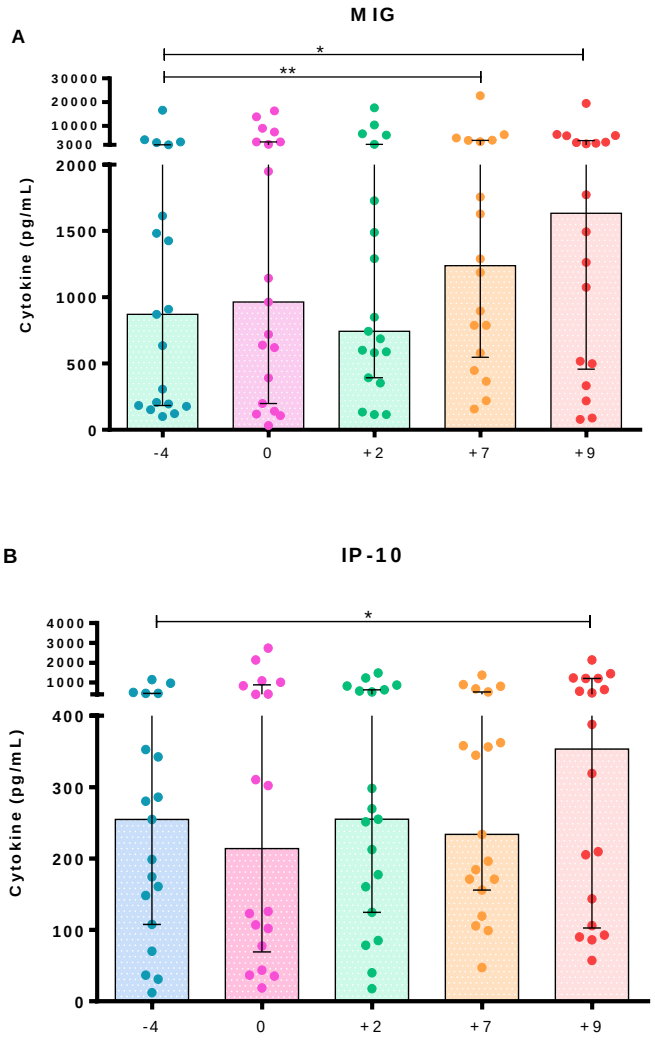

Figure 32. Non-colonised volunteers show induction of pro-inflammatory cytokines MIG and IP-10 in the nose . Levels of (A) MIG and (B) IP-10 were measured in nasal lining of volunteers in the control group that cleared Spn in the nasopharynx (TIV Spn-, n=18-19) and compared at each timepoint to baseline (day -4) by Wilcoxon test with *p≤0.05 and **p<0.01. Medians and interquartile range of each timepoint are shown.

Antecedent LAIV-induction of cytokines that regulate adaptive immunity diverge between Spn colonised and non-colonised volunteers

We assessed the levels of cytokines related to regulation of the adaptive immune responses after concurrent influenza vaccine administration in volunteers previously challenged with Spn, but not colonised.

LAIV-vaccinated volunteers showed increase in cytokines IFN-γ and IL-15 3 days after vaccination (day +6, IFN-γ: median 1.4-fold increase, p=0.035, Figure 33A; IL.15: median 1.3-fold increase, p=0.041, Figure 33B). Six days after LAIV (day +9), the vaccine induced a moderate increase in IL-2R, IL-2 and IL-12 levels (IL-2R: median 1.1-fold increase, p=0.018, Figure 34A; IL-2: median 1.2-fold increase, p=0.073, Figure 34B; IL-12: median 1.5-fold increase, p=0.012, Figure 34C), as well as for IFN-γ and IL-15 (IFN-γ: median 1.4-fold increase, p=0.047, Figure 33A; IL-15: median 1.1-fold increase, p=0.025, Figure 33B). Volunteers who received TIV had a slight increase in IL-4 6 days after vaccination (day +9, median 1.1-fold increase, p=0.018, Figure 35A). A similar increase of IL-4 was also observed in the LAIV-vaccinated group (median 1.1-fold increase, p=0.039, Figure 35B).

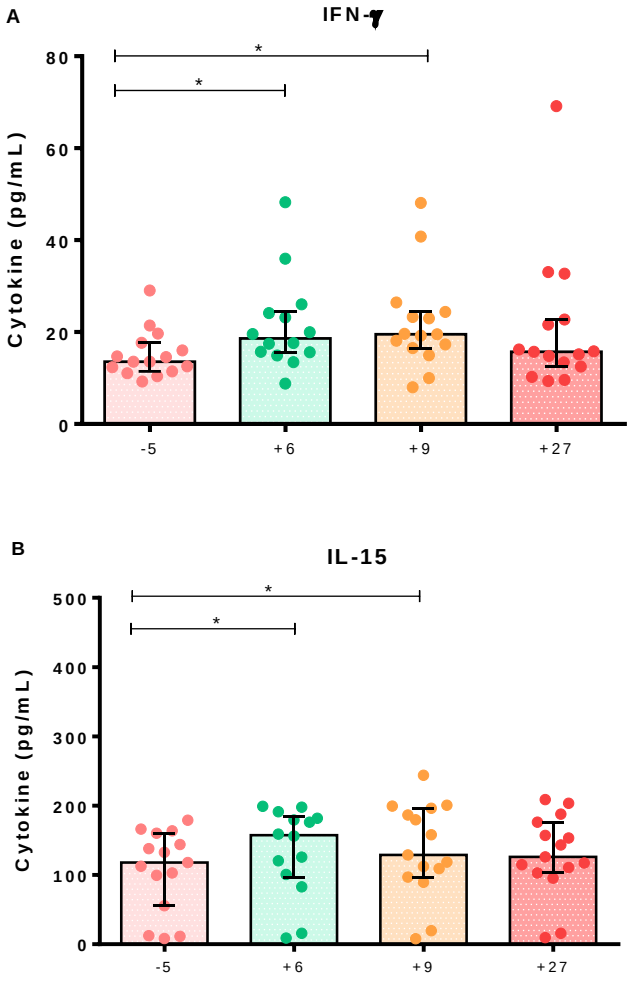

Figure 33. LAIV vaccinated show increase in cytokines IFN-γ and IL-15 in the nasopharynx.

Levels of (A) IFN-γ and (B) IL-15 measured in nasal lining of volunteers vaccinated with LAIV (n=28) were compared at each timepoint to baseline (day -5) by Wilcoxon test with *p≤0.05. Medians and interquartile range of each timepoint are shown.

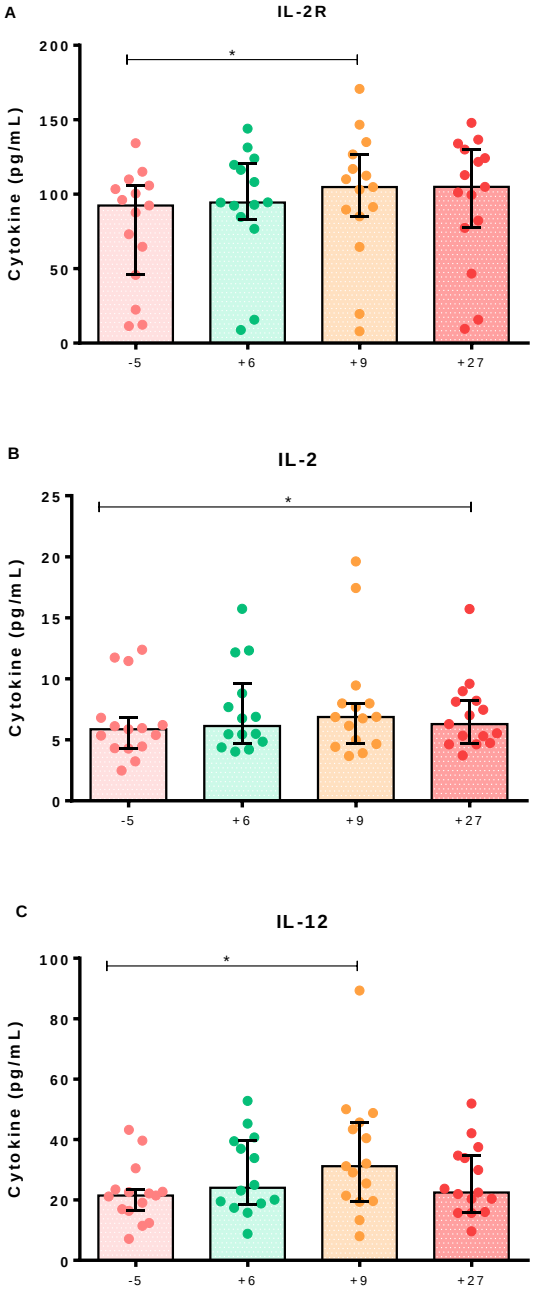

Figure 34. LAIV vaccinated show increase in cytokines that regulate adaptive responses in the nasopharynx. Levels of (A) IL-2R, (B) IL-2 and (C) IL-12 measured in nasal lining of volunteers vaccinated with LAIV (n=28) were compared at each timepoint to baseline (day -5) by Wilcoxon test with *p≤0.05. Medians and interquartile range of each timepoint are shown.

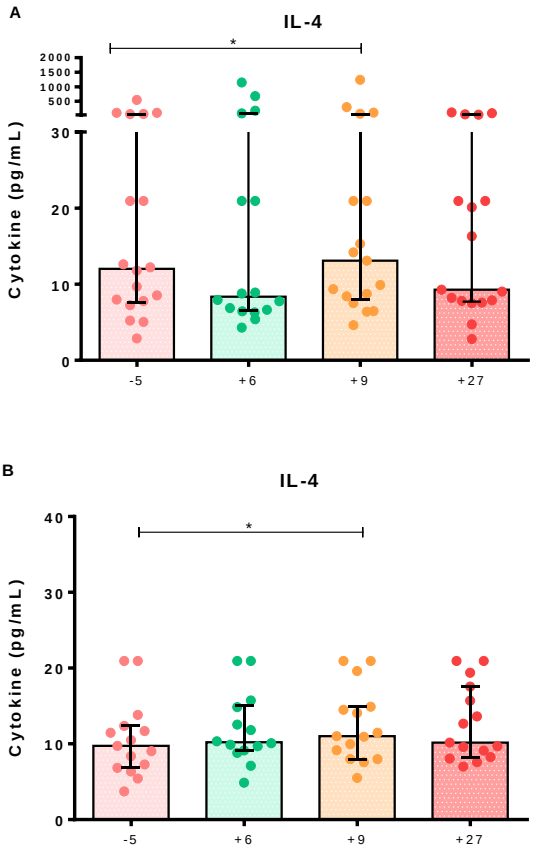

Figure 35. TIV- and LAIV-vaccinated show increase in the cytokine IL-4 in the nasopharynx. Levels of IL-4 was measured in nasal lining of volunteers vaccinated with (A) TIV (n=28) and (B) LAIV (n=28). Results compared at each timepoint to baseline (day -4) by Wilcoxon test with *p≤0.05 and medians and interquartile range of each timepoint are shown.

Further, we analysed a second set of volunteers, vaccinated with influenza vaccines followed by Spn challenge, and stratified into groups regarding vaccination and colonisation status.

Before Spn inoculation (day +0), both groups of volunteers vaccinated with LAIV demonstrated a moderate induction of IL-4 (LAIV Spn-, median 1.2-fold increase, p=0.021, Figure 36A; LAIV Spn+, median 1.4-fold increase, p=0.008, Figure 36B), IL-2 (LAIV Spn-, median 1.1-fold increase, p=0.026, Figure 36C; LAIV Spn+, median 1.4-fold increase, p=0.001, Figure 36D), IFN-γ (LAIV Spn-, median 1.2-fold increase, p=0.009, Figure 37A; LAIV Spn+, median 1.3-fold increase, p<0.001, Figure 37B) and IL-15 (LAIV Spn-, median 1.3-fold increase, p=0.001; Figure 37C; LAIV Spn+, median 1.2-fold increase, p=0.024, Figure 37D).

Interestingly, in LAIV Spn- the vaccine also conferred a slight increase in IL-12 (median 1.2-fold increase, p=0.035, Figure 38A) and IL-2R levels (p=0.021, Figure 38B), which was not observed in LAIV Spn+.

Two days after Spn inoculation, LAIV continued to similarly induce cytokines expressed at day +0 in both non-colonised and Spn colonised. Importantly and similarly to the first set of volunteers vaccinated with antecedent vaccines, we observed that volunteers vaccinated with LAIV showed increase in IL-4 levels (LAIV Spn-: p=0.003, Figure 36A; LAIV Spn+: p=0.025, Figure 36B). Moreover, TIV Spn- demonstrated instead a median 1.3-fold decrease in IL-4 (p=0.042, Figure 39).

Only in the groups vaccinated with LAIV we observed moderate inductions of IL-2, IFN-γ, IL-15 2 days after inoculation (LAIV Spn-: IL-2, median 1.1-fold increase, p=0.010, Figure 36C; IFN-γ, median 1.1-fold increase, p<0.001, Figure

37A; IL-15, median 1.5-fold increase, p=0.006, Figure 37C; LAIV Spn+: IL-2, median 1.3-fold increase, p=0.004, Figure 36D; IFN-γ, median 1.3-fold increase, p=0.002, Figure 37B; IL-15, median 1.4-fold increase, p=0.021, Figure 37D).

In addition, in non-colonised group IL-12 induction by LAIV was similar to this observed before the Spn challenge (day +0) (median 1.5-fold increase, p=0.035, Figure 38A). In the LAIV Spn+ group such an induction was observed only early post the challenge (median 1.2-fold increase, p=0.012, Figure 40A). Importantly, LAIV Spn+ were the only group to elicit a median increase of 1.5-fold in IL-17A levels (p=0.018, Figure 40B).

Nine days after inoculation, LAIV in LAIV Spn blocked an increase in IL-4 (1.2-fold increase, p=0.021, Figure 36A), first induced by LAIV in non-colonised even before Spn challenge.

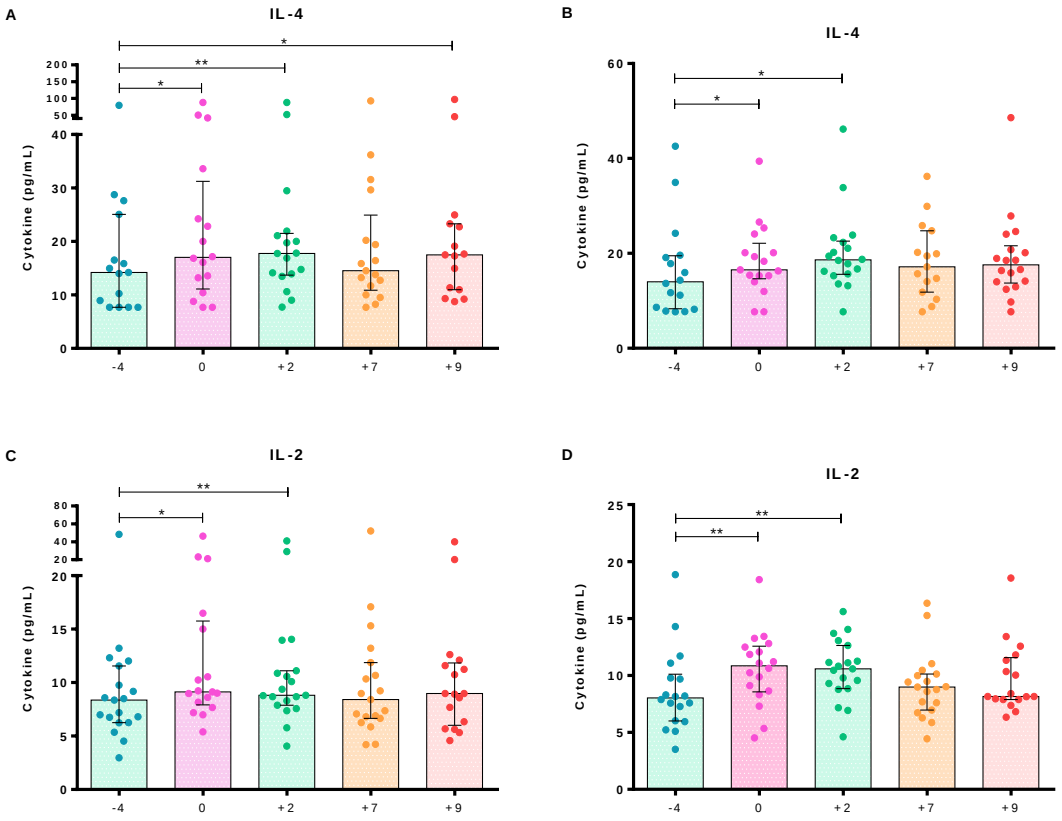

Figure 36. Antecedent LAIV induce cytokines IL-2 and IL-4 in the nose. Levels of cytokines measured in nasal lining of volunteers vaccinated with LAIV (n=18-19). IL-4 was measured in volunteers (A) not colonised by Spn (LAIV Spn-) and (B) colonised by Spn (LAIV Spn+). Similarly, IL-2 was measured in (C) not colonised and (D) colonised. Results were compared at each timepoint to baseline (day -4) by Wilcoxon test with *p≤0.05 and **p<0.01 and medians and interquartile range of each timepoint are shown.

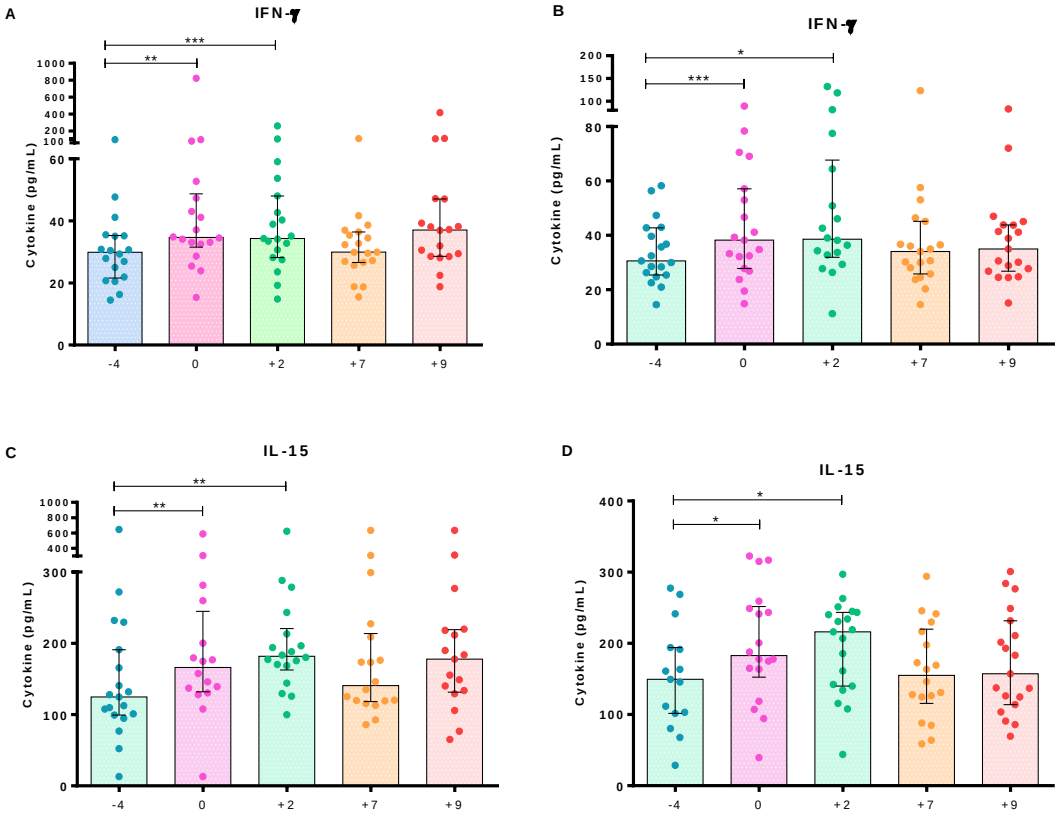

Figure 37. Antecedent LAIV induce cytokines IFN-γ and IL-15 in the nose. Levels of cytokines measured in nasal lining of volunteers vaccinated with LAIV (n=18-19). IFN-γ was measured in volunteers (A) not colonised by Spn (LAIV Spn-) and (B) colonised by Spn LAIV Spn) . IL-15 was measured in (C) not colonised and (D) colonised. Results were compared at each timepoint to baseline (day -4) by Wilcoxon test with *p≤0.05, **p<0.01 and ***p<0.001 and medians and interquartile range of each timepoint are shown.

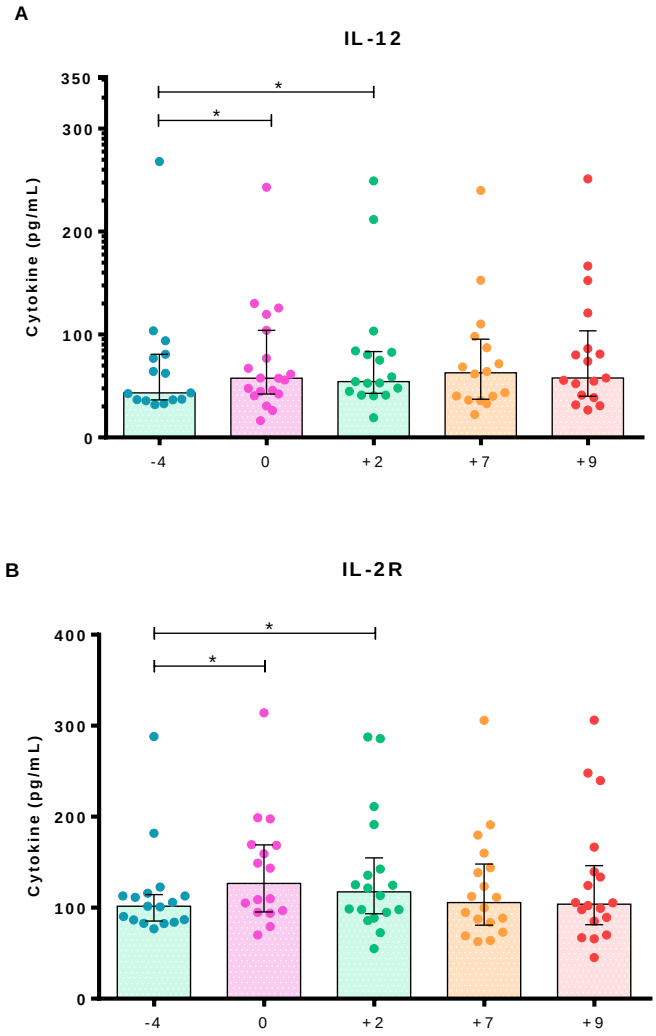

Figure 38. Antecedent LAIV induce cytokines IL-12 and IL-2R in the nose of non-colonised volunteers. Levels of (A) IL-12 and (B) IL-2R were measured in nasal lining of volunteers vaccinated with LAIV and not colonised by Spn (LAIV Spn-, n=18-19) and compared at each timepoint to baseline (day -4) by Wilcoxon test with *p≤0.05. Medians and interquartile range of each timepoint are shown.

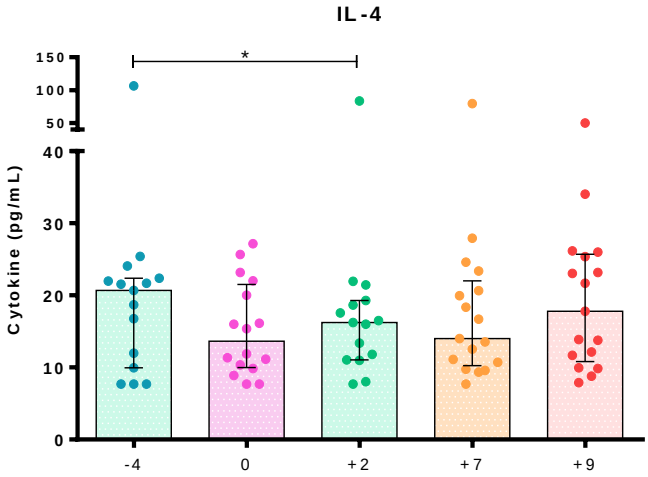

Figure 39. Non-colonised volunteers show induction of IL-4 in the nose . Levels of IL-4 was measured in nasal lining of volunteers in the control group that cleared Spn in the nasopharynx (TIV Spn-, n=18-19) and compared at each timepoint to baseline (day -4) by Wilcoxon test with *p≤0.05. Medians and interquartile range of each timepoint are shown.

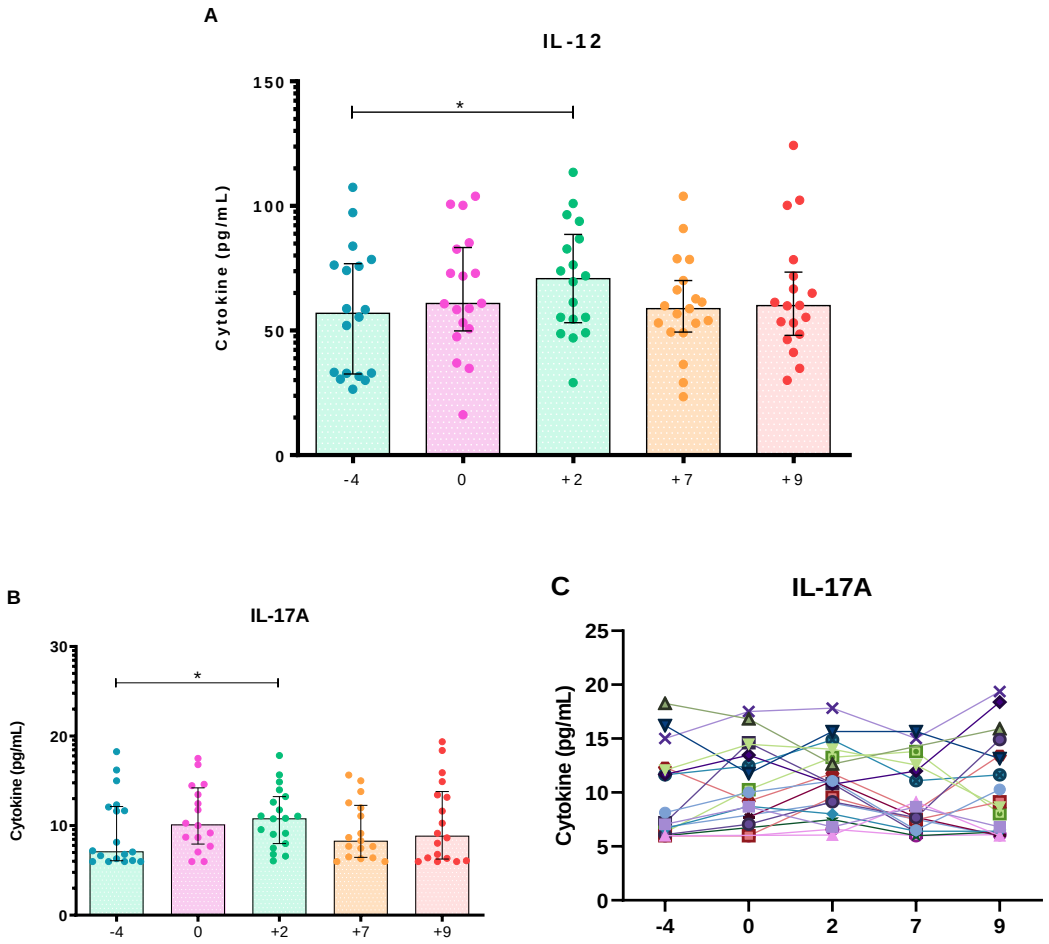

Figure 40. Antecedent LAIV induce cytokine IL-12 and IL-17A in the nose of volunteers colonised by Spn. Levels of (A) IL-12 and (B, C) IL-17A were measured in nasal lining of volunteers vaccinated with LAIV and colonised by Spn (LAIV Spn+, n=18-19) and compared at each timepoint to baseline (day -4) by Wilcoxon test with *p≤0.05. In A and B, medians and interquartile range of each timepoint are shown. In C each line represents one volunteer and each point the result of a single volunteers in a specific timepoint.

LAIV induces cytokines that regulate cell growth in the nasopharynx especially in Spn colonised.

Here, we investigated how concurrent LAIV and TIV in volunteers previously challenged with Spn but not colonised affect the production of cytokines that regulate growth of granulocytes, neuronal, epidermal, epithelial B, T-, NK- and hematopoietic stem cells, as well as embryonic development, morphogenesis, tissue repair, angiogenesis.

Three days after vaccination (day +6), LAIV induced a median 2-fold increase in EGF (p=0.035, Figure 41A) while TIV-vaccinated showed a median 1.3-fold decrease (p=0.033, Figure 41B). In addition, LAIV induced a median 1.4-fold increase in VEGF (p=0.021, Figure 42A). Further, at 6 days post-vaccination (day +9) TIV-vaccinated volunteers did not show increased cytokine levels while LAIV continued to induce a median 1.5 and 1.4-fold increase in EGF and VEGF, respectively (p=0.010, Figure 41B, and p=0.047, Figure 42B). Interestingly, TIV vaccinated showed decrease in levels of FGF-Basic 24 days after vaccination (median 1.3-fold decrease, p=0.021, Figure 43).

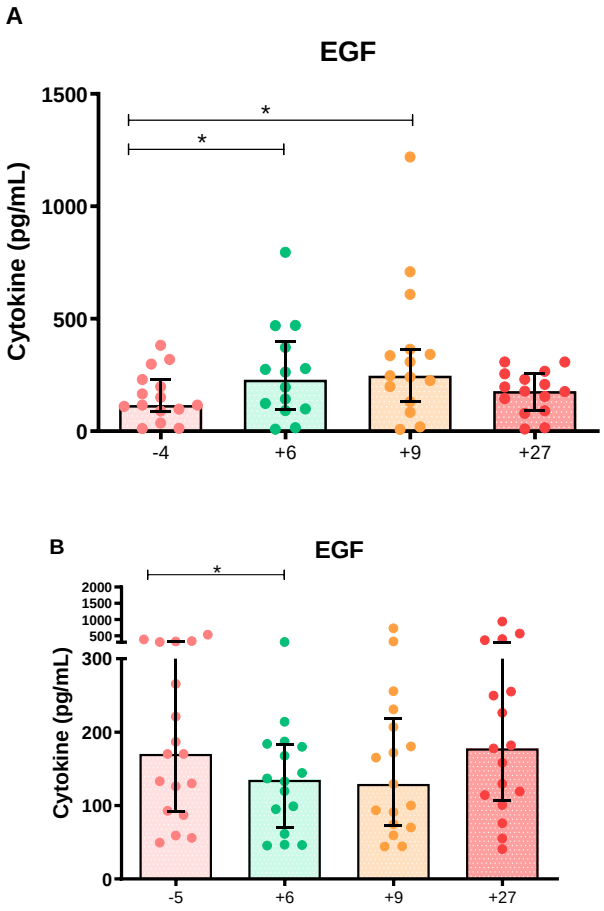

Figure 41. LAIV- and TIV- vaccinated show increase in cytokines EGF in the nasopharynx. Levels of EGF were measured in nasal lining of volunteers vaccinated with (A) LAIV (n=28) and (B) TIV (n=28). Results were compared at each timepoint to baseline (day -5) by Wilcoxon test with *p≤0.05 and medians and interquartile range of each timepoint are shown

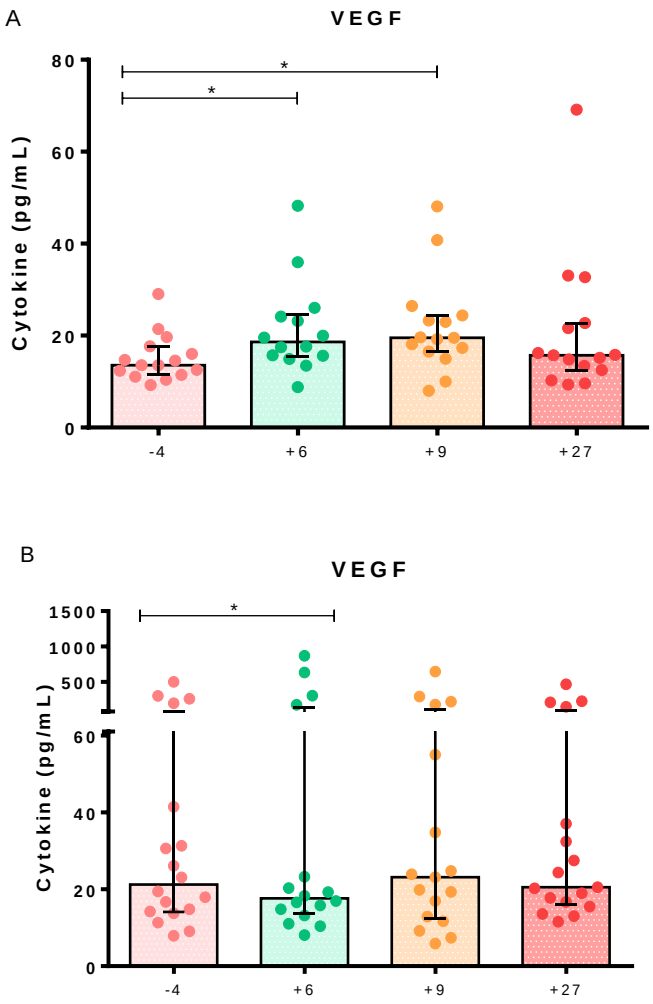

Figure 42. LAIV vaccinated show increase in cytokines VEGF in the nasopharynx. Levels of VEGF were measured in nasal lining of volunteers vaccinated with (A) LAIV (n=28) and (B) TIV (n=28) and compared at each timepoint to baseline (day -5) by Wilcoxon test with *p≤0.05. Medians and interquartile range of each timepoint are shown.

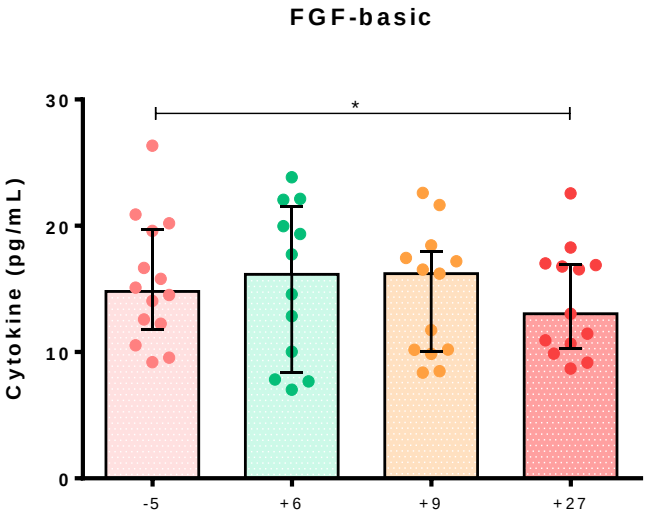

Figure 43. LAIV vaccinated show increase in cytokine FGF-Basic in the nasopharynx. Levels of FGF-Basic were measured in nasal lining of volunteers vaccinated with LAIV (n=28). Results were compared at each timepoint to baseline (day -5) by Wilcoxon test with *p≤0.05 and medians and interquartile range of each timepoint are shown.

Additionally, we measured cytokine production in a second set of volunteers, vaccinated with antecedent LAIV and TIV followed by Spn challenge, and stratified in vaccination and colonisation status.

Before Spn inoculation (day +0), LAIV induced only a median 1.4-fold increase in G-CSF in those volunteers who would not achieve colonisation post the bacterial challenge (p=0.001, Figure 44A). On the other hand, vaccination in LAIV Spn+ induced a median 1.6-fold increase in G-CSF (p=0.001, Figure 44B) along with median 1.2-fold increase in FGF-Basic and VEGF (p=0.010 and p=0.044, Figure 45A and 45B, respectively).

Two days after Spn inoculation, LAIV in LAIV Spn- continued to induce G-CSF (median 1.2-fold increase, p=0.009, Figure 44A) and started to express higher levels of FGF-Basic and EGF (FGF-Basic: median 1.8-fold increase, p=0.023, Figure 46A; EGF, median 1.7-fold increase, p=0.010, Figure 46B). In LAIV colonised volunteers induction of G-CSF, FGF-Basic and VEGF persisted in this timepoint (G-CSF, median 1.4-fold increase, p=0.009, Figure 44B; FGF-Basic, median 1.2-fold increase, p=0.004, Figure 45A; VEGF, median 1.1-fold increase, p=0.040, Figure 45B) with additional induction of IL-7, only elicited in LAIV vaccinated after Spn challenge (median 1.3-fold increase, p=0.027, Figure 47).

Importantly, only EGF was elicited exclusively by TIV Spn+ (median 1.7-fold increase, p=0.005, Figure 48) showing similar induction in LAIV Spn- (median 1.3-fold increase, p=0.048, Figure 46B). Further, at 7 and 9 days post-inoculation, the control group did not show increase in EGF, however, LAIV Spn- demonstrated a LAIV-induced median 2-fold increase (p=0.020, Figure 46B). In addition, in both non-colonised and colonised volunteers, LAIV induced a late

increase of a median 1.2-fold of G-CSF (LAIV Spn-: p=0.026, Figure 44A, LAIV Spn+: p=0.026, Figure 44B). Interestingly, this cytokine was induced by the attenuated influenza viruses since before Spn challenge at day +0.

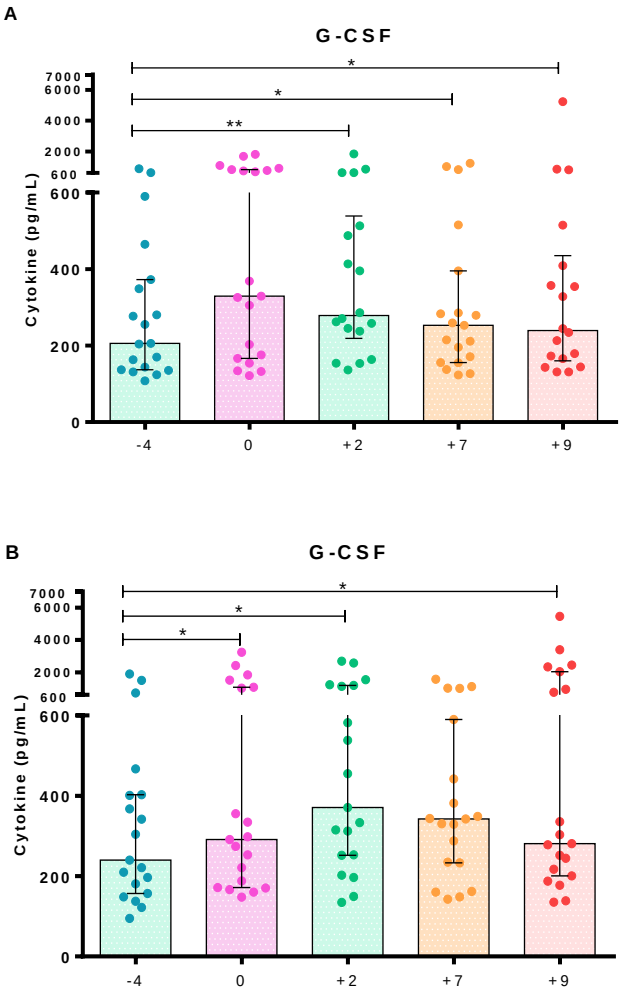

Figure 44. Antecedent LAIV induce cytokine G-CSF in the nose of volunteers. Levels of G-CSF was measured in nasal lining of volunteers vaccinated with LAIV and (A) not colonised and (B) colonised by Spn (n=18-19) and compared at each timepoint to baseline (day -4) by Wilcoxon test with *p≤0.05 and **p<0.01. Medians and interquartile range of each timepoint are shown.

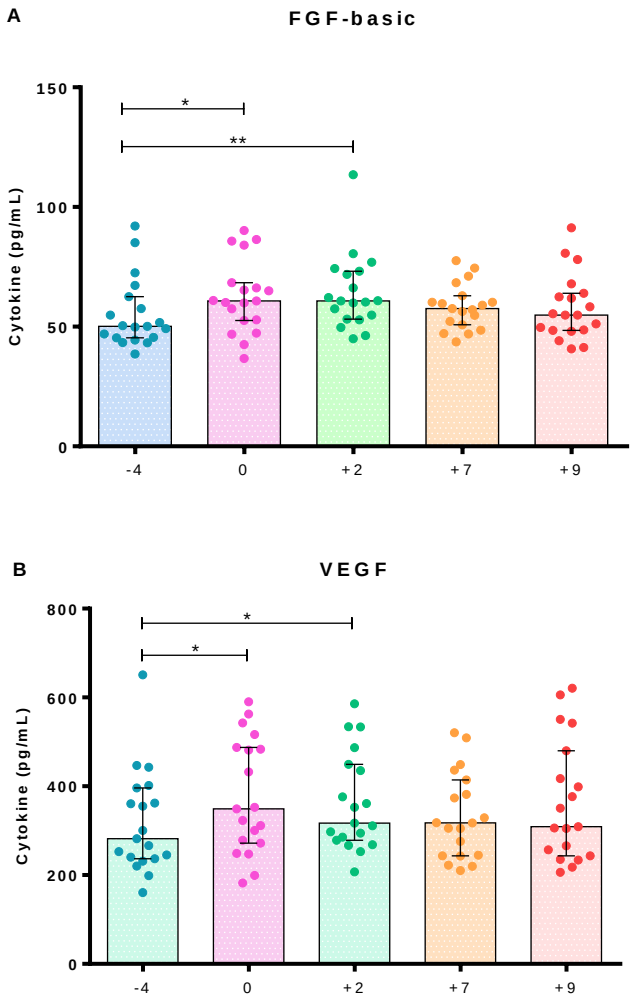

Figure 45. LAIV induces FGF-Basic and VEGF in the nasopharynx of Spn colonised volunteers. Levels of (A) FGF-Basic and (B) VEGF were measured in nasal lining of volunteers vaccinated with LAIV and colonised by Spn (LAIV Spn+, n=18-19) and compared at each timepoint to baseline (day -4) by Wilcoxon test with *p≤0.05 and **p<0.01. Medians and interquartile range of each timepoint are shown.

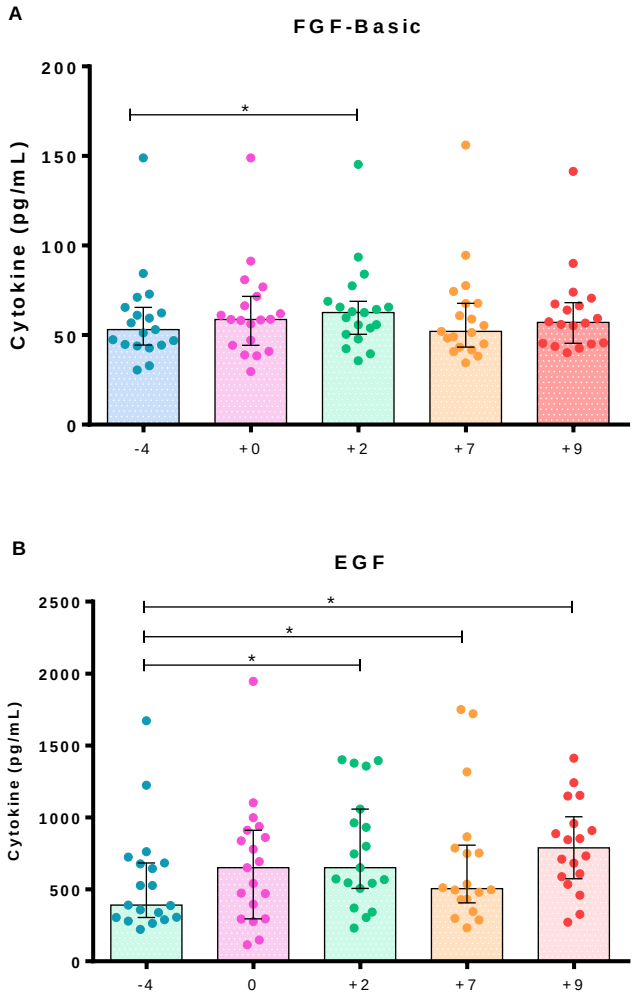

Figure 46. LAIV induces cytokine FGF-Basic and EGF in the nasopharynx of non-colonised volunteers. Levels of (A) FGF-Basic and (B) EGF were measured in nasal lining of volunteers vaccinated with LAIV and not colonised by Spn (LAIV Spn-, n=18-19) and compared at each timepoint to baseline (day -4) by Wilcoxon test with *p≤0.05. Medians and interquartile range of each timepoint are shown.

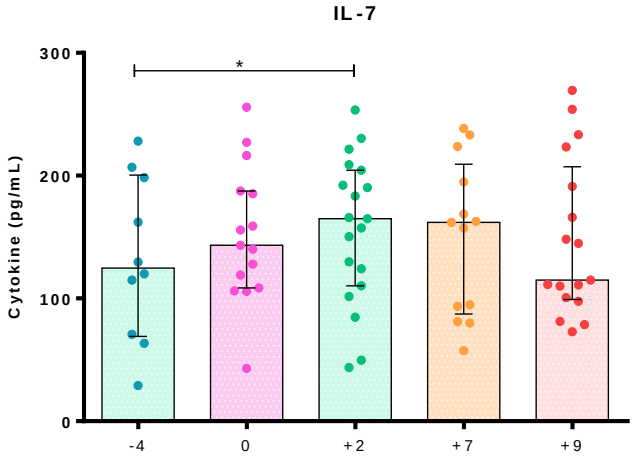

Figure 47. LAIV induces cytokine IL-7 in the nasopharynx of Spn colonised volunteers. Levels of IL-7 was measured in nasal lining of volunteers vaccinated with LAIV and colonised by Spn (LAIV Spn+, n=18-19) and compared at each timepoint to baseline (day -4) by Wilcoxon test with *p≤0.05. Medians and interquartile range of each timepoint are shown.

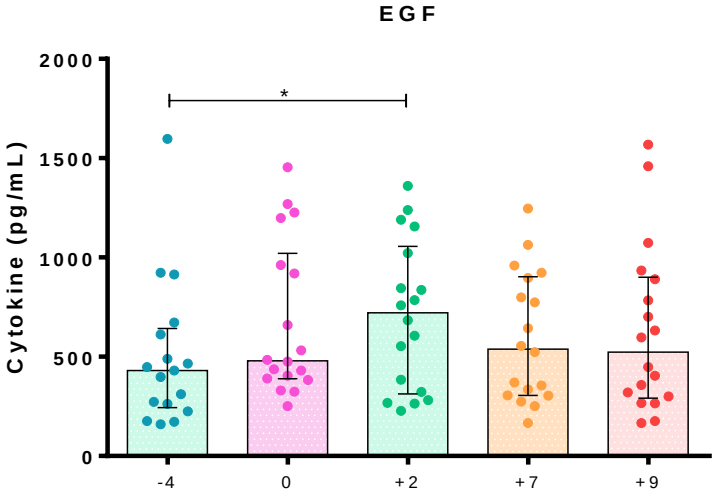

Figure 48. Spn colonised show increased levels of cytokine EGF in the nasopharynx. Levels of EGF was measured in nasal lining of volunteers in the control group colonised by Spn (TIV Spn+, n=18-19) and compared at each timepoint to baseline (day -4) by Wilcoxon test with *p≤0.05. Medians and interquartile range of each timepoint are shown.

LAIV induction of anti-inflammatory cytokine response is impaired in the Spn colonised nasal mucosa

Firstly, we assessed anti-inflammatory cytokines in the nasopharynx of volunteers challenged with Spn and not colonised, followed with vaccination with concurrent LAIV and TIV. Here, we demonstrated that LAIV induces only HGF, 6 days after vaccination (median 1.3-fold increase, p=0.030, Figure 49).

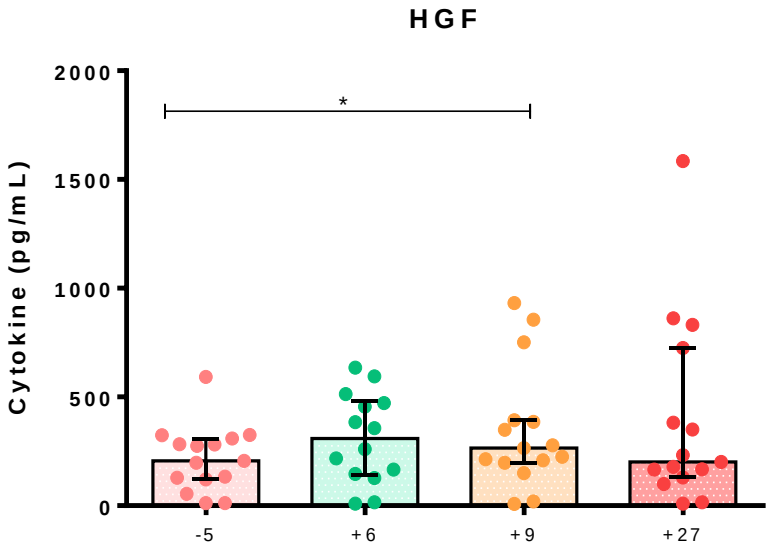

Figure 49. TIV-vaccinated show increase in cytokine HGF in the nasopharynx. Levels of HGF was measured in nasal lining of volunteers vaccinated with TIV (n=28). Results were compared at each timepoint to baseline (day -5) by Wilcoxon test with *p≤0.05 and medians and interquartile range of each timepoint are shown.

Secondly, another set of volunteers vaccinated with antecedent influenza vaccines followed with Spn challenge, were stratified into groups regarding vaccination and colonisation status.

After vaccination but before inoculation (day +0), LAIV resulted in a considerable increase in IL-10 levels (median 3.7-fold increase, p=0.006, Figure 50A) in LAIV Spn-. This induction was unique, as was not observed in any other group. Two days after challenge, a median 2.8-fold increase in IL-10 was still observed (p=0.002). Additionally, an induction of HGF (median 1.3-fold increase, p=0.009, Figure 50B) was observed in the same group post Spn inoculation.

Interestingly, in the control group, Spn colonisation prevented an increase of anti-inflammatory cytokine IL-10 7 days post-inoculation, observed in TIV Spn- (median 1.4-fold increase, p=0.026, Figure 51), but not in the first set of volunteers vaccinated with concurrent vaccines. Further, at 9 days post-inoculation, LAIV Spn- showed an induction of IL-10, similarly demonstrated at day +0 and +2 (median 2.3-fold increase, p=0.034, Figure 50A).

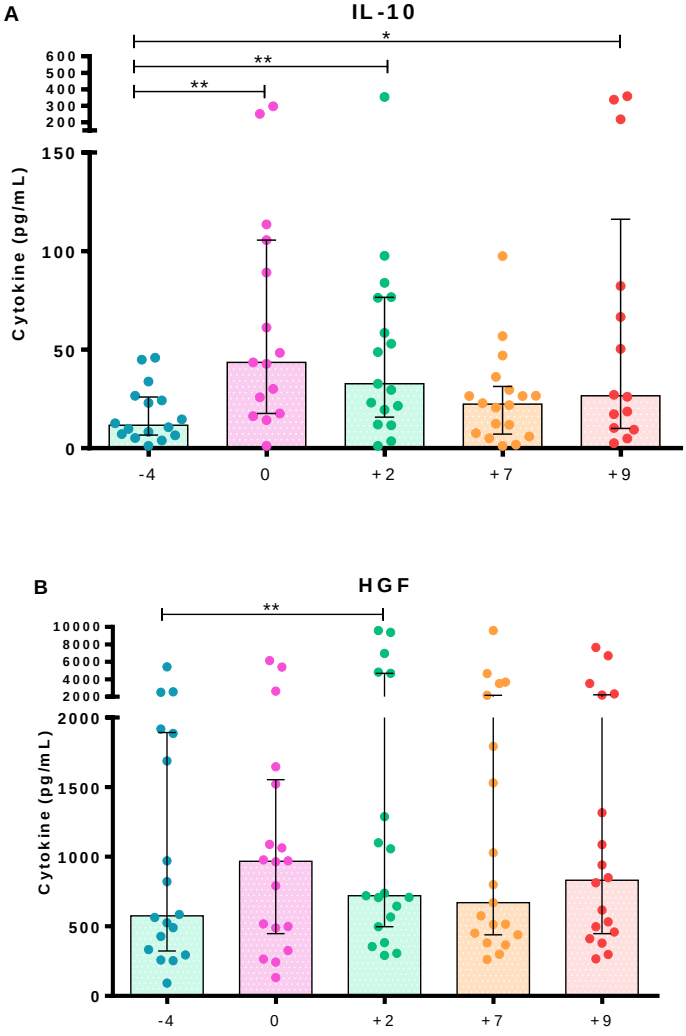

Figure 50. LAIV induces anti-inflammatory cytokines IL-10 and HGF in the nasopharynx of non-colonised volunteers. Levels of (A) IL-10 and (B) HGF were measured in nasal lining of volunteers vaccinated with LAIV and not colonised by Spn (LAIV Spn-, n=18-19) and compared at each timepoint to baseline (day -4) by Wilcoxon test with *p≤0.05 and **p<0.01. Medians and interquartile range of each timepoint are shown.

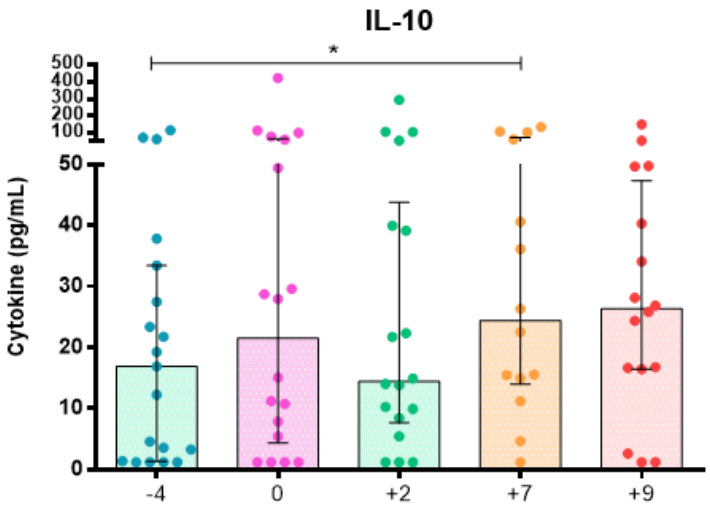

Figure 51. Non-colonised volunteers show increased levels of IL-10 in the nose. Levels of IL-10 was measured in nasal lining of volunteers in the control group that cleared Spn in the nasopharynx (TIV Spn-, n=18-19) and compared at each timepoint to baseline (day -4) by Wilcoxon test with *p≤0.05. Medians and interquartile range of each timepoint are shown.

5.4 Discussion

Here, we assessed cytokine responses elicited by Spn and/or LAIV in the human nasal mucosa by collecting nasal lining fluid (nasosorption, NS) from volunteers from 2 consecutives randomised controlled clinical trials and measured cytokine levels with 30-plex human cytokine panel. Our results have demonstrated that the control group, vaccinated with TIV and not colonised by Spn, was associated with less general inflammation. The detectable levels of inflammatory cytokines in this group corroborate the view of Spn as a commensal bacterium that can asymptomatically be present in the nose of healthy adults [336].

Conversely, the attenuated influenza viruses contained in the LAIV elicited induction of pro-inflammatory cytokines, as well as cytokines that regulate adaptive immunity and cell growth. However, we observed divergent cytokine profiles promoted by antecedent LAIV vaccination in non-colonised and Spn colonised, which imply that differences in the host responses to Spn after primary influenza infection may affect susceptibility to Spn colonisation. As described in wild-type influenza virus and secondary Spn co-infection [271], antecedent LAIV vaccination induces an exacerbated immune response that is regulated by a loop of positive feedback between immune cells and cytokines. In our results this response was characterised mainly by increases in IL-6, IFN-α, IP-10, MIP-1α, MIP-1β and IL-17A in the LAIV/Spn+ group.

The cytokine IL-6 is considered one of the major physiological mediators of acute infection and plays an important role on acquired immune response by stimulation of antibody production and of effector T-cell development *in vitro* and *in vivo*. In mice colonised with Spn, antecedent LAIV-induced IL-6 is shown to be increased in the upper respiratory tract [186,188], which is corroborated by our results

in humans. Additionally, LAIV in Spn colonised induced an increase in IL-17A, a cytokine observed in murine models following colonisation and critical for defence against bacterial infections, especially Spn [327,333,337]. IL-17A is known to induce neutrophil recruitment and enhance macrophage capacity to kill Spn *in vitro* [287]. The increased IL-17A levels observed in Spn colonised volunteers suggest that proliferation of neutrophils may occur in the nasal mucosa after bacterial challenge, in order to facilitate control and clearance of growing Spn colonisation.

On the other hand, although LAIV induced a high pro-inflammatory profile in Spn colonised, vaccination reduced specific responses to Spn in humans, mainly confined in the chemoattractant cytokines MCP-1, RANTES, MIG, TNF-α, IL-1β and IL-10. In mice models it has been shown that MIG and TNF-α possess anti-viral activities [188,199,338,339], whereas IL-1β is known for mediate Spn clearance [295].

In addition, LAIV-induced anti-inflammatory IL-10 was reduced by Spn colonisation, cytokine that is a central factor for regulating immune responses to virus and bacteria [340,129]. Here we showed that the attenuated influenza viruses contained in the LAIV do not show similar effect to murine models of antecedent wild-type influenza and Spn co-infection that have associated impact on the nasal epithelium with induction of IL-10 [127,341].

In this study we addressed important questions about how immune responses are elicited by LAIV as well as how the attenuated influenza viruses contained in the LAIV can alter Spn control in the host. By using for the first time 2 double human infection challenge model with LAIV and Spn, we revealed that Spn colonisation caused minimal alteration in the nasal cytokine milieu, whereas LAIV trigger a robust pro-inflammatory response, which was impaired in Spn

colonised. Our findings are in agreement with studies shown that antecedent LAIV increases susceptibility to Spn acquisition and promotes bacterial growth [284]. Secondary Spn infection following wild-type viral respiratory tract infection has a large burden of disease worldwide, therefore it is essential to understand how LAIV can affect cytokine immune responses that, in turn, lead to increased bacterial loads and transmission.

CHAPTER SIX

EFFECT OF SPN AND LAIV ON INNATE AND ADAPTIVE IMMUNITY TO INFLUENZA

6.1 Introduction

Each year, 5–15% of the world's population will suffer from an influenza infection, with up to 5 million cases of severe disease and 5 hundred thousand deaths [342]. Influenza viruses have the ability to mutate [343] and hence escape immune defence mechanisms of the host [344], which require annual vaccine updates. As described before, the current available vaccines are the TIV and LAIV.

The different vaccination routes of TIV and LAIV trigger distinct immune mechanisms and pathways of protection. TIV leads to host protection by inducing neutralising antibodies to strain-specific glycoproteins HA and NA [345]. LAIV is a cold-adapted vaccine that replicates only in the nasopharynx - mimicking a natural influenza infection [346]. The nasal replication of the attenuated influenza viruses leads to recognition of its pathogen-associated molecular patterns (PAMPs) by host pattern recognition receptors (PRRs), which initiates a cascade of cellular immune responses [344].

In mice, LAIV vaccination increases the frequency of $CD4^+$ and $CD8^+$ T-cells in the lung and cytokine production upon influenza re-stimulation compared to the inactivated virus vaccination or no vaccine administration [186,188,347,348]. Moreover, LAIV seeds the murine lung with both $CD4^+$ and virus-specific $CD8^+$ TRM T-cells. TRM have been shown to provide long-term cross-strain protection to influenza [347]. In humans, the immune responses elicited by LAIV have been found to provide broader clinical protection in children compared to the inactivated influenza vaccines [349]. However, the detailed immunological mechanisms of this remain incompletely understood.

Each year influenza vaccines are formulated according to circulating strains, but their effectiveness varies as influenza replication often results in antigenic drift and shift, changing the viral genome [350]. Estimates from the World Health Organisation (WHO) suggest that influenza vaccines effectiveness rarely exceeds 60% and has fallen below 30% in some years [351,352]. Due to poor effectiveness among 2 to 17 year-olds, during previous seasons, the CDC Advisory Committee on Immunisation Practices (ACIP) suggested the exclusion of LAIV from US national childhood influenza immunisation programme during 2016-2017 and 2017-2018 season [353], whereas it expressed no preference for the LAIV vaccine over the inactivated one for the 2018-2019 influenza season. Many underlying causes for this variation have been suggested, including poor matching with circulating strains [354], differential ability of some LAIV to induce immunity, in particular against H_1N_1 strains, and interactions with the existing microbiome during LAIV replication in the nose [355].

Despite several reports about the microbiota and its impact on vaccination responses [356–359] - including responses to influenza vaccine [358,360] - it is still unclear how the microbiome effects LAIV immunogenicity in humans [277]. In murine models, Spn colonisation altered the anti-viral B-cell responses during co-infection with wild-type influenza virus, potentially compromising long-term antiviral antibody-mediated immunity [277]. As colonisation of the nasopharynx with Spn is very common during childhood, with 50% of infants in resource-rich settings and up to 90% in low and middle income countries colonised at any time [361], it is essential to continue the investigation on how Spn could affect immune responses elicited by the currently available influenza vaccines.

Here, we showed that in humans, concurrent LAIV elicits immune responses primarily at mucosal sites- both nose and lung. Interestingly, pre-existing nasal Spn colonisation impacted on LAIV immunogenicity, dampening the LAIV-mediated nasal and lung immune responses

6.2 Methods

6.2.1 Volunteer recruitment, vaccination and inoculation

170 healthy adult volunteers were recruited to the Concurrent study (Section 2.2). In the Concurrent study, subjects were inoculated with Spn using EHPC and 3 days later, vaccinated with either LAIV (n=80) or TIV as control (n=90) (Section 2.5.4 and 2.6.4).

To investigate the immune responses to influenza vaccination, samples of NW and serum were collected at baseline and 24 days after vaccination. For comparisons within the lung datasets, BAL fluid and lung lymphocytes were collected at 1 timepoint, as well as from an unvaccinated subset of volunteers (Spn-, n=10 and Spn+, n=10) which were used as a control since each volunteer was only able to provide a single time point sample.

6.2.2 Nasal wash, BAL and serum collection and processing

NW was collected by washing volunteers' nostrils with saline (Section 2.5.5) while BAL was collected by washing off the lungs with saline (Section 2.5.9). In addition, serum was also collected (Section 2.5.8). NW and serum samples were stored in a -80^{o}C freezer for future analysis (Section 2.6.5), whereas BAL was processed for experiments immediately (Section 2.6.8).

6.2.3 Intracellular cytokine staining of BAL cells

As described in Section 2.6.8.2, non-adherent BAL cells were counted and stimulated with TIV or left unstimulated as negative control, and incubated. Cells were washed and stained with extracellular markers CD3, TCR-γδ, CD4, CD8, CD69, CD25, CD103 and CD49a and intracellular markers FOXP3, IFN-γ, TNF-α, IL-10 and IL-17A for analysis in a flow cytometer.

6.2.4 Enzyme-linked immunosorbent assay (ELISA)

ELISA was used to quantify levels of IgG and IgA antibodies to influenza in the serum, NW and BAL supernatant of volunteers vaccinated with TIV or LAIV as described in Section 2.6.9.5. The average blank corrected value was calculated for each sample and the data analysed using Omega Analysis.

6.2.5 Statistical analysis

The levels of antibodies were analysed by comparing baseline and 24 days after vaccination (D24) by Wilcoxon test. As described in Section 2.7, the number of positive cells were compared between mock and flu-stimulated within groups by Wilcoxon test and between groups by Mann-Whitney test.

6.3 Results

To assess and compare the immune responses elicited by influenza in volunteers inoculated with Spn and vaccinated with concurrent LAIV, we collected a series of samples. Mucosal samples, such as NW and BAL, as well as serum samples, were collected from the groups TIV Spn- (n=21), TIV Spn+ (n=19), LAIV Spn- (n=37) and LAIV Spn+ (n=43). For the assessment of lung immune responses, we included a non-vaccinated cohort (Spn-, n=10 and Spn+, n=10), as we were only able to sample the human lung only post challenge and vaccination.

Concurrent LAIV increases the frequency of influenza-specific TNF-α and IFN-γ producing CD4$^+$ and TRM CD4$^+$ T-cells in the lung

Data from animal models suggest that concurrent LAIV, but not TIV, elicits protective cellular responses in the lung [324,362]. To assess if influenza vaccination induced cellular responses in humans, BAL cells were stimulated with influenza antigens. T-cell subsets (CD4$^+$, CD8$^+$ and TCR-γδ$^+$) were immunophenotyped and the frequency of positive IFN-γ, IL-17A and TNF-α-producing, influenza-specific T-cells measured. Frequencies of total CD4$^+$, CD8$^+$ and TCR-γδ$^+$ T-cells were not affected by vaccination status (Figure 52).

Furthermore, we investigated the presence of TRM T-cell responses to influenza, using the extracellular markers CD69, CD103 and CD49a. As over 1/3 of CD4$^+$ CD69$^+$ cells, commonly defined as TRM [294] did not express the additional resident memory markers CD103 and CD49a, we defined TRM only as CD69$^+$. In contrast, nearly all CD8$^+$CD69$^+$ cells also expressed CD103 and CD49a.

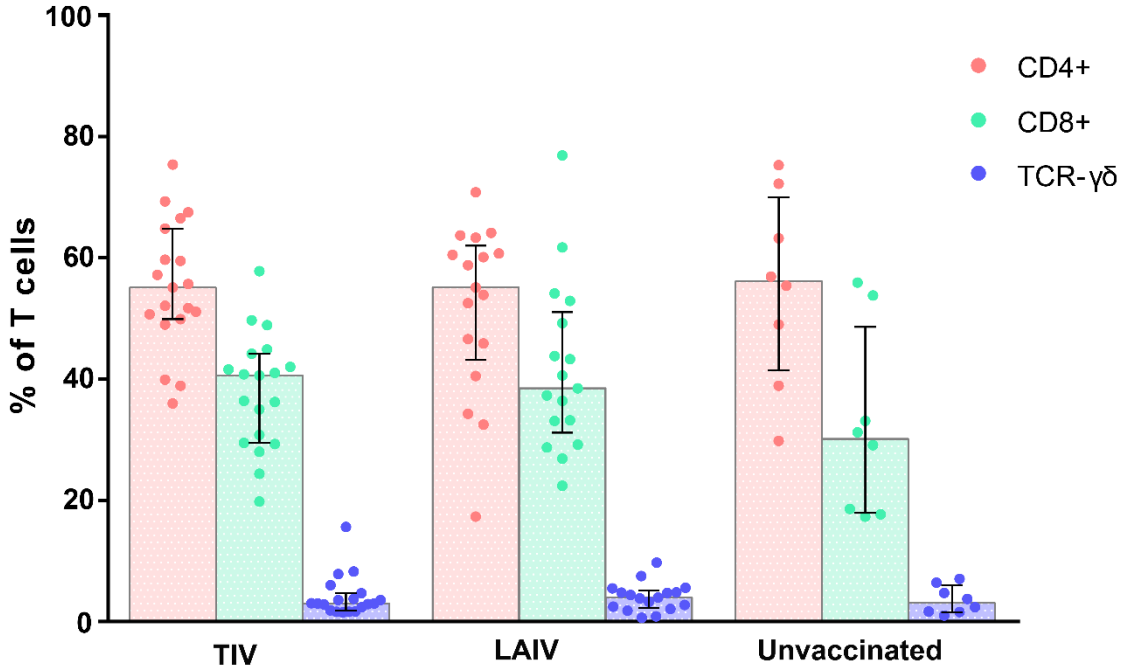

Figure 52. Concurrent LAIV and TIV do not affect frequencies of total CD4$^+$, CD8$^+$ and TCRγδ$^+$ T-cell subsets in the lung. Subset frequencies among viable T-cells were measured after an overnight incubation of isolated BAL cells. Bars depict the median proportion of T-cell subsets among total T-cells for TIV Spn- (n=9), TIV Spn+ (n=11), LAIV Spn- (n=11) and LAIV Spn+ (n=9). Also, unvaccinated Spn- (n=3) and unvaccinated Spn+ (n=5).

$CD4^+$ TNF-α production upon influenza stimulation was observed in both TIV and LAIV recipients regardless of colonisation status, but not in unvaccinated individuals (Figure 61A and 61B). However, levels of influenza-specific TNF-α were significantly increased in LAIV Spn- when compared to the unvaccinated (mean 2.6-fold increase, p=0.015, Figure 61B).

Following stimulation with influenza antigens, $CD4^+$ TRM T-cells produced TNF-α in all vaccinated groups but not in the unvaccinated group (Figure 61C). The induction of TNF-α producing $CD4^+$ TRM following stimulation did not significantly differ between TIV and LAIV, but was more pronounced in the LAIV-vaccinated, in both Spn colonised and non-colonised individuals (LAIV Spn+: mean 6.5-fold change to unvaccinated, p=0.004; LAIV Spn-: mean 7.7-fold change to unvaccinated, p=0.024) compared to the unvaccinated group (Figure 61D).

We also assessed IFN-γ production by total $CD4^+$ and TRM $CD4^+$ T-cells residing in the human lung. IFN-γ production by total $CD4^+$ T-cells was observed in all groups upon stimulation, including the unvaccinated group (Figure 61E). The levels of IFN-γ producing $CD4^+$ T-cells were not different when comparing vaccinated and unvaccinated groups. However, the induction of IFN-γ producing $CD4^+$ TRM T-cells was greater in the LAIV-vaccinated volunteers (Figure 61F). In contrast to total $CD4^+$ T-cells, stimulation of TRMs of unvaccinated individuals did not elicit an IFN-γ response (Figure 61F).

Furthermore, the proportion of IL-17A producing $CD4^+$ T-cells or $CD4^+$ TRM T-cells was not affected by concurrent vaccination with either TIV or LAIV (Figure 62).

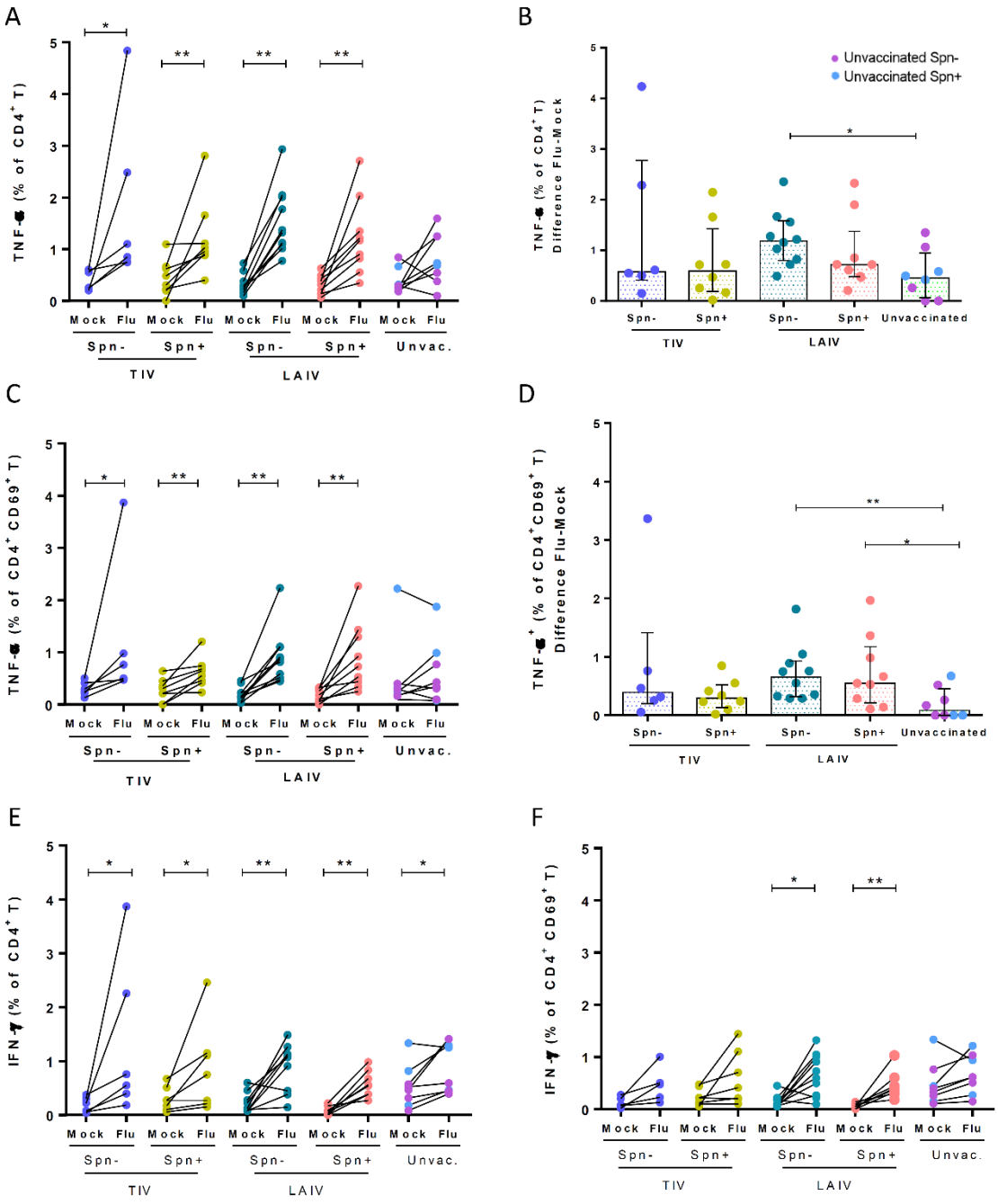

Figure 53. Concurrent LAIV increases frequency of influenza-specific TNF-α and IFN-γ-producing CD4⁺ and TRM CD4⁺ T-cells in the lung. Frequencies of cytokine-producing CD4⁺ and TRM CD4⁺ T-cells were measured in human BAL samples with and without (mock) *in vitro* influenza antigen stimulation. Volunteers were divided by vaccine and colonisation status in TIV Spn- (n=6), TIV Spn+ (n=8), LAIV Spn- (n=10), LAIV Spn+ (n=9), unvaccinated (Spn-, n=3 and Spn+, n=5) group. (A) Production of TNF-α by total CD4⁺ T-cells in each group [paired unstimulated (mock) and stimulated condition (flu)]. (B) influenza-specific production of TNF-α by total CD4⁺ T-cells (Difference between influenza-stimulated and unstimulated) in each group. (C) Production of TNF-α by CD4⁺ CD69⁺ T-cells in each group. (D) Production of influenza-specific TNF-α by CD4⁺ CD69⁺ T-cells in each group. (E) Production of IFN-γ by total CD4⁺ T-cells and (F) CD4⁺ CD69⁺ T-cells in each group. Each individual dot represents a single volunteer and the conditions from one individual are connected. Medians with IQR are depicted for influenza-specific responses, *p<0.05, **p<0.01 by Wilcoxon test for comparisons within the same group and by Mann-Whitney test for between-group comparisons.

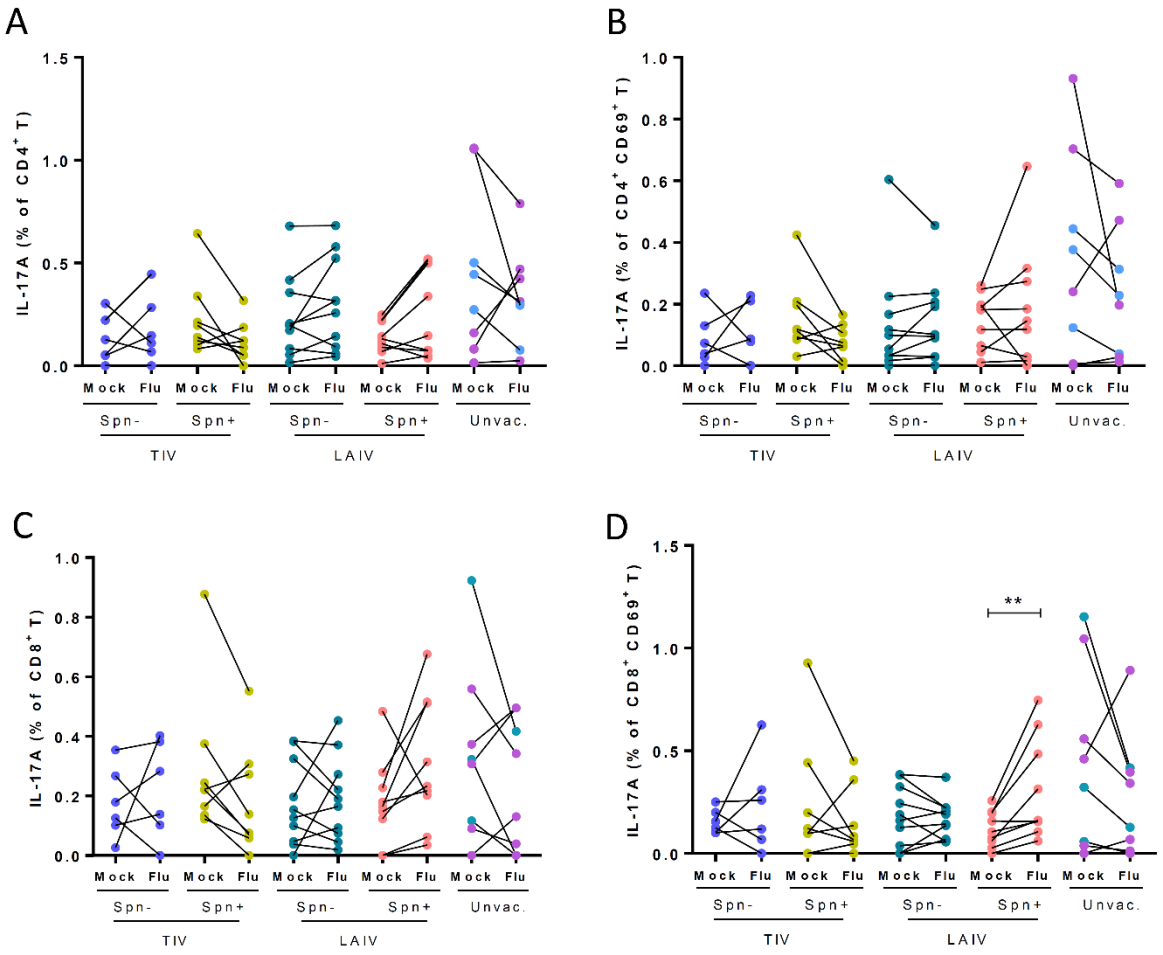

Figure 54. Concurrent LAIV and TIV do not increase the frequency of influenza-specific, IL-17A-producing T-cells in the lung. The frequency of cytokine-producing cells was measured by intracellular staining flow cytometry analysis after stimulation with influenza antigens or unstimulated for TIV Spn- (n=8), TIV Spn+ (n=6), LAIV Spn- (n=10), LAIV Spn+ (n=9), unvaccinated Spn- (n=3), unvaccinated Spn+ (n=5). IL-17A production in (A) total $CD4^+$ T-cells, (B) $CD4^+$ $CD69^+$ T-cells, (C) total $CD8^+$ T-cells and (D) $CD8^+$ $CD69^+$ T-cells. Each individual dot represents a single volunteer and the conditions from one individual are connected. **p<0.01. The unstimulated and influenza antigen-stimulated responses were compared within each group by Wilcoxon test. Influenza-specific responses (influenza-stimulated - unstimulated) were compared between the groups using Mann-Whitney test.

Concurrent LAIV increases the frequency of TNF-α producing influenza-specific CD8$^+$ and TRM CD8$^+$ T-cells in the lungs

Re-stimulation induced increased production of TNF-α producing CD8$^+$ T-cells in LAIV but not TIV or unvaccinated group. LAIV-vaccinated non-colonised had a mean 2.3-fold increase of TNF-α producing CD8$^+$T-cells post stimulation compared to the non-stimulated condition (p=0.030), whereas the same type of cellular response was less pronounced in the Spn colonised volunteers (mean 1.9-fold increase, p=0.007, Figure 63A). Similarly, TNF-α production by TRM CD8$^+$ cells was only observed in the LAIV-vaccinated group, increased by median 3.1- (p=0.006) and 2.1- (p=0.004) fold change in non-colonised and colonised subjects, respectively (Figure 63B).

In contrast to CD4$^+$ responses, production of IFN-γ by stimulated CD8$^+$ T-cells was only induced in the LAIV-vaccinated colonised by Spn (mean 1.6-fold change with IQR: 1.5x-2.7x, p=0.007, Figure 63C). TIV and control group had no significant increase in the proportion of IFN-γ producing CD8$^+$ T-cell post stimulation with influenza antigens. In addition, IFN-γ production by lung TRM CD8$^+$ T-cells not significantly altered post stimulation in any of the groups (Figure 63D).

Stimulation did not elicit production of IL-17A producing CD8$^+$ T-cells, except for IL-17A production by TRM CD8$^+$ T-cells in the Spn colonised group (mean 2.6-fold increase, p=0.008, Figure 64).

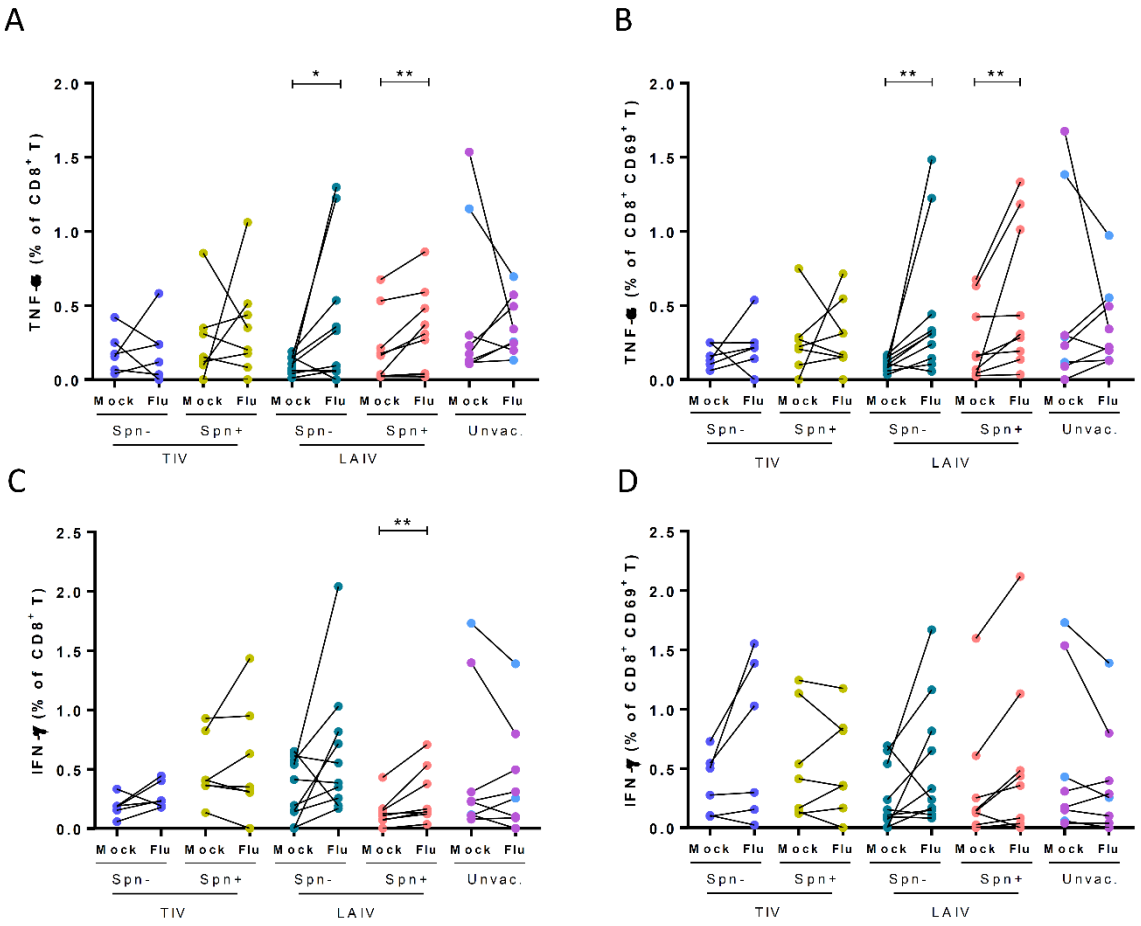

Figure 55. Concurrent LAIV increases frequency of TNF-α producing influenza-specific CD8⁺ and TRM CD8⁺ T-cells in the lungs. Frequencies of cytokine-producing CD8⁺ T-cells were measured in human BAL samples by intracellular staining flow cytometry analysis following stimulation with influenza antigens or non-stimulation (mock) in each group. Volunteers were divided by vaccine and colonisation status in TIV Spn- (n=6), TIV Spn+ (n=8), LAIV Spn- (n=10), LAIV Spn+ (n=9) and unvaccinated (Spn-, n=3 and Spn+, n=5) group. Production of TNF-α by (A) total CD8⁺ T-cells and (B) TRM CD8⁺ T-cells in each group (paired unstimulated [mock] and stimulated condition [Flu]). Production of IFN-γ production by (C) total CD8⁺ T-cells and (D) TRM CD8⁺ T-cells in each group. Each individual dot represents a single volunteer and the conditions per individual are connected. *p<0.05, **p<0.01 by Wilcoxon test.

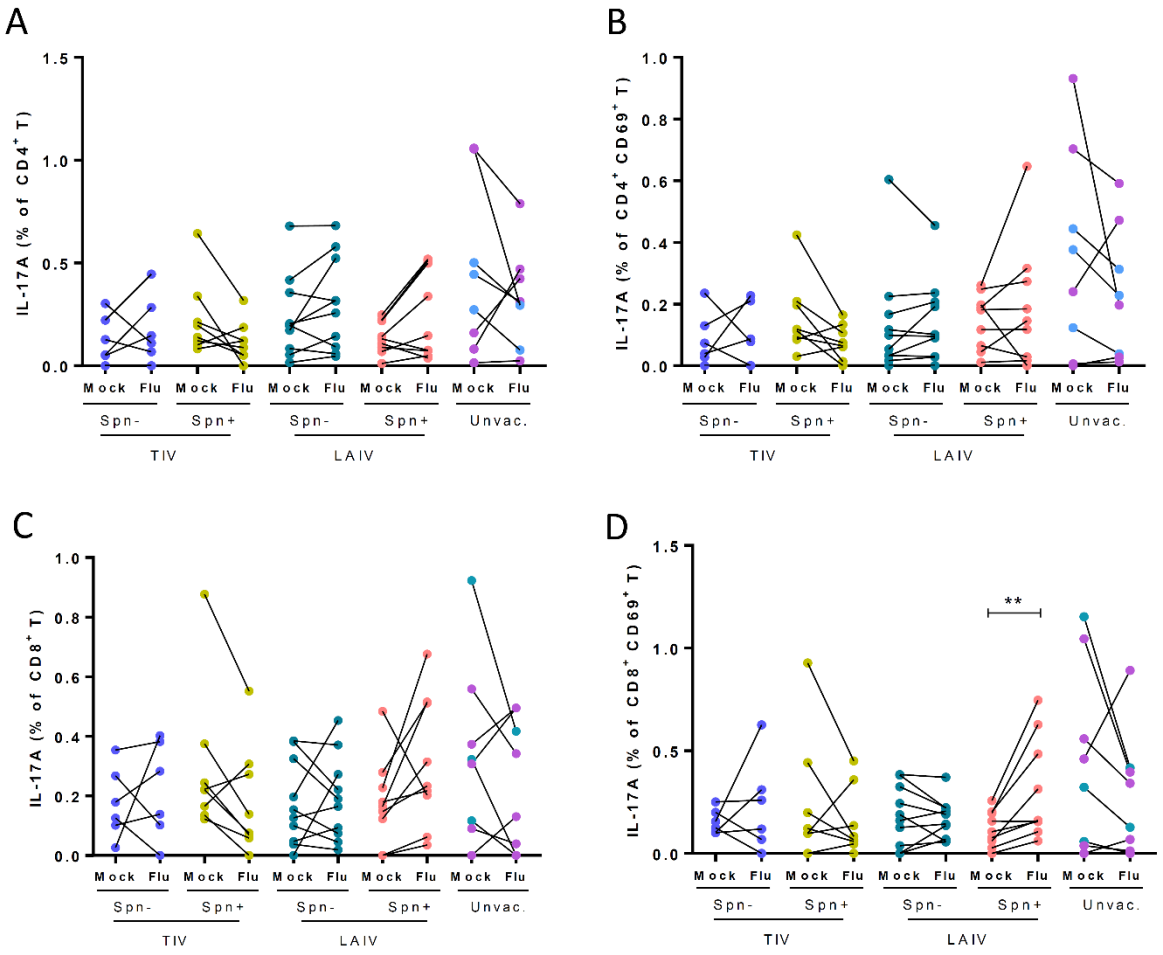

Figure 56 Concurrent LAIV and TIV do not increase the frequency of influenza-specific, IL-17A-producing T-cells in the lung. The frequency of cytokine-producing cells was measured by intracellular staining flow cytometry analysis after stimulation with influenza antigens or unstimulated for TIV Spn- (n=8), TIV Spn+ (n=6), LAIV Spn- (n=10), LAIV Spn+ (n=9), unvaccinated Spn- (n=3), unvaccinated Spn+ (n=5). IL-17A production in (A) total $CD4^+$ T-cells, (B) $CD4^+$ $CD69^+$ T-cells, (C) total $CD8^+$ T-cells and (D) $CD8^+$ $CD69^+$ T-cells. Each individual dot represents a single volunteer and the conditions from one individual are connected. **p<0.01. The unstimulated and influenza antigen-stimulated responses were compared within each group by Wilcoxon test. Influenza-specific responses (influenza-stimulated - unstimulated) were compared between the groups using Mann-Whitney test.

Concurrent LAIV increases frequency of influenza-responding IFN-γ producing TCR-γδ$^+$ in the lungs of non-colonised individuals

TCR-γδ$^+$ cells, a subset of specialised innate-like T-cells that can exert effector functions immediately upon activation, play an important role in pulmonary infection [363,364]. Therefore, we assessed whether TCR-γδ$^+$ T-cell responses to influenza antigens were induced following vaccination. No significant increase in TNF-α producing TCR-γδ$^+$ was observed after stimulation in any of the groups (Figure 65A). However, the proportion of IFN-γ producing TCR-γδ$^+$ was significantly greater in LAIV vaccinated non-colonised (median 2.9-fold increase upon stimulation compared to the unstimulated condition, (p=0.002, Figure 65B). None of the other vaccinated or unvaccinated groups showed a significant induction of IFN-γ production. Similar to the other T-cell subsets, IL-17A producing TCR-γδ$^+$ cells did not significantly increase after stimulation with influenza antigens (Figure 65C).

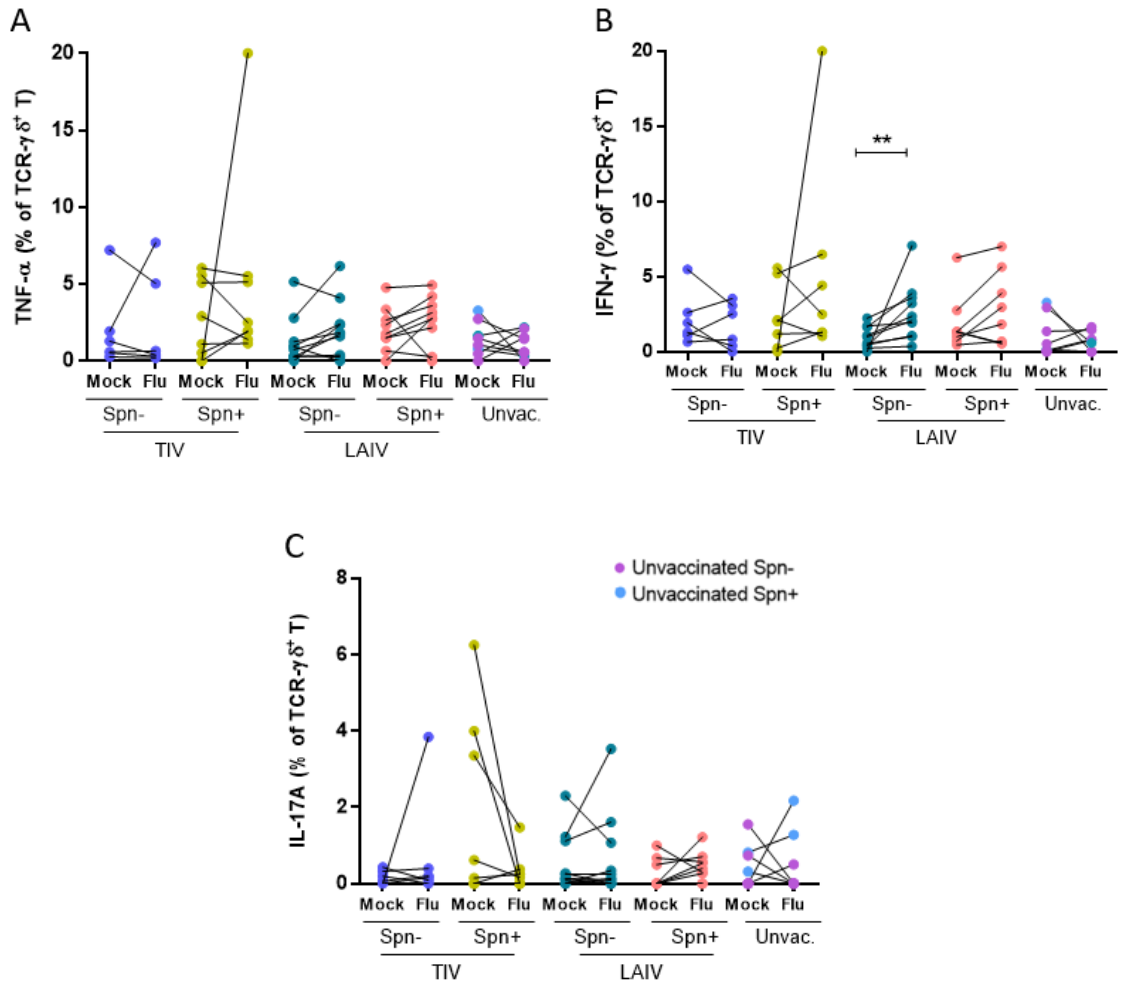

Figure 57. Concurrent LAIV increases frequency of IFN-γ producing influenza-specific TCR-γδ⁺ in the lungs of Spn non-colonised individuals. Frequency of cytokine-producing TCR-γδ⁺ T-cells was measured in human BAL samples by intracellular staining flow cytometry analysis after *in vitro* stimulation with influenza antigens or non-stimulation (mock). Volunteers were divided by vaccine and colonisation status in TIV Spn- (n=6), TIV Spn+ (n=8), LAIV Spn- (n=10), LAIV Spn+ (n=9) and unvaccinated (Spn-, n=3 and Spn+, n=5) group. Production of (A) TNF-α, (B) IFN-γ and (C) IL-17A by lung TCR-γδ⁺ T-cells. Individual dot represents a single volunteer and the conditions per individual are connected. **p<0.01 by Wilcoxon test.

Concurrent LAIV increases frequency of $CD4^+$ regulatory T-cells in the lung of non-colonised individuals

A balanced immune response in the lung has been demonstrated to be important in preventing pneumonia [132]. To investigate whether concurrent LAIV could alter frequency of T-regs in the lung, we measured the frequency of $CD25^{hi}$ $FOXP3^+$ T-regs among $CD4^+$ T-cells using intracellular staining. Increased levels of $CD4^+$ T-regs were only significantly different in BAL samples of LAIV non-colonised when compared to unvaccinated individuals (mean 1.5-fold increase, p=0.039, Figure 66).

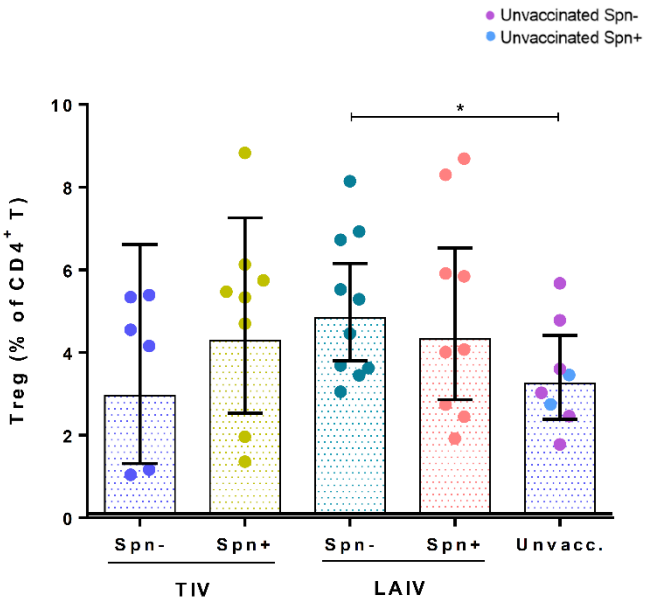

Figure 58. Concurrent LAIV increases frequency of CD4⁺ regulatory T-cells in the lung of Spn non-colonised individuals. Frequency of unstimulated CD4⁺ T-regs (CD3⁺ CD4⁺ CD25⁺ FOXP3⁺) was measured by flow cytometry in human BAL samples from TIV Spn- (n=6), TIV Spn- (n=8), LAIV Spn- (n=10), LAIV Spn+ (n=9) and unvaccinated (Spn-, n=3 and Spn+, n=5). Each individual dot represents a single volunteer and geometric means with 95% CI are shown. *p<0.05 by unpaired t test.

TIV but not concurrent LAIV vaccination increases levels of IgG to influenza in serum

In addition to cellular responses, we sought to assess humoral responses elicited by TIV and concurrent LAIV vaccination both systemically and at the mucosal sites (nasal and lung). In serum samples, IgG levels against influenza antigens were measured at baseline (prior to bacterial challenge and influenza immunisation) and at 24 days post vaccination. TIV induced a median 5.9-fold increase ($p<0.0001$) of influenza-specific IgG, while LAIV intranasal administration did not confer increase of sera IgG levels (Figure 59A). Prior colonisation of the nasopharynx with Spn did not alter influenza-specific IgG levels induced in response to either vaccine. (Figure 59B).

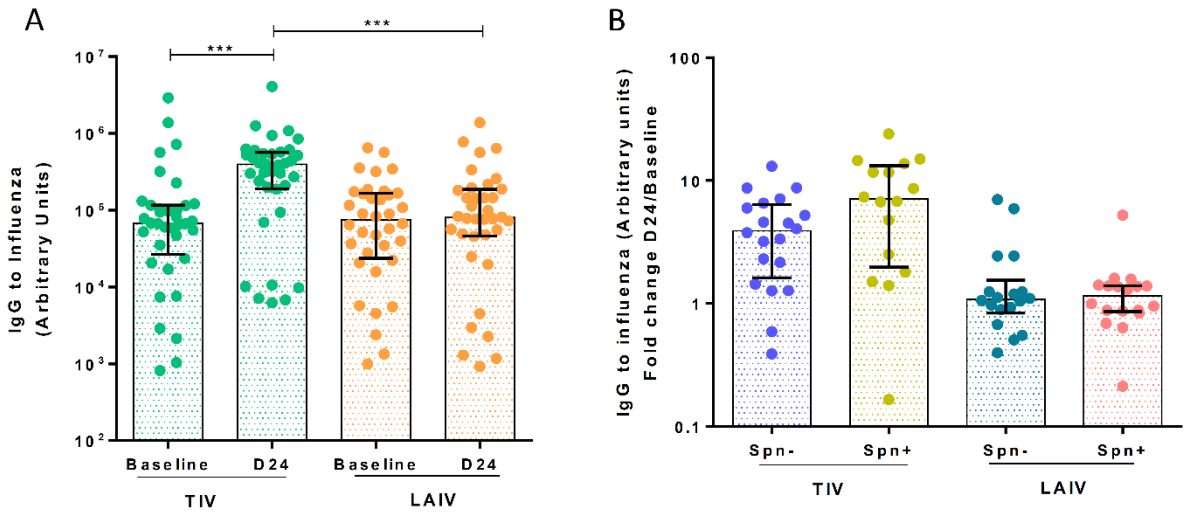

Figure 59. TIV but not concurrent LAIV vaccination increases levels of IgG to influenza in serum. (A) Geometric mean of IgG levels to influenza, measured by ELISA, in serum of LAIV (n=36) and TIV (n=36) vaccinated subjects at baseline (8 days pre-vaccination) and D24 (24 days post-vaccination). (B) Fold change (D24/Baseline) of paired IgG titres to influenza in serum following TIV or LAIV vaccination. TIV Spn- (n=20), TIV Spn+ (n=16), LAIV Spn- (n=18); LAIV Spn+ (n=18). Medians with IQR are shown. ****p<0.0001 by Wilcoxon test for comparisons within the same group, and ***p<0.001 by Mann-Whitney test for comparisons between groups.

IgG but not IgA is induced by influenza vaccines in the lung, with concurrent LAIV mediated-responses being impaired by Spn colonisation

Humoral responses in the lung following TIV or concurrent LAIV vaccination were assessed in BAL samples collected between 26 to 46 days post influenza vaccination. Due to the single timepoint sampling of the lung, 20 unvaccinated subjects (10 Spn-colonised and 10 non-colonised) were used as a control group.

Levels of IgA to influenza in the lung did not differ between TIV, LAIV and control groups (Figure 60A). In terms of IgG levels, TIV was associated with a high IgG response (median 5.8-fold increase compared to control, $p<0.0001$), whereas LAIV conferred a modest IgG induction (median 1.6-fold change compared to control, $p=0.028$, Figure 60B). TIV elicited influenza-specific IgG levels were 3.7x greater than LAIV-induced responses in the pulmonary mucosa (Figure 60B).

IgA levels were not significantly increased in the lung by vaccination and were not affected by Spn colonisation (Figure 61C). Spn colonisation affected IgG titres in the LAIV vaccinated group, but not in the TIV group. IgG to influenza was higher in LAIV Spn- compared to colonised (LAIV Spn+, median 1.35-fold increase, $p=0.010$), however the colonised group was not different from the control group ($p=0.006$, Figure 60D).

Spn colonisation impairs nasal IgA induction following LAIV but does not alter responses to TIV

To assess antibody responses at the nasal mucosa, we measured influenza-specific IgA and IgG levels in NW samples at baseline and 24 days following influenza immunisation. TIV induced a median 2.2- and a 5.2-fold increase in influenza virus-specific IgA and IgG levels, respectively, 24 days post-vaccination (Figure 61A and 61B). On the other hand, LAIV-induced IgG antibody responses were weakened compared to those induced by TIV. LAIV nasal administration resulted to increase of IgA titres to influenza (median 1.3-fold increase, IQR: 0.7x-2.1x, Figure 61A), whereas the induction of IgG levels (median 1.4-fold increase) was moderated if seen alongside the corresponding induction resulted by TIV. (Figure 61B).

Reduced LAIV-mediated immunogenicity, as observed for lung cellular responses, was also observed for humoral responses at the nasal mucosa of Spn colonised volunteers. Concurrent colonisation of the nasopharynx with Spn affected IgA titres, but not IgG, in the LAIV-vaccinated (Figure 61C and 61D). At day 24 post-vaccination, LAIV-vaccinated non-colonised had significantly greater levels of IgA to influenza circulating in the nasal lumen, compared to the colonised group (LAIV Spn- median=1.69, IQR: 0.98-2.65 vs LAIV Spn+ median=1.24, IQR: 0.66-1.81, p=0.020, Figure 60C). Concurrent Spn colonisation did not alter antibody responses to influenza in the TIV-vaccinated individuals (Figure 61C and 61D).

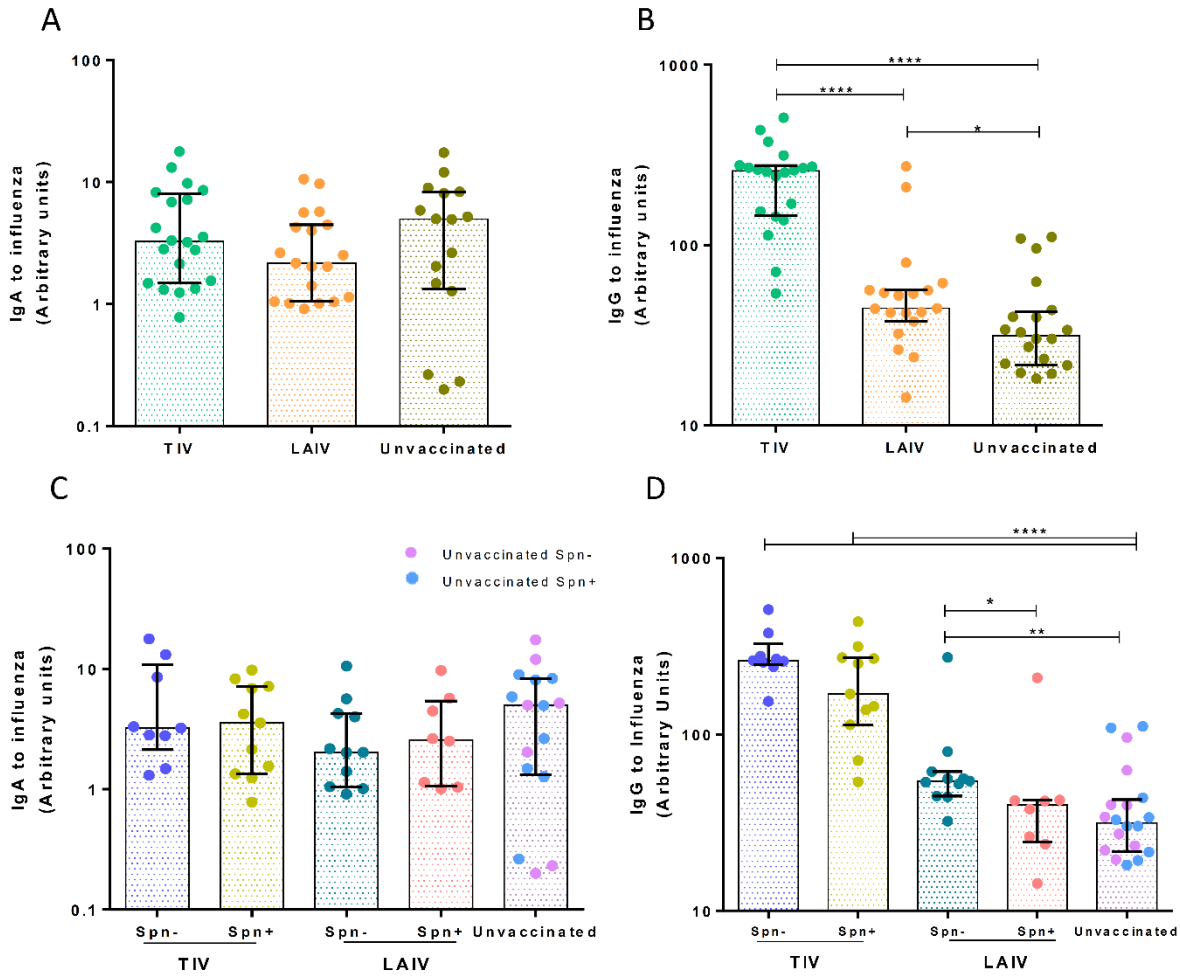

Figure 60. IgG but not IgA is induced by influenza vaccines in the lung, with concurrent LAIV responses being reduced during Spn colonisation. (A)-(B) Geometric mean of IgA and IgG titres to influenza for TIV (n=20), LAIV (n=19) vaccinated subjects and unvaccinated (n=20) was measured by ELISA in BAL fluid. (C)-(D) Geometric mean of IgA and IgG titres grouped based on vaccination and colonisation status, as TIV Spn- (n=9), TIV Spn+ (n=11), LAIV Spn- (n=11), LAIV Spn+ (n=8), unvaccinated (n=20). *p<0.05, **p<0.01, ****p<0.0001 by Wilcoxon test for comparisons within the same group and by Mann-Whitney test for comparisons between groups.

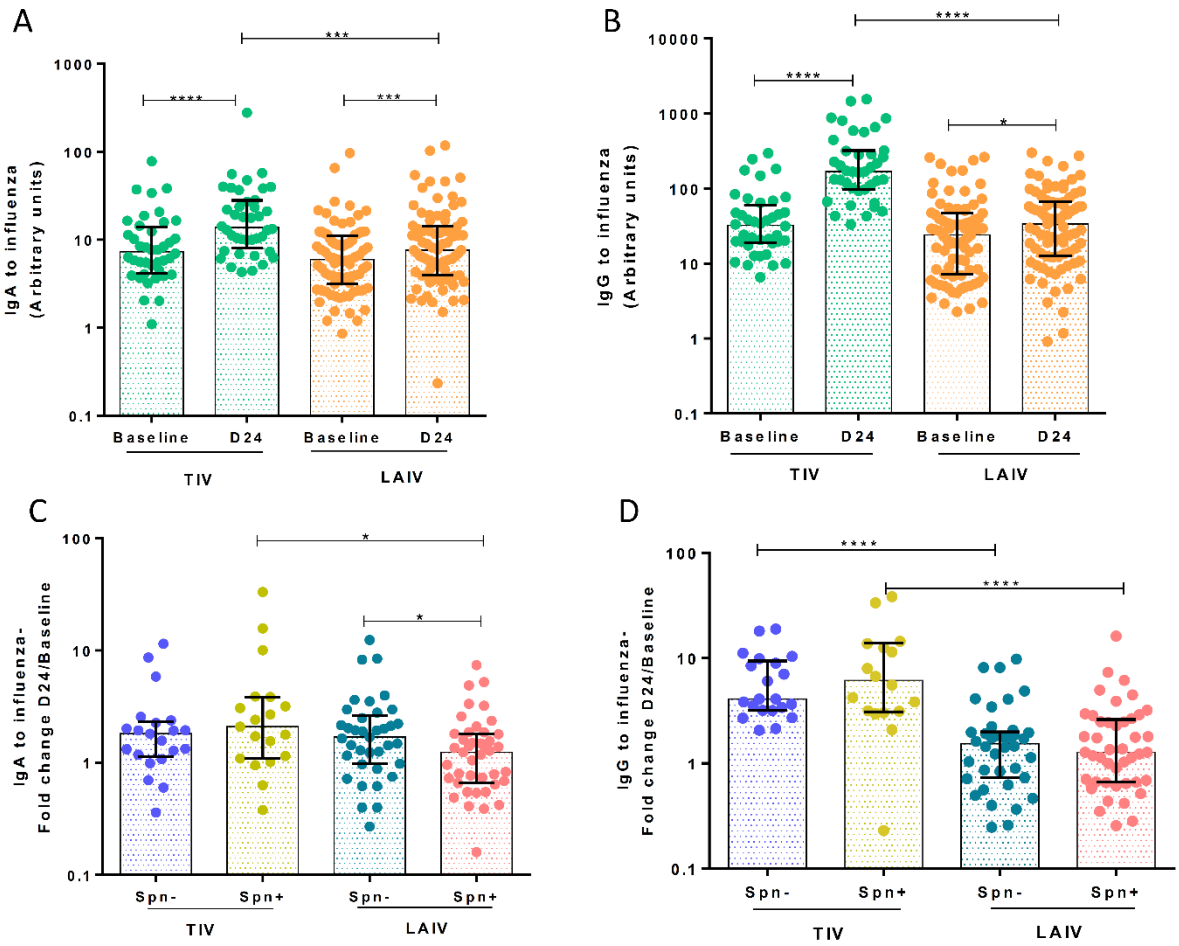

Figure 61. Spn colonisation impairs nasal IgA induction following concurrent LAIV but does not alter responses to TIV. (A) Geometric mean of IgA and (B) IgG titres to influenza measured by ELISA in NW of TIV (n=40) and LAIV (n=80) vaccinated subjects at baseline (8 days pre-vaccination) and D24 (24 days post-vaccination). (C) Fold change (D24/Baseline) of paired IgA and (D) IgG titres to influenza in NW following vaccination with TIV Spn- (n=21), TIV Spn+ (n=19), LAIV Spn- (n=37), LAIV Spn+ (n=43). Medians with IQR are shown. *p<0.05, ***p<0.001, ****p<0.0001 by Wilcoxon test for comparisons within the same group and by Mann-Whitney test for comparisons between groups.

6.4 Discussion

As we demonstrated here, the 2 available influenza vaccines (TIV and LAIV), induce immune responses in the host via the innate and adaptive pathways [365]. However, there is a lack of detailed understanding of the immunological mechanisms induced by these vaccines and how this induction affects Spn colonisation.

In this study, we sought to investigate the cellular and humoral immune responses elicited by TIV and concurrent LAIV, focusing on respiratory tract mucosal sites, in addition to assessing whether colonisation of the nasopharynx with Spn influences vaccine immunogenicity. The results indicate that TIV and LAIV confer differential immunity to adults. TIV mainly induces high levels of influenza-specific antibodies in the serum and mucosal sites, while LAIV combines less pronounced mucosal humoral responses with enhanced cellular immunity in the lung. Importantly, LAIV immunogenicity is diminished by the nasal presence of Spn and this important confounder should be considered when assessing LAIV efficacy.

TIV, as well as other inactivated influenza vaccines [366,367], is known to induce higher titres of serum hemagglutination-inhibiting IgG and IgA antibodies when compared to LAIV [366–368]. On the other hand, LAIV was demonstrated to induce higher levels of nasal mucosa IgA to influenza when compared to TIV [14], which mainly elicits IgG antibodies in the nasal mucosa [222].

Mucosal lymphocytes are considered the dominant source of IgA [29] and IgG [369] in the nose, with IgG also originated by transudation or diffusion from plasma [369]. It is important to be mentioned that IgA is the most abundant immunoglobulin in the upper respiratory tract and both bacteria and viruses

express signals that play immunomodulatory role in these mucosal surfaces [29]. In the nose, IgA performs critical functions such as neutralisation of antigens [370], modulation of B-cell responses [188,270,277]. While IgA is the predominant immunoglobulin found in the upper airways, IgG titers increase progressively in the lower respiratory tract - being the predominant immunoglobulin in the lung [29] – and IgG deficiency is associated with susceptibility to pneumonia and respiratory tract infections [29].

In agreement with previous studies[213], TIV vaccination induced high systemic and mucosal antibody responses, whereas LAIV elicited both mucosal (mainly IgA) influenza virus-specific antibodies and cell-mediated immune responses. Interestingly, pre-existing Spn colonisation of the nasopharynx reduced these LAIV-mediated immune responses but left TIV-induced responses unaltered which is corroborated by studies that show that Spn can impact LAIV-induced responses in the human nasal microbiome as it develops cellular and adaptive immunity to the bacteria in the site of the attenuated influenza viruses replication [49,62.]

We also showed that – in contrary to published results of immune responses 10 days [371] and 6 weeks after vaccination in mice [372]– the numbers of $CD3^+$, $CD4^+$ and $CD8^+$ T-cells in the lung were similar between TIV and LAIV vaccinated subjects.

In the lungs, LAIV prevailed over TIV on cellular induced responses. LAIV nasal administration led to increased lung levels of TNF-α and IFN-γ producing $CD4^+$ T-cells, including TRMs, as well TNF-α producing $CD8^+$ T-cells, upon in vitro stimulation. Interestingly, we observed that influenza-specific $CD4^+$ T-cell lung responses were more pronounced in individuals not colonised with Spn at

the time of vaccination. Similarly, there was a higher proportion of IFN-γ producing TCR-γδ$^+$ T-cells in the non-colonised LAIV recipients. Moreover, LAIV was associated with increased frequencies of lung regulatory T-cells only in the absence of nasal Spn colonisation. On the other hand, LAIV-vaccinated individuals who were colonised by Spn elicited IL-17A-producing CD8$^+$ T-cells which is consistent with findings in previous studies with humans [287] and mice in which this cytokine was essential for bacterial control and clearance during wild-type influenza and Spn co-infection [256].

Humoral responses were highly induced by TIV, whereas LAIV conferred an overall modest antibody induction. Systemically, TIV elicited influenza virus-specific IgG responses, which were not observed in the LAIV vaccinated arm. In the nose, TIV conferred predominantly IgG induction, while LAIV was mainly associated with high levels of IgA. Colonisation of the nasopharynx with Spn at the time of LAIV administration impaired the induction of mucosal IgA to influenza in the nose, but not in the lung.

LAIV in adults, unlike children, does not confer superior protection compared to TIV [14]. This is probably related to the life-long accumulation of influenza immunity through natural exposure and previous vaccinations, which can prevent the nasal replication of the attenuated virus and shorten the viral replication cycle [373]. Consequently, LAIV may elicit less potent responses in adults compared to children, thus any extrapolation from findings in adults to children, the target population for this vaccine, must be done with caution.

Our finding that concurrent Spn colonisation could inhibit LAIV-induced immune responses is a variable that should be taken into account when evaluating LAIV efficacy, as children display high rates of Spn colonisation [374,375].

This finding would potentially explain why one study in Senegal showed no efficacy of vaccination with LAIV [376], as Spn colonisation rates are higher in low-income countries (up to 93% of children colonised by Spn) [377]. The impaired LAIV-induced immunity during established Spn colonisation was associated with a lack of a pro-inflammatory response in the nasal lumen following LAIV vaccination. A possible explanation for this is that Spn colonisation affects local immune and epithelial cell responses upon LAIV vaccination, which could diminish immune cells infiltration and antigen presenting cells (APC) activation, impacting on the downstream memory responses [282,378]. Alternatively, it is possible that Spn colonisation interferes with the viral replication cycle [256,379].

Ideally, an effective and broadly protective influenza vaccine should induce both humoral and cellular immunity. Whereas antibody responses to influenza show some degree of strain cross-reactivity [380,381] they are insufficient to provide heterosubtypic, cross-strain influenza protection [382,383] Recent data from natural history cohort studies have focused on the potential of T-cells as key players in mediating heterosubtypic immunity in humans [124,384]. We observed that even in the absence of vaccination, healthy adults showed $CD4^+$ T-cell responses to influenza stimulation, which likely reflects their lifelong exposure to influenza viruses. The use of purified influenza antigens included adjuvant, to measure cellular responses, would possibly lead to greater T-cells responses. Our results demonstrated that LAIV induced influenza-specific cytokine-producing $CD8^+$ and $CD4^+$ T-cells, including TRM in the lung. As part of T-cell immune response to influenza, recent studies have elucidated the importance of TRM in protection of mucosal barrier tissues against pathogen challenge by producing chemokines for cell recruitment [385]. It has been shown that TRM T-cells provide superior

protection to influenza infection when compared with circulating T-cells[386]. By seeding the lungs with these cells, it is possible to establish long-term heterosubtypic protection to influenza [387,388].

We have also demonstrated that, in volunteers not colonised by Spn, LAIV increased levels of T-regs in the lung compared to unvaccinated individuals. $CD4^+$ T-regs contribute to homeostasis of the immune system, controlling infection by respiratory viruses and avoiding tissue damage[92] and secondary bacterial infection [389]. As a result of recurrent exposure to virus and bacteria, $CD4^+$ T-regs increase in frequency with age [390]. For this reason, our findings in adults might underestimate the effect of LAIV on frequency of T-regs in the lung of children.

In conclusion, using a controlled human infection model at a known time relative to vaccination, this study was able to highlight differences in immunogenicity between LAIV and TIV at relevant mucosal sites. Moreover, we identified Spn colonisation as an important variable in LAIV-induced immunity.

CHAPTER SEVEN

GENERAL DISCUSSION

7.1 Introduction

The investigation of host immune responses to pathogens during attenuated influenza virus (LAIV) and Spn co-infection in the nasopharynx is essential for deeper understanding of interactions between host, attenuated virus and bacteria. These insights clarify effects of co-infection on viral and bacterial clearance, acquisition, replication, transmission as well as efficacy of the attenuated influenza vaccine to ultimately to promote better immunisation approaches to the population. In humans, wild-type influenza virus can predispose to secondary Spn colonisation and, likewise, the bacteria can induce viral shedding [391] which, in turn, deregulates inflammatory responses and leads to loss of control of Spn density [392]. Interestingly, in murine models of co-infection, sialic acid cleaved from host's epithelium by the influenza virus is consumed by Spn promoting growth [75] which indicates the need for further research in humans in order to corroborate the hypothesis.

In this study, LAIV-induced influenza-specific immune responses were compared to TIV using nasal wash, nasosorption, nasal cells, BAL and serum of healthy adult volunteers. In addition, by inoculating individuals with live Spn (EHPC), we assessed how responses to LAIV affect Spn colonisation as well as how the bacteria affects vaccine immunogenicity. The primary underlying mechanisms of cell recruitment, cytokine and antibody production were also investigated. Altogether, this thesis is part of the largest conducted vaccine testing studies using a controlled human infection model as well as the first controlled challenge studies in humans using two live pathogens to directly assess the impact of a vaccine on microbiota. In short, we observed that different profiles of responses are observed in TIV- and LAIV-vaccinated as well as LAIV-

vaccinated volunteers who became colonised by Spn when compared to non-colonised.

7.2 Summary and discussion of findings

To gather knowledge about LAIV and how the attenuated influenza virus vaccination affects Spn colonisation is essential for accurate assessment of vaccine immunogenicity in humans and its effect on the nasopharyngeal microbiota and host immunity. This work was based on previous studies with wild-type influenza virus and Spn co-infection that show it to be associated with increased bacterial nasal load in mice [275,281,393] and humans [256,266]. Importantly, LAIV administration has also been correlated with increase in Spn density in mice [75,76] and humans [318,394], however the underlying mechanisms were still not elucidated.

Here, we showed that LAIV in healthy adults induces inflammatory responses in the nasopharynx, including cytokines that regulate adaptive immunity as previously described in other studies [395]. Importantly, our results confirm that the attenuated virus elicits many of the same immune responses as the wild-type influenza, such as induction of cytokines IL-6 [262] and IL-1β [396].

In addition, in the lung LAIV alone still induced moderate antibody levels against influenza after 30 days post-vaccination. Moreover, T-cell responses were increased with higher percentages of $CD4^+$ T-cells producing IFN-γ as well as $CD4^+$ and $CD8^+$ T-cells producing TNF-α, two cytokines also induced in the murine lung by LAIV administration [372]. Additionally, TRM T-cells, which persist in the lung for long periods after infection [388] - were shown to have an important role for lung cytokine response against the attenuated influenza virus, similar to results from studies with the wild type virus [386,397] .

Models of co-infection with the attenuated influenza virus and Spn show induction of adaptive immune responses in the nose and lung to achieve viral and bacterial clearance to be necessary during co-infection [188]. In this thesis, we showed that LAIV induces a state of inflammation in the nasopharynx independent of Spn that predisposes the host to colonisation. In addition, is possible that vaccination also causes epithelial damage, dysfunction or denudation in the colonised nose as indicated by increases in cytokines that regulate cell growth and that corroborates results in murine models with wild-type influenza and Spn co-infection [193,271,398]. Furthermore, we have shown that in the lung co-infection of attenuated influenza virus and Spn does not elicit IL-17A-producing $CD8^+$ T-cells, which opposes previous studies with wild-type influenza [256,287].

However, when compared to non-colonised volunteers, our results show that some of the host immune responses were affected by LAIV which corroborated murine models of wild-type influenza virus and Spn co-infection that, likewise, demonstrate impaired induction of anti-inflammatory cytokines [127,399], of monocyte recruitment [186], of influenza specific antibodies [188] and of neutrophils activation [192]. Notably, we demonstrated for the first time that LAIV vaccination in Spn colonised volunteers impaired activation of T-cells in the nasopharynx.

Additionally, LAIV impaired the induction of chemoattractant to monocytes such as MCP-1 [400] and RANTES [401,402] in the Spn colonised nasopharynx, possibly limiting monocyte recruitment to the nose as observed in our results. These results confirm the hypothesis that unbalanced cytokines affect recruitment of immune cells during Spn co-infection with the attenuated influenza

virus similarly to the wild-type virus [403]. Furthermore, the impairment of MCP-1 can also explain decreased induction in levels of IL-1β whereas the impairment of RANTES can clarify reduced T-cell activation [404] demonstrated in our results.

It is important to notice that previous studies have demonstrated that Spn colonisation induces cytokines TNF-α [122] in the nasopharynx, however our results show that LAIV affects Spn-induced TNF-α, possibly affecting control of the infection. Moreover, here we confirmed that LAIV induces specific antibody responses against influenza characterised by IgG and particularly IgA in nasal wash [240], however, we showed for the first time that LAIV-induced IgA production was reduced in the Spn colonised nasopharynx which adds to the uncontrolled bacterial growth [370] by neither neutralising antigens or preventing the attenuated influenza virus and Spn adherence to epithelial cells [29]

Importantly, the detected LAIV impact in the lung of colonised volunteers corroborate wild-type influenza virus and Spn co-infection animal models in which influenza-specific IgG and $CD4^+$ T-cells were impaired when compared to non-colonised individuals [270]. However, our results in adults do not confirm murine studies in which $CD4^+$ T-reg are increased in co-infected animals [188].

In summary, we demonstrated that LAIV induces an inflammatory state in the nasopharynx of healthy adults. On one hand, the immune deregulation caused by LAIV vaccination in Spn colonised individuals caused substantial inflammation but, on the other hand, impaired responses that control viral and bacterial clearance.

7.3 Limitations of LAIV research in humans

Despite its proven success in preventing infectious diseases, the vast majority of vaccines used today for human immunisation have been developed empirically, at first often with little understanding of the immune mechanisms by which they induce protective immunity. This work has uncovered some limitations in the assessment of immunogenicity and research of LAIV-induced responses which will contribute to more accurate understanding of the vaccine as well as to shed a light on the impact of vaccination on other pathogens, specifically Spn.

Importantly, LAIV vaccination in adults induces different immune responses compared to children [14] - the target population for this vaccine - as a result of induced neutralising antibodies due to previous exposure to influenza virus during their lives. Continuous exposure to influenza virus can prevent the nasal replication of the attenuated virus contained in the LAIV [373] as well as elicit natural immunity against influenza antigens. Consequently, extrapolation of these results to the paediatric population must be done with caution especially regarding T-regs, recruitment of monocytes and activation of neutrophils, already proven to not be analogous to adults.

In addition, the relationship between LAIV and Spn colonisation rates presented in this thesis uses nasal wash and classical microbiology. Other techniques or devices for assessment of colonisation rates such as nasosorption [405], nasal curettage [406] and bronchoabsorption [407] were not validated and should be compared to this thesis results with caution.

7.4 Future research directions and questions derived from this thesis

Most studies on co-infection of LAIV and Spn have been done in animal models. Here we have shown discrepancies in results when comparing LAIV or wild-type influenza virus and Spn co-infection results from murine models with human models. The extrapolation of immune responses between murine models and humans can be problematic since mice doesn't represent the complexity of microbiome and immunity of the human host [408]. As noted by Ferreira, Jambo and Gordon [53], humans are natural hosts of Spn and each individual has been in contact and colonised numerous times throughout their life while mice models are pathogen-free and only susceptible to the bacteria in laboratorial conditions, skewing the results. In addition, chinchillas and ferrets are also used as co-infection models but likewise present great difficulties to confirm similar results in humans [260].

Moreover, to study co-infection of the attenuated influenza virus and Spn, we have used only one Spn serotype 6B isolate, which limits the scope of our results. Future studies using other isolates with variable invasive phenotypes could answer how generalizable these findings are across serotypes.

In addition, further studies into mucosal immunology using different techniques for sample collection can validate the results found in this thesis and enable further discoveries on patient groups who are unable to undergo the procedures proposed. Importantly, methods for collection of nasal lavage, nasal cells and lung lavage used in this thesis (NW, nasal curettage, nasosorption and BAL) can be compared to alternative techniques such as nasosorption with different synthetic absorptive matrix, bronchial brush and bronchoabsorption.

Moreover, serial nasosorption can be performed for assessment of nasal cytokines, antibodies and viral load kinetics. For instance, studies of early hours kinetics can be done with this technique as well as NW and saliva 409 for further understanding mucosal events since many cytokine responses are concentrated in the first 48 hours of immunological response.

Notably, it is critical to continue the assessment of the best sampling device and site to measure Spn colonisation and bacterial density. This can be done by comparison of sampling and analysis methods, for instance nasosorption results against NW, nasal transcriptomics of nasal curettage results against flow cytometry as well as bronchoabsorption results against BAL.

It is important to notice that methodologies such as nasal curettage can take into consideration the rate of ciliated respiratory epithelial cells to squamous stratified epithelial cells as to assure the results accuracy of cell recruitment to the mucosa.

Continuous development of the EHPC method as well as other multiple pathogens challenge models are essential to elucidate the potential for viral pandemics, secondary infections and interactions between bacteria, virus and human host as well as identification of health indicators and therapies for prevention of high morbidity scenarios. Furthermore, studies focused on analysis of the 4 specific virus strains contained in the LAIV can validate novel combinations of viruses to be added in the annual influenza vaccine.

Table 9. Suggestions for future studies, method validation and development of challenge models.

Field of research	Suggested studies	Specific suggestions
Alternative methods for sample collection	Development of alternative sampling methods for different patient groups (smokers, elderly, asthmatics, children).	• Bronchial brush. • Bronchoabsorption; • Serial nasosorption;
Challenge models for vaccine testing	Development of a human nasal challenge model with LAIV and viral vaccines to measure responses to nasal strain-specific viruses with more detailed mucosal kinetics.	• Focus on collection of serial nasosorption, nasal scrape and bronchoabsorption; • LAIV and novel viral vaccines studies; • Topical nasal vaccines.
Pneumococcal serotypes	Validation of results in this thesis using different serotypes of Spn.	• Serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F.
Point-of-care assays	Development of antibody, cytokines, viral and bacterial load indicators of healthy and unhealthy patients.	• Cytokines and chemokines; • Nasal mucosal antibodies (IgG and IgA);

		• Spn, bacteria and microbiome; • Viral infections.
Secondary viral infections	Study of the relationship between respiratory and gastrointestinal pathogens and human microbiome in diseases in which virus are the main cause of hospitalising exacerbations and mortality. In addition, studies on the propensity of different viruses to alter innate respiratory mucosal immunity.	• Asthma; • Chronic obstructive pulmonary disease; • Cystic fibrosis. • Idiopathic pulmonary fibrosis;
Spn density assessment	Validation of novel sampling and measurement methods of Spn colonisation and density.	• BAL; • Bronchial brushing; • Nasal curettage; • Nasal curettage followed by transcriptomics.
Validation of results	Validation of this thesis results using different sampling techniques.	▪ Bronchoabsorption; ▪ Cough plates;

		▪ Nasal curettage assessed by transcriptomics; ▪ Nasal swabs; ▪ Nasopharyngeal aspirates; ▪ Nasosorption for measurement of Spn density; ▪ Nasosorption with different synthetic absorptive matrix; ▪ Other methods of nasal lavage; ▪ Collection of saliva.
Viral challenge models	Development of experimental challenge models using virus besides influenza.	• HRV; • RSV.

7.5 Conclusion

LAIV vaccination remains efficient in the long term as it protects children and elderly from flu with subclinical side effects that usually only affects hosts that

lack preformed virus antibodies [395]. Importantly, LAIV vaccination protects the host from future virus infections thus preventing a secondary Spn colonisation [274].

Our finding that Spn colonisation could inhibit LAIV-induced immune responses is a variable that should be taken into account when evaluating LAIV efficacy, as children display high rates of Spn colonisation [374,375]. As colonisation rates are higher in low-income countries (up to 93% of children colonised by Spn) [377], these results could potentially explain one study in Senegal that showed no efficacy of vaccination with LAIV [376] , although mismatch of circulating influenza virus strains with the vaccine was a limitation to accurate assessment.

In short, this thesis provides new topics for continuity of human research into LAIV and its relationship with the microbiota, which is necessary to further elucidate the impacts of mass immunisation with live attenuated virus vaccines especially regarding its impact on Spn density and transmission.

8. References

1. Garcia-Bustos JF, Chait BT, Tomasz A. Structure of the peptide network of pneumococcal peptidoglycan. *J Biol Chem*. 1987;262(32):15400-15405. http://www.ncbi.nlm.nih.gov/pubmed/2890629. Accessed November 7, 2019.
2. Dziarski R, Tapping RI, Tobias PS. Binding of bacterial peptidoglycan to CD14. *J Biol Chem*. 1998;273(15):8680-8690. doi:10.1074/jbc.273.15.8680
3. Braido F, Bellotti M, De Maria A, Cazzola M, Canonica GW. The role of Pneumococcal vaccine. *Pulm Pharmacol Ther*. 2008;21(4):608-615. doi:10.1016/j.pupt.2008.04.001
4. Sjostrom K, Spindler C, Ortqvist A, et al. Clonal and Capsular Types Decide Whether Pneumococci Will Act as a Primary or Opportunistic Pathogen. *Clin Infect Dis*. 2006;42(4):451-459. doi:10.1086/499242
5. van der Poll T, Opal SM. Pathogenesis, treatment, and prevention of pneumococcal pneumonia. *Lancet*. 2009;374(9700):1543-1556. doi:10.1016/S0140-6736(09)61114-4
6. Catterall JR. Streptococcus pneumoniae. *Thorax*. 1999;54(10):929-937. doi:10.1136/thx.54.10.929
7. Hausdorff WP, Bryant J, Paradiso PR, Siber GR. Which Pneumococcal Serogroups Cause the Most Invasive Disease: Implications for Conjugate Vaccine Formulation and Use, Part I. *Clin Infect Dis*. 2000;30(1):100-121. doi:10.1086/313608
8. Wasfy MO, Pimentel G, Abdel-Maksoud M, et al. Antimicrobial susceptibility and serotype distribution of Streptococcus pneumoniae

causing meningitis in Egypt, 1998-2003. *J Antimicrob Chemother*. 2005;55(6):958-964. doi:10.1093/jac/dki101

9. Mandell LA. Antimicrobial resistance and treatment of community-acquired pneumonia. *Clin Chest Med*. 2005;26(1):57-64. doi:10.1016/j.ccm.2004.10.005
10. van Dam JEG, Fleer A, Snippe H. Immunogenicity and immunochemistry of Streptococcus pneumoniae capsular polysaccharides. *Antonie Van Leeuwenhoek*. 1990;58(1):1-47. doi:10.1007/BF02388078
11. Calix JJ, Nahm MH. A New Pneumococcal Serotype, 11E, Has a Variably Inactivated wcjE Gene . *J Infect Dis*. 2010;202(1):29-38. doi:10.1086/653123
12. Kuch A, Sadowy E, Skoczyńska A, Hryniewicz W. First report of Streptococcus pneumoniae serotype 6D isolates from invasive infections. *Vaccine*. 2010;28(39):6406-6407. doi:10.1016/j.vaccine.2010.07.051
13. In HP, Pritchard DG, Cartee R, Brandao A, Brandileone MCC, Nahm MH. Discovery of a new capsular serotype (6C) within serogroup 6 of Streptococcus pneumoniae. *J Clin Microbiol*. 2007;45(4):1225-1233. doi:10.1128/JCM.02199-06
14. Hoft DF, Lottenbach KR, Blazevic A, et al. Comparisons of the humoral and cellular immune responses induced by live attenuated influenza vaccine and inactivated influenza vaccine in adults. *Clin Vaccine Immunol*. 2017;24(1):e00414-16. doi:10.1128/CVI.00414-16
15. Mitchell AM, Mitchell TJ. Streptococcus pneumoniae: Virulence factors and variation. *Clin Microbiol Infect*. 2010;16(5):411-418. doi:10.1111/j.1469-0691.2010.03183.x

16. Gamez G, Hammerschmidt S. Combat Pneumococcal Infections: Adhesins as Candidates for Protein- Based Vaccine Development. *Curr Drug Targets*. 2012;13(3):323-337. doi:10.2174/138945012799424697

17. Simell B, Auranen K, Käyhty H, Goldblatt D, Dagan R, O'Brien KL. The fundamental link between pneumococcal carriage and disease. *Expert Rev Vaccines*. 2012;11(7):841-855. doi:10.1586/erv.12.53

18. TUOMANEN EI, MASURE HR. Molecular and Cellular Biology of Pneumococcal Infection. *Microb Drug Resist*. 1997;3(4):297-308. doi:10.1089/mdr.1997.3.297

19. Ejstrud PER, Kristensen B, Hansen JB, Madsen KM, Schønheyder HC, Sørensen HT. Risk and patterns of bacteraemia after splenectomy: A population-based study. *Scand J Infect Dis*. 2000;32(5):521-525. doi:10.1080/003655400458811

20. Feldman C, Anderson R, Cockeran R, et al. The effects of pneumolysin and hydrogen peroxide, alone and in combination, on human ciliated epithelium in vitro. *Respir Med*. 2002;96(2002):580-585. doi:10.1053/rmed.2002

21. Barocchi MA, Ries J, Zogaj X, et al. A pneumococcal pilus influences virulence and host inflammatory responses. *Proc Natl Acad Sci U S A*. 2006;103(8):2857-2862. doi:10.1073/pnas.0511017103

22. Sanchez CJ, Kumar N, Lizcano A, et al. Streptococcus pneumoniae in Biofilms Are Unable to Cause Invasive Disease Due to Altered Virulence Determinant Production. Biswas I, ed. *PLoS One*. 2011;6(12):e28738. doi:10.1371/journal.pone.0028738

23. Moore RA, Wiffen PJ, Lipsky BA. Are the pneumococcal polysaccharide

vaccines effective? Meta-analysis of the prospective trials. *BMC Fam Pract*. 2000;1:1. doi:10.1186/1471-2296-1-1

24. Meats E, Brueggemann AB, Enright MC, et al. Stability of serotypes during nasopharyngeal carriage of Streptococcus pneumoniae. *J Clin Microbiol*. 2003;41(1):386-392. doi:10.1128/jcm.41.1.386-392.2003
25. Sleeman KL, Daniels L, Gardiner M, et al. Acquisition of Streptococcus pneumoniae and nonspecific morbidity in infants and their families: A cohort study. *Pediatr Infect Dis J*. 2005;24(2):121-127. doi:10.1097/01.inf.0000151030.10159.b1
26. Hill PC, Townend J, Antonio M, et al. Transmission of Streptococcus pneumoniae in Rural Gambian Villages: A Longitudinal Study . *Clin Infect Dis*. 2010;50(11):1468-1476. doi:10.1086/652443
27. Donkor ES. Understanding the pneumococcus: Transmission and evolution. *Front Cell Infect Microbiol*. 2013;4(MAR). doi:10.3389/fcimb.2013.00007
28. 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. doi:10.1038/nrmicro1871
29. Lionel Mandell, Mark Woodhead, Antoni Torres, Santiago Ewig. *Respiratory Infections*. London: Taylor & Francis Ltd; 2006. https://www.bookdepository.com/Respiratory-Infections-Lionel-Mandell/9780340816943.
30. Hussain M, Melegaro A, Pebody RG, et al. A longitudinal household study of Streptococcus pneumoniae nasopharyngeal carriage in a UK setting. *Epidemiol Infect*. 2005;133(5):891-898. doi:10.1017/S0950268805004012

31. Francis JP, Richmond PC, Pomat WS, et al. Maternal antibodies to pneumolysin but not to pneumococcal surface protein a delay early pneumococcal carriage in high-risk Papua New Guinean infants. *Clin Vaccine Immunol*. 2009;16(11):1633-1638. doi:10.1128/CVI.00247-09

32. Regev-Yochay G, Dagan R, Raz M, et al. Association between carriage of Streptococcus pneumoniae and Staphylococcus aureus in Children. *JAMA*. 2004;292(6):716-720. doi:10.1001/jama.292.6.716

33. Nicoletti C, Brandileone MCC, Guerra MLS, Levin AS. Prevalence, serotypes, and risk factors for pneumococcal carriage among HIV-infected adults. *Diagn Microbiol Infect Dis*. 2007;57(3):259-265. doi:10.1016/j.diagmicrobio.2006.08.021

34. Berezin EN, Cardenuto MD, Ferreira LL, Otsuka M, Guerra ML, Brandileone MCC. Distribution of Streptococcus pneumoniae serotypes in nasopharyngeal carriage and in invasive pneumococcal disease in Sao Paulo, Brazil. *Pediatr Infect Dis J*. 2007;26(7):643-645. doi:10.1097/INF.0b013e3180616d0f

35. Darboe MK, Fulford AJC, Secka O, Prentice AM. The dynamics of nasopharyngeal streptococcus pneumoniae carriage among rural Gambian mother-infant pairs. *BMC Infect Dis*. 2010;10. doi:10.1186/1471-2334-10-195

36. Abdullahi O, Karani A, Tigoi CC, et al. The Prevalence and Risk Factors for Pneumococcal Colonization of the Nasopharynx among Children in Kilifi District, Kenya. Ratner AJ, ed. *PLoS One*. 2012;7(2):e30787. doi:10.1371/journal.pone.0030787

37. Greiff L, Wollmer P, Erjefalt I, Pipkorn U, Persson CGA. Clearance of

99mTc DTPA from guinea pig nasal, tracheobronchial, and bronchoalveolar airways. *Thorax*. 1990;45(11):841-845. doi:10.1136/thx.45.11.841

38. Wang DY, Clement P. Pathogenic Mechanisms Underlying the Clinical Symptoms of Allergic Rhinitis. *Am J Rhinol*. 2000;14(5):325-333. doi:10.2500/105065800781329483

39. Yamazaki M, Kimura K, Watanabe S, et al. Use of a rapid detection assay for influenza virus, on nasal aspirate specimens. *Kansenshogaku Zasshi*. 1999;73(10):1064-1068. doi:10.11150/kansenshogakuzasshi1970.73.1064

40. Watelet JB, Gevaert P, Holtappels G, Van Cauwenberge P, Bachert C. Collection of nasal secretions for immunological analysis. *Eur Arch Oto-Rhino-Laryngology*. 2004;261(5):242-246. doi:10.1007/s00405-003-0691-y

41. Lü FX, Esch RE. Novel nasal secretion collection method for the analysis of allergen specific antibodies and inflammatory biomarkers. *J Immunol Methods*. 2010;356(1-2):6-17. doi:10.1016/j.jim.2010.03.004

42. Ruocco L, Fattori B, Romanelli A, et al. A new collection method for the evaluation of nasal mucus proteins. *Clin Exp Allergy*. 1998;28(7):881-888. doi:10.1046/j.1365-2222.1998.00312.x

43. Klimek L, Reske-Kunz AB, Malling HJ. [Methods for monitoring of therapeutic efficacy in immunotherapy of allergic rhinitis]. *Wien Med Wochenschr*. 1999;149(14-15):394-402. http://www.ncbi.nlm.nih.gov/pubmed/10584282. Accessed April 20, 2020.

44. Walsh EE, Falsey AR. A Simple and Reproducible Method for Collecting

Nasal Secretions in Frail Elderly Adults, for Measurement of Virus-Specific IgA. *J Infect Dis*. 1999;179(5):1268-1273. doi:10.1086/314726

45. Naclerio RM, Creticos PS, Norman PS, Lichtenstein LM. Mediator release after nasal airway challenge with allergen. *Am Rev Respir Dis*. 1986;134(5):1102.
46. Glennie S, Gritzfeld JF, Pennington SH, et al. Modulation of nasopharyngeal innate defenses by viral coinfection predisposes individuals to experimental pneumococcal carriage. *Mucosal Immunol*. 2015;9(1):56-67. doi:10.1038/mi.2015.35
47. Gritzfeld JF, Wright AKAD, Collins AM, et al. Experimental human pneumococcal carriage. *J Vis Exp*. 2013;(72):1-6. doi:10.3791/50115
48. Shak JR, Cremers AJH, Gritzfeld JF, et al. Impact of experimental human pneumococcal carriage on nasopharyngeal bacterial densities in healthy adults. *PLoS One*. 2014;9(6):e98829. doi:10.1371/journal.pone.0098829
49. Ferreira DM, Neill DR, Bangert M, et al. Controlled human infection and rechallenge with Streptococcus pneumoniae reveals the protective efficacy of carriage in healthy adults. *Am J Respir Crit Care Med*. 2013;187(8):855-864. doi:10.1164/rccm.201212-2277OC
50. Rylance J, de Steenhuijsen Piters WAA, Mina MJ, Bogaert D, French N, Ferreira DM. Two randomized trials of the effect of live attenuated influenza vaccine on pneumococcal colonization. *Am J Respir Crit Care Med*. 2019;199(9):1160-1163. doi:10.1164/rccm.201811-2081LE
51. Collins AM, Wright AD, Mitsi E, et al. First Human Challenge Testing of a Pneumococcal Vaccine. Double-Blind Randomized Controlled Trial. *Am J Respir Crit Care Med*. 2015;192(7):853-858. doi:10.1164/rccm.201503-

0542OC

52. Collins A, Wright A, Mitsi E, et al. Pneumococcal Conjugate Vaccine Reduces Rate, Density And Duration Of Experimental Human Pneumococcal Colonisation: First Human Challenge Testing Of A Pneumococcal Vaccine. *Thorax*. 2014;69(Suppl 2):A2-A2. doi:10.1136/thoraxjnl-2014-206260.4

53. Ferreira DM, Jambo KC, Gordon SB. Experimental human pneumococcal carriage models for vaccine research. *Trends Microbiol*. 2011;19(9):464-470. doi:10.1016/j.tim.2011.06.003

54. Belda J, Parameswaran K, Keith PK, Hargreave FE. Repeatability and validity of cell and fluid-phase measurements in nasal fluid: A comparison of two methods of nasal lavage. *Clin Exp Allergy*. 2001;31(7):1111-1115. doi:10.1046/j.1365-2222.2001.01133.x

55. Nikasinovic-Fournier L, Just J, Seta N, et al. Nasal lavage as a tool for the assessment of upper-airway inflammation in adults and children. *J Lab Clin Med*. 2002;139(3):173-180. doi:10.1067/mlc.2002.121661

56. Garcia S, Levine OS, Cherian T, Gabastou JM, Andrus J, Working Group Members. Pneumococcal disease and vaccination in the Americas: an agenda for accelerated vaccine introduction. *Rev Panam Salud Publica*. 2006;19(5):340-348. doi:10.1590/s1020-49892006000500007

57. Cope EK, Goldstein-Daruech N, Kofonow JM, et al. Regulation of virulence gene expression resulting from streptococcus pneumoniae and nontypeable haemophilus influenzae interactions in chronic disease. *PLoS One*. 2011;6(12). doi:10.1371/journal.pone.0028523

58. Bogaert D, De Groot R, Hermans PWM. Streptococcus pneumoniae

colonisation: the key to pneumococcal disease. *Lancet Infect Dis*. 2004;4(3):144-154. doi:10.1016/S1473-3099(04)00938-7

59. Regev-Yochay G, Raz M, Dagan R, et al. Nasopharyngeal Carriage of Streptococcus pneumoniae by Adults and Children in Community and Family Settings . *Clin Infect Dis*. 2004;38(5):632-639. doi:10.1086/381547
60. Hermans PW, Sluijter M, Dejsirilert S, et al. Molecular epidemiology of drug-resistant pneumococci: toward an international approach. *Microb Drug Resist*. 1997;3(3):243-251. doi:10.1089/mdr.1997.3.243
61. Henriques Normark B, Kalin M, Ortqvist A, et al. Dynamics of penicillin-susceptible clones in invasive pneumococcal disease. *J Infect Dis*. 2001;184(7):861-869. doi:10.1086/323339
62. Wright AK a, Ferreira DM, Gritzfeld JF, et al. Human nasal challenge with Streptococcus pneumoniae is immunising in the absence of carriage. *PLoS Pathog*. 2012;8(4):e1002622. doi:10.1371/journal.ppat.1002622
63. Musher DM, Groover JE, Reichler MR, et al. Emergence of Antibody to Capsular Polysaccharides of Streptococcus pneumoniae During Outbreaks of Pneumonia : Association with Nasopharyngeal Colonization. *Clin Infect Dis*. 1997;24:441-446.
64. Dowell SF, Whitney CG, Wright C, Rose CE, Schuchat A. Seasonal patterns of invasive pneumococcal disease. *Emerg Infect Dis*. 2003;9(5):573-579. doi:10.3201/eid0905.020556
65. Centers for Disease Control and Prevention (CDC). Outbreak of pneumococcal pneumonia among unvaccinated residents of a nursing home--New Jersey, April 2001. *MMWR Morb Mortal Wkly Rep*. 2001;50(33):707-710. http://www.ncbi.nlm.nih.gov/pubmed/11787578.

Accessed November 7, 2019.

66. Gleich S, Morad Y, Echague R, et al. Streptococcus pneumoniae serotype 4 outbreak in a home for the aged: report and review of recent outbreaks. *Infect Control Hosp Epidemiol*. 2000;21(11):711-717. doi:10.1086/501717

67. Gray GC, Callahan JD, Hawksworth AW, Fisher CA, Gaydos JC. Respiratory diseases among U.S. military personnel: countering emerging threats. *Emerg Infect Dis*. 5(3):379-385. doi:10.3201/eid0503.990308

68. DeMaria A, Browne K, Berk SL, Sherwood EJ, McCabe WR. An outbreak of type 1 pneumococcal pneumonia in a men's shelter. *JAMA*. 1980;244(13):1446-1449. http://www.ncbi.nlm.nih.gov/pubmed/7420632. Accessed November 7, 2019.

69. Subramanian D, Sandoe JAT, Keer V, Wilcox MH. Rapid spread of penicillin-resistant Streptococcus pneumoniae among high-risk hospital inpatients and the role of molecular typing in outbreak confirmation. *J Hosp Infect*. 2003;54(2):99-103. doi:10.1016/s0195-6701(03)00110-5

70. Martin M, Turco JH, Zegans ME, et al. An outbreak of conjunctivitis due to atypical Streptococcus pneumoniae. *N Engl J Med*. 2003;348(12):1112-1121. doi:10.1056/NEJMoa022521

71. Proulx JF, Déry S, Jetté LP, Ismaël J, Libman M, De Wals P. Pneumonia epidemic caused by a virulent strain of Streptococcus pneumoniae serotype 1 in Nunavik, Quebec. *Can Commun Dis Rep*. 2002;28(16):129-131. http://www.ncbi.nlm.nih.gov/pubmed/12387098. Accessed November 7, 2019.

72. Luchi M. Pneumonia before Antibiotics: Therapeutic Evolution and Evaluation in Twentieth-Century America. *J Hist Med Allied Sci*.

2007;63(1):130-132. doi:10.1093/jhmas/jrm040

73. World Health Organization, Tessa M Wardlaw, Emily White Johansson, Matthew Hodge, UNICEF. *Pneumonia: The Forgotten Killer of Children*.; 2006.

74. Brueggemann AB, Griffiths DT, Meats E, Peto T, Crook DW, Spratt BG. Clonal relationships between invasive and carriage Streptococcus pneumoniae and serotype- and clone-specific differences in invasive disease potential. *J Infect Dis*. 2003;187(9):1424-1432. doi:10.1086/374624

75. Siegel SJ, Roche AM, Weiser JN. Influenza promotes pneumococcal growth during co-infection by providing host sialylated substrates as a nutrient source. *Cell Host Microbe*. 2014;16(1):55-67. doi:10.1016/j.chom.2014.06.005

76. Mina MJ, Klugman KP, McCullers J a. Live attenuated influenza vaccine, but not pneumococcal conjugate vaccine, protects against increased density and duration of pneumococcal carriage after influenza infection in pneumococcal colonized mice. *J Infect Dis*. 2013;208(8):1281-1285. doi:10.1093/infdis/jit317

77. McCullers JA. The co-pathogenesis of influenza viruses with bacteria in the lung. *Nat Rev Microbiol*. 2014;12(4):252-262. doi:10.1038/nrmicro3231

78. Weiser JN, Roche AM, Hergott CB, et al. Macrophage Migration Inhibitory Factor Is Detrimental in Pneumococcal Pneumonia and a Target for Therapeutic Immunomodulation. *J Infect Dis*. 2015;212(10):1677-1682. doi:10.1093/infdis/jiv262

79. Jetté LP, Delage G, Ringuette L, et al. Surveillance of invasive

Streptococcus pneumoniae infection in the province of Quebec, Canada, from 1996 to 1998: Serotype distribution, antimicrobial susceptibility, and clinical characteristics. *J Clin Microbiol*. 2001;39(2):733-737. doi:10.1128/JCM.39.2.733-737.2001

80. Feikin DR, Schuchat A, Kolczak M, et al. Mortality from invasive pneumococcal pneumonia in the era of antibiotic resistance, 1995-1997. *Am J Public Health*. 2000;90(2):223-229. doi:10.2105/ajph.90.2.223
81. Robinson KA, Baughman W, Rothrock G, et al. Epidemiology of invasive Streptococcus pneumoniae infections in the United States, 1995-1998 opportunities for prevention in the conjugate vaccine era. *J Am Med Assoc*. 2001;285(13):1729-1735. doi:10.1001/jama.285.13.1729
82. Shimada J, Yamanaka N, Hotomi M, et al. Household transmission of Streptococcus pneumoniae among siblings with acute otitis media. *J Clin Microbiol*. 2002;40(5):1851-1853. doi:10.1128/jcm.40.5.1851-1853.2002
83. Feldman C, Mitchell TJ, Andrew PW, et al. The effect of Streptococcus pneumoniae pneumolysin on human respiratory epithelium in vitro. *Microb Pathog*. 1990;9(4):275-284. doi:10.1016/0882-4010(90)90016-J
84. Murphy BR, Clements ML. The systemic and mucosal immune response of humans to influenza A virus. *Curr Top Microbiol Immunol*. 1989;146:107-116. doi:10.1007/978-3-642-74529-4_12
85. Paterson GK, Mitchell TJ. Innate immunity and the pneumococcus. *Microbiology*. 2006;152(Pt 2):285-293. doi:10.1099/mic.0.28551-0
86. Whitsett JA. Surfactant proteins in innate host defense of the lung. *Biol Neonate*. 2005;88(3):175-180. doi:10.1159/000087580
87. Rijneveld AW, Florquin S, Branger J, Speelman P, Van Deventer SJ, van

der Poll T. TNF-alpha compensates for the impaired host defense of IL-1 type I receptor-deficient mice during pneumococcal pneumonia. *J Immunol*. 2001;167(9):5240-5246. doi:10.4049/jimmunol.167.9.5240

88. Lauw FN, Branger J, Florquin S, et al. IL-18 Improves the Early Antimicrobial Host Response to Pneumococcal Pneumonia. *J Immunol*. 2002;168(1):372-378. doi:10.4049/jimmunol.168.1.372

89. Ramos-Sevillano E, Ercoli G, Brown JS. Mechanisms of naturally acquired immunity to streptococcus pneumoniae. *Front Immunol*. 2019;10(MAR). doi:10.3389/fimmu.2019.00358

90. Henriques-Normark B, Tuomanen EI. The pneumococcus: Epidemiology, microbiology, and pathogenesis. *Cold Spring Harb Perspect Med*. 2013;3(7). doi:10.1101/cshperspect.a010215

91. Abbas AK, Lichtman AHH, Pillai S. *Cellular and Molecular Immunology, Eighth Edition*.; 2014. https://www-clinicalkey-com.ep.fjernadgang.kb.dk/#!/browse/book/3-s2.0-C20130013230. Accessed November 7, 2019.

92. Neill DR, Coward WR, Gritzfeld JF, et al. Density and duration of pneumococcal carriage is maintained by transforming growth factor β1 and T regulatory cells. *Am J Respir Crit Care Med*. 2014;189(10):1250-1259. doi:10.1164/rccm.201401-0128OC

93. Briles DE, Miyaji E, Fukuyama Y, Ferreira DM, Fujihashi K. Elicitation of mucosal immunity by proteins of Streptococcus pneumoniae. *Adv Otorhinolaryngol*. 2011;72:25-27. doi:10.1159/000324589

94. Zhang Z, Clarke TB, Weiser JN. Cellular effectors mediating Th17-dependent clearance of pneumococcal colonization in mice. *J Clin Invest*.

2009;119(7):1899-1909. doi:10.1172/JCI36731

95. Khan MN, Pichichero ME. The host immune dynamics of pneumococcal colonization: Implications for novel vaccine development. *Hum Vaccin Immunother*. 2015;10(March):3688-3699. doi:10.4161/21645515.2014.979631

96. Periselneris J, José RJ, Brown J. Targeting Inflammatory Responses to Streptococcus pneumoniae. *New Horizons Transl Med*. 2015;2(6-7):167-174. doi:10.1016/j.nhtm.2015.09.002

97. 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. doi:10.1073/pnas.0435928100

98. Echchannaoui H, Frei K, Schnell C, Leib SL, Zimmerli W, Landmann R. Toll-like receptor 2-deficient mice are highly susceptible to Streptococcus pneumoniae meningitis because of reduced bacterial clearing and enhanced inflammation. *J Infect Dis*. 2002;186(6):798-806. doi:10.1086/342845

99. Lee KS, Scanga CA, Bachelder EM, Chen Q, Snapper CM. TLR2 synergizes with both TLR4 and TLR9 for induction of the MyD88-dependent splenic cytokine and chemokine response to Streptococcus pneumoniae. *Cell Immunol*. 2007;245(2):103-110. doi:10.1016/j.cellimm.2007.04.003

100. Mogensen TH. 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. doi:10.1189/jlb.1105626

101. Paterson GK, Orihuela CJ. Pneumococci: Immunology of the innate host response. *Respirology*. 2010;15(7):1057-1063. doi:10.1111/j.1440-1843.2010.01814.x

102. Van Rossum AMC, Lysenko ES, Weiser JN. Host and bacterial factors contributing to the clearance of colonization by Streptococcus pneumoniae in a murine model. *Infect Immun*. 2005;73(11):7718-7726. doi:10.1128/IAI.73.11.7718-7726.2005

103. Schröder NWJ, 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. doi:10.1074/jbc.M212829200

104. 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. doi:10.1128/iai.72.2.788-794.2004

105. Tomlinson G, Chimalapati S, Pollard T, et al. TLR-Mediated Inflammatory Responses to Streptococcus pneumoniae Are Highly Dependent on Surface Expression of Bacterial Lipoproteins . *J Immunol*. 2014;193(7):3736-3745. doi:10.4049/jimmunol.1401413

106. Krishnan J, Selvarajoo K, Tsuchiya M, Lee G, Choi S. Toll-like receptor signal transduction. *Exp Mol Med*. 2007;39(4):421-438. doi:10.1038/emm.2007.47

107. Dockrell DH, Whyte MKB, Mitchell TJ. Pneumococcal pneumonia: Mechanisms of infection and resolution. *Chest*. 2012;142(2):482-491. doi:10.1378/chest.12-0210

108. Puchta A, Naidoo A, Verschoor CP, et al. TNF Drives Monocyte Dysfunction with Age and Results in Impaired Anti-pneumococcal Immunity. *PLoS Pathog*. 2016;12(1). doi:10.1371/journal.ppat.1005368

109. Colino J, Shen Y, Snapper CM. Dendritic cells pulsed with intact Streptococcus pneumoniae elicit both protein- and polysaccharide-specific immunoglobulin isotype responses in vivo through distinct mechanisms. *J Exp Med*. 2002;195(1):1-13. doi:10.1084/jem.20011432

110. Leverkus M, Walczak H, McLellan A, et al. Maturation of dendritic cells leads to up-regulation of cellular FLICE-inhibitory protein and concomitant down-regulation of death ligand-mediated apoptosis. *Blood*. 2000;96(7):2628-2631. http://www.ncbi.nlm.nih.gov/pubmed/11001921. Accessed November 7, 2019.

111. Lundqvist A, Nagata T, Kiessling R, Pisa P. Mature dendritic cells are protected from Fas/CD95-mediated apoptosis by upregulation of Bcl-X(L). *Cancer Immunol Immunother*. 2002;51(3):139-144. doi:10.1007/s00262-002-0265-7

112. Li Q, Li YX, Stahl GL, Thurman JM, He Y, Tong HH. Essential role of factor B of the alternative complement pathway in complement activation and opsonophagocytosis during acute pneumococcal otitis media in mice. *Infect Immun*. 2011;79(7):2578-2585. doi:10.1128/IAI.00168-11

113. Melin M, Trzciński K, Antonio M, et al. Serotype-related variation in susceptibility to complement deposition and opsonophagocytosis among clinical isolates of Streptococcus pneumoniae. *Infect Immun*. 2010;78(12):5252-5261. doi:10.1128/IAI.00739-10

114. McCool TL, Cate TR, Moy G, Weiser JN. The Immune Response to

Pneumococcal Proteins during Experimental Human Carriage. *J Exp Med* ©. 2002;195(3):359–365.

115. Cohen JM, Khandavilli S, Camberlein E, Hyams C, Baxendale HE, Brown JS. Protective contributions against invasive Streptococcus pneumoniae pneumonia of antibody and Th17-cell responses to nasopharyngeal colonisation. *PLoS One*. 2011;6(10):e25558. doi:10.1371/journal.pone.0025558
116. Rapola S, Jäntti V, Haikala R, et al. Natural Development of Antibodies to Pneumococcal Surface Protein A, Pneumococcal Surface Adhesin A, and Pneumolysin in Relation to Pneumococcal Carriage and Acute Otitis Media. *J Infect Dis*. 2000;182(4):1146-1152. doi:10.1086/315822
117. Mitsi E, Roche AM, Reiné J, et al. Agglutination by anti-capsular polysaccharide antibody is associated with protection against experimental human pneumococcal carriage. *Mucosal Immunol*. 2017;10(2):385-394. doi:10.1038/mi.2016.71
118. Roche AM, Richard AL, Rahkola JT, Janoff EN, Weiser JN. Antibody blocks acquisition of bacterial colonization through agglutination. *Mucosal Immunol*. 2015;8(1):176-185. doi:10.1038/mi.2014.55
119. Weiser JN, Bae D, Fasching C, Scamurra RW, Ratner AJ, Janoff EN. Antibody-enhanced pneumococcal adherence requires IgA1 protease. *Proc Natl Acad Sci U S A*. 2003;100(7):4215-4220. doi:10.1073/pnas.0637469100
120. Janoff EN, Rubins JB, Fasching C, et al. Pneumococcal IgA1 protease subverts specific protection by human IgA1. *Mucosal Immunol*. 2014;7(2):249-256. doi:10.1038/mi.2013.41

121. Khan AQ, Shen Y, Wu Z-Q, Wynn TA, Snapper CM. Endogenous pro- and anti-inflammatory cytokines differentially regulate an in vivo humoral response to Streptococcus pneumoniae. *Infect Immun*. 2002;70(2):749-761. doi:10.1128/iai.70.2.749-761.2002

122. Marriott HM, Gascoyne KA, Gowda R, et al. Interleukin-1β 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. doi:10.1128/IAI.05697-11

123. Matthew Marks, Tamika Burns, Maria Abadi, et al. Influence of Neutropenia on the Course of Serotype 8 Pneumococcal Pneumonia in Mice. *Infect Immun*. 2007;75(4):1586–1597. https://iai.asm.org/content/iai/75/4/1586.full.pdf. Accessed October 16, 2019.

124. McCullers J a. Insights into the interaction between influenza virus and pneumococcus. *Clin Microbiol Rev*. 2006;19(3):571-582. doi:10.1128/CMR.00058-05

125. Seki M, Yanagihara K, Higashiyama Y, et al. Immunokinetics in severe pneumonia due to influenza virus and bacteria coinfection in mice. *Eur Respir J*. 2004;24(1):143-149. http://www.ncbi.nlm.nih.gov/pubmed/15293617. Accessed February 26, 2015.

126. Smith MW, Schmidt JE, Rehg JE, Orihuela CJ, McCullers JA. Induction of pro- and anti-inflammatory molecules in a mouse model of pneumococcal pneumonia after influenza. *Comp Med*. 2007;57(1):82-89.

http://www.ncbi.nlm.nih.gov/pubmed/17348295. Accessed October 16, 2019.

127. Van Der Poll Florquin T, Goldman M, Jansen HM, et al. IL-10 is an important mediator of the enhanced susceptibility to pneumococcal pneumonia after influenza infection. *J Immunol*. 2004;172(12):7603-7609. doi:10.4049/jimmunol.172.12.7603

128. van der Poll T, Marchant A, Keogh C V., Goldman M, Lowry SF. Interieukin-10 Impairs Host Defense in Murine Pneumococcal Pneumonia. *J Infect Dis*. 1996;174(5):994-1000. doi:10.1093/infdis/174.5.994

129. Jeong D-G, Jeong E-S, Seo J-H, Heo S-H, Choi Y-K. Difference in Resistance to Streptococcus pneumoniae Infection in Mice. *Lab Anim Res*. 2011;27(2):91-98. doi:10.5625/lar.2011.27.2.91

130. Mizrachi-Nebenzahl Y, Lifshitz S, Teitelbaum R, et al. Differential activation of the immune system by virulent Streptococcus pneumoniae strains determines recovery or death of the host. *Clin Exp Immunol*. 2003;134(1):23-31. doi:10.1046/j.1365-2249.2003.02261.x

131. Lundgren A, Bhuiyan TR, Novak D, et al. Characterization of Th17 responses to Streptococcus pneumoniae in humans: comparisons between adults and children in a developed and a developing country. *Vaccine*. 2012;30(26):3897-3907. doi:10.1016/j.vaccine.2012.03.082

132. Neill DR, Fernandes VE, Wisby L, et al. T regulatory cells control susceptibility to invasive pneumococcal pneumonia in mice. *PLoS Pathog*. 2012;8(4):e1002660. doi:10.1371/journal.ppat.1002660

133. Garvy BA, Harmsen AG. The importance of neutrophils in resistance to pneumococcal pneumonia in adult and neonatal mice. *Inflammation*.

1996;20(5):499-512. doi:10.1007/BF01487042

134. U. Boehm, T. Klamp, M. Groot A, Howard JC, EF W. Cellular responses to Interferon-gamma. *Annu Rev Immunol*. 1997;15(1):749-795. doi:doi:10.1146/annurev.immunol.15.1.749

135. Thorburn AN, Brown AC, Nair PM, et al. Pneumococcal components induce regulatory T cells that attenuate the development of allergic airways disease by deviating and suppressing the immune response to allergen. *J Immunol*. 2013;191(8):4112-4120. doi:10.4049/jimmunol.1201232

136. Wang R, Jaw JJ, Stutzman NC, Zou Z, Sun PD. Natural killer cell-produced IFN-γ and TNF-α induce target cell cytolysis through up-regulation of ICAM-1. *J Leukoc Biol*. 2012;91(2):299-309. doi:10.1189/jlb.0611308

137. Naganuma H, Kiessling R, Patarroyo M, Hansson M, Handgretinger R, Grönberg A. Increased susceptibility of ifn-γ-treated neuroblastoma cells to lysis by lymphokine-activated killer cells: Participation of ICAM-1 induction on target cells. *Int J Cancer*. 1991;47(4):527-532. doi:10.1002/ijc.2910470410

138. Corinti S, Albanesi C, la Sala A, Pastore S, Girolomoni G. Regulatory activity of autocrine IL-10 on dendritic cell functions. *J Immunol*. 2001;166(7):4312-4318. doi:10.4049/jimmunol.166.7.4312

139. Igietseme JU, Ananaba GA, Bolier J, et al. Suppression of endogenous IL-10 gene expression in dendritic cells enhances antigen presentation for specific Th1 induction: potential for cellular vaccine development. *J Immunol*. 2000;164(8):4212-4219. doi:10.4049/jimmunol.164.8.4212

140. Grayson KM, Blevins LK, Oliver MB, Ornelles DA, Swords WE, Alexander-Miller MA. Activation-dependent modulation of Streptococcus pneumoniae-

mediated death in human lymphocytes. *Pathog Dis*. 2017;75(2). doi:10.1093/femspd/ftx008

141. Cole TS, Cant AJ. Clinical experience in T cell deficient patients. *Allergy, Asthma Clin Immunol*. 2010;6(1):9. doi:10.1186/1710-1492-6-9
142. Ochs HD, Thrasher AJ. The Wiskott-Aldrich syndrome. doi:10.1016/j.jaci.2006.02.005
143. Torgerson TR, Ochs HD. Regulatory T cells in primary immunodeficiency diseases. *Curr Opin Allergy Clin Immunol*. 2007;7(6):515-521. doi:10.1097/ACI.0b013e3282f1a27a
144. Malley R, Trzcinski K, Srivastava A, Thompson CM, Anderson PW, Lipsitch M. CD4+ T cells mediate antibody-independent acquired immunity to pneumococcal colonization. *Proc Natl Acad Sci U S A*. 2005;102(13):4848-4853. doi:10.1073/pnas.0501254102
145. Basset A, Thompson CM, Hollingshead SK, et al. Antibody-independent, CD4+ T-cell-dependent protection against pneumococcal colonization elicited by intranasal immunization with purified pneumococcal proteins. *Infect Immun*. 2007;75(11):5460-5464. doi:10.1128/IAI.00773-07
146. Lamb RA, Krug RM. Orthomyxoviridae: The viruses and their replication. In: Lippincott-Raven Press; 2001:1216-1253.
147. Vincent AL, Ma W, Lager KM, Janke BH, Richt JA. Swine influenza viruses a North American perspective. In: KNIPE D, HOWLEY P, GRIFFIN D, LAMB R, MARTIN M, ROIZMAN B, eds. *Fields Virology*. 4th editio. Philadelphia: Lippincott Williams & Wilkins; 2008:127-154. doi:10.1016/S0065-3527(08)00403-X
148. Nayak DP, Hui EKW, Barman S. Assembly and budding of influenza virus.

Virus Res. 2004;106(2 SPEC.ISS.):147-165. doi:10.1016/j.virusres.2004.08.012

149. Skehel JJ, Wiley DC. Receptor Binding and Membrane Fusion in Virus Entry: The Influenza Hemagglutinin. *Annu Rev Biochem*. 2000;69(1):531-569. doi:10.1146/annurev.biochem.69.1.531
150. Rumschlag-Booms E, Rong L. Influenza A virus entry: Implications in virulence and future therapeutics. *Adv Virol*. 2013;2013. doi:10.1155/2013/121924
151. Drake JW. Rates of spontaneous mutation among RNA viruses. *Proc Natl Acad Sci U S A*. 1993;90(9):4171-4175. doi:10.1073/pnas.90.9.4171
152. Hampson AW. Influenza virus antigens and “antigenic drift.” *Perspect Med Virol*. 2002;7:49-85. doi:10.1016/s0168-7069(02)07004-0
153. Cox RJ, Brokstad KA, Ogra P. Influenza Virus: Immunity and Vaccination Strategies. Comparison of the Immune Response to Inactivated and Live, Attenuated Influenza Vaccines. *Scand J Immunol*. 2004;59(1):1-15. doi:10.1111/j.0300-9475.2004.01382.x
154. Centers for Disease Control and Prevention (CDC). How the Flu Virus Can Change: “Drift” and “Shift” | CDC. https://www.cdc.gov/flu/about/viruses/change.htm. Published 2019. Accessed November 11, 2019.
155. Reeth K Van. Avian and swine influenza viruses : our current understanding of the zoonotic risk Kristen Van Reeth To cite this version : Review article Avian and swine influenza viruses : our current understanding of the zoonotic risk. 2007. doi:10.1051/vetres
156. Salazar M, López-Ortega O, León-Avila G, Ramírez-Gónzalez J, Castro-

Mussot M. The origin of the genetic variability of influenza viruses. *Gac Med Mex*. 2010;146(3):199-206. https://www.ncbi.nlm.nih.gov/pubmed/20957816. Accessed November 6, 2019.

157. Cox NJ, Subbarao K. Influenza. *Lancet*. 1999;354(9186):1277-1282. doi:10.1016/S0140-6736(99)01241-6
158. Barik S. New treatments for influenza. *BMC Med*. 2012;10. doi:10.1186/1741-7015-10-104
159. Tong S, Li Y, Rivailler P, et al. A distinct lineage of influenza A virus from bats. *Proc Natl Acad Sci U S A*. 2012;109(11):4269-4274. doi:10.1073/pnas.1116200109
160. George F. Gripe. In: Almeida F, R M, eds. *História de Doenças Infecciosas*. ; 2014:87-126.
161. Shaman J, Kohn M. Absolute humidity modulates influenza survival, transmission, and seasonality. *Proc Natl Acad Sci U S A*. 2009;106(9):3243-3248. doi:10.1073/pnas.0806852106
162. Cox NJ, Fukuda K. INFLUENZA. *Infect Dis Clin North Am*. 1998;12(1):27-38. doi:10.1016/S0891-5520(05)70406-2
163. Taubenberger JK, Morens DM. The pathology of influenza virus infections. *Annu Rev Pathol*. 2008;3:499-522. doi:10.1146/annurev.pathmechdis.3.121806.154316
164. Principi N, Esposito S, Gasparini R, Marchisio P, Crovari P, Principi N. Burden of influenza in healthy children and their households. doi:10.1136/adc.2003.045401
165. Principi N, Esposito S, Gasparini R, Marchisio P, Crovari P. Burden of

influenza in healthy children and their households (Retraction in: Archives of Disease in Childhood (2006) 91:9 (797)). *Arch Dis Child*. 2004;89(11):1002-1007. doi:10.1136/adc.2003.045401

166. Iskander M, Booy R, Lambert S. The burden of influenza in children. *Curr Opin Infect Dis*. 2007;20(3):259-263. doi:10.1097/QCO.0b013e3280ad4687

167. Esposito S, Marchisio P, Principi N. The global state of influenza in children. *Pediatr Infect Dis J*. 2008;27(11 Suppl):S149-53. doi:10.1097/INF.0b013e31818a542b

168. Poehling KA, Edwards KM, Weinberg GA, et al. The underrecognized burden of influenza in young children. *N Engl J Med*. 2006;355(1):31-40. doi:10.1056/NEJMoa054869

169. Poehling KA, Edwards KM, Griffin MR, et al. The burden of influenza in young children, 2004-2009. *Pediatrics*. 2013;131(2):207-216. doi:10.1542/peds.2012-1255

170. Neuzil KM, Mellen BG, Wright PF, Mitchel EF, Griffin MR. The effect of influenza on hospitalizations, outpatient visits, and courses of antibiotics in children. *N Engl J Med*. 2000;342(4):225-231. doi:10.1056/NEJM200001273420401

171. Salleras L, Navas E, Torner N, et al. Economic benefits of inactivated influenza vaccines in the prevention of seasonal influenza in children. In: *Human Vaccines and Immunotherapeutics*. Vol 9. Landes Bioscience; 2013:707-711. doi:10.4161/hv.23269

172. Toplak N, Avčin T. Influenza and autoimmunity. In: *Annals of the New York Academy of Sciences*. Vol 1173. Blackwell Publishing Inc.; 2009:619-626.

doi:10.1111/j.1749-6632.2009.04759.x

173. Neuzil KM, Wright PF, Mitchel EF, Griffin MR. The burden of influenza illness in children with asthma and other chronic medical conditions. *J Pediatr*. 2000;137(6):856-864. doi:10.1067/mpd.2000.110445

174. Lee J, Boutz DR, Chromikova V, et al. Molecular-level analysis of the serum antibody repertoire in young adults before and after seasonal influenza vaccination. *Nat Med*. 2016;22(12):1456-1468. https://www.nature.com/nm/journal/v22/n12/pdf/nm.4224.pdf. Accessed May 25, 2017.

175. Rizzo C, Rota MC, Bella A, et al. Cross-reactive antibody responses to the 2009 A/H1N1v influenza virus in the Italian population in the pre-pandemic period. *Vaccine*. 2010;28(20):3558-3562. doi:10.1016/j.vaccine.2010.03.006

176. Hancock K, Veguilla V, Lu X, et al. Cross-reactive antibody responses to the 2009 pandemic H1N1 influenza virus. *N Engl J Med*. 2009;361(20):1945-1952. doi:10.1056/NEJMoa0906453

177. Morens DM, Taubenberger JK, Fauci AS. Predominant role of bacterial pneumonia as a cause of death in pandemic influenza: implications for pandemic influenza preparedness. *J Infect Dis*. 2008;198(7):962-970. doi:10.1086/591708

178. Morris DE, Cleary DW, Clarke SC. Secondary bacterial infections associated with influenza pandemics. *Front Microbiol*. 2017;8(JUN). doi:10.3389/fmicb.2017.01041

179. World Health Organization. Influenza (Seasonal). https://www.who.int/news-room/fact-sheets/detail/influenza-(seasonal).

Published 2018. Accessed November 11, 2019.

180. Richard M, Fouchier RAM. Influenza A virus transmission via respiratory aerosols or droplets as it relates to pandemic potential. *FEMS Microbiol Rev*. 2015;40(1):68-85. doi:10.1093/femsre/fuv039

181. Belshe RB, Gruber WC, Mendelman PM, et al. Correlates of immune protection induced by live, attenuated, cold-adapted, trivalent, intranasal influenza virus vaccine. *J Infect Dis*. 2000;181(3):1133-1137. doi:10.1086/315323

182. Webster RG, Monto AS, Braciale TJ, Lamb RA. *Textbook of Influenza*.; 2013.

183. Lui KJ, Kendal AP. Impact of influenza epidemics on mortality in the United States from October 1972 to May 1985. *Am J Public Health*. 1987;77(6):712-716. doi:10.2105/AJPH.77.6.712

184. Simonsen L, Clarke MJ, Williamson GD, Stroup DF, Arden NH, Schonberger LB. The impact of influenza epidemics on mortality: Introducing a severity index. *Am J Public Health*. 1997;87(12):1944-1950. doi:10.2105/AJPH.87.12.1944

185. Caini S, Kroneman M, Wiegers T, El Guerche-Séblain C, Paget J. Clinical characteristics and severity of influenza infections by virus type, subtype, and lineage: A systematic literature review. *Influenza Other Respi Viruses*. 2018;12(6):780-792. doi:10.1111/irv.12575

186. 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. doi:10.1172/JCI57762

187. Korenkov D, Isakova-Sivak I, Rudenko L. Basics of CD8 T-cell immune responses after influenza infection and vaccination with inactivated or live attenuated influenza vaccine. *Expert Rev Vaccines*. 2018;17(11):977-987. doi:10.1080/14760584.2018.1541407

188. Blevins LK, Wren JT, Holbrook BC, et al. Coinfection with Streptococcus pneumoniae Negatively Modulates the Size and Composition of the Ongoing Influenza-Specific CD8+ T Cell Response. *J Immunol*. 2014;193(10):5076-5087. doi:10.4049/jimmunol.1400529

189. Brandtzaeg P. Potential of nasopharynx-associated lymphoid tissue for vaccine responses in the airways. *Am J Respir Crit Care Med*. 2011;183(12):1595-1604. doi:10.1164/rccm.201011-1783OC

190. Chen X, Liu S, Goraya MU, Maarouf M, Huang S, Chen JL. Host immune response to influenza A virus infection. *Front Immunol*. 2018;9(MAR). doi:10.3389/fimmu.2018.00320

191. Ghoneim HE, Thomas PG, McCullers J a. Depletion of alveolar macrophages during influenza infection facilitates bacterial superinfections. *J Immunol*. 2013;191:1250-1259. doi:10.4049/jimmunol.1300014

192. McNamee L a., Harmsen AG. Both influenza-induced neutrophil dysfunction and neutrophil-independent mechanisms contribute to increased susceptibility to a secondary Streptococcus pneumoniae infection. *Infect Immun*. 2006;74(12):6707-6721. doi:10.1128/IAI.00789-06

193. Pittet LA, Hall-Stoodley L, Rutkowski MR, Harmsen AG. Influenza virus infection decreases tracheal mucociliary velocity and clearance of Streptococcus pneumoniae. *Am J Respir Cell Mol Biol*. 2010;42(4):450-

460. doi:10.1165/rcmb.2007-0417OC

194. Lee LN, Baban D, Ronan EO, Ragoussis J, Beverley PCL, Tchilian EZ. Chemokine gene expression in lung CD8 T cells correlates with protective immunity in mice immunized intra-nasally with Adenovirus-85A. *BMC Med Genomics*. 2010;3:46. doi:10.1186/1755-8794-3-46

195. Harty JT, Tvinnereim AR, White DW. CD8+ T Cell Effector Mechanisms in Resistance to Infection. *Annu Rev Immunol*. 2000;18(1):275-308. doi:10.1146/annurev.immunol.18.1.275

196. Vareille M, Kieninger E, Edwards MR, Regamey N. The airway epithelium: soldier in the fight against respiratory viruses. *Clin Microbiol Rev*. 2011;24(1):210-229. doi:10.1128/CMR.00014-10

197. Topham DJ, Tripp RA, Doherty PC. CD8+ T cells clear influenza virus by perforin or Fas-dependent processes. *J Immunol*. 1997;159(11):5197-5200. http://www.ncbi.nlm.nih.gov/pubmed/9548456. Accessed October 16, 2019.

198. Doherty PC, Topham DJ, Tripp RA, Cardin RD, Brooks JW, Stevenson PG. Effector CD4+ and CD8+ T-cell mechanisms in the control of respiratory virus infections. *Immunol Rev*. 1997;159(1):105-117. doi:10.1111/j.1600-065X.1997.tb01010.x

199. Sridhar S, Begom S, Bermingham A, et al. Cellular immune correlates of protection against symptomatic pandemic influenza. *Nat Med*. 2013;19(10):1305-1312. doi:10.1038/nm.3350

200. Welliver TP, Garofalo RP, Hosakote Y, et al. Severe Human Lower Respiratory Tract Illness Caused by Respiratory Syncytial Virus and Influenza Virus Is Characterized by the Absence of Pulmonary Cytotoxic

Lymphocyte Responses. *J Infect Dis*. 2007;195(8):1126-1136. doi:10.1086/512615

201. Gerdil C. The annual production cycle for influenza vaccine. *Vaccine*. 2003;21(16):1776-1779. doi:10.1016/S0264-410X(03)00071-9
202. Pickering LK, Baker CJ, Freed GL, et al. Immunization programs for infants, children, adolescents, and adults: clinical practice guidelines by the Infectious Diseases Society of America.[Erratum appears in Clin Infect Dis. 2009 Nov 1;49(9):1465]. *Clin Infect Dis*. 2009;49(6):817-840. http://acs.hcn.com.au?acc=36422&url=http://ovidsp.ovid.com/ovidweb.cgi?T=JS&CSC=Y&NEWS=N&PAGE=fulltext&D=med5&AN=19659433. Accessed November 6, 2019.
203. Shane AL. Red Book: 2006 Report of the Committee on Infectious Diseases, 27th Edition. *Emerg Infect Dis*. 2006;12(12):2003-2004. doi:10.3201/eid1212.061045
204. Prosser LA, Bridges CB, Uyeki TM, et al. Health benefits, risks, and cost-effectiveness of influenza vaccination of children. *Emerg Infect Dis*. 2006;12(10):1548-1558. doi:10.3201/eid1210.051015
205. Weycker D, Edelsberg J, Elizabeth Halloran M, et al. Population-wide benefits of routine vaccination of children against influenza. *Vaccine*. 2005;23(10):1284-1293. doi:10.1016/j.vaccine.2004.08.044
206. Simonsen L, Reichert TA, Viboud C, Blackwelder WC, Taylor RJ, Miller MA. Impact of influenza vaccination on seasonal mortality in the US elderly population. *Arch Intern Med*. 2005;165(3):265-272. doi:10.1001/archinte.165.3.265
207. Belshe RB, Gruber WC. Prevention of otitis media in children with live

attenuated influenza vaccine given intranasally. *Pediatr Infect Dis J*. 2000;19(5 Suppl):S66-71. doi:10.1097/00006454-200005001-00010

208. Vesikari T, Fleming DM, Aristegui JF, et al. Safety, efficacy, and effectiveness of cold-adapted influenza vaccine-trivalent against community-acquired, culture-confirmed influenza in young children attending day care. *Pediatrics*. 2006;118(6):2298-2312. doi:10.1542/peds.2006-0725
209. Block SL, Heikkinen T, Toback SL, Zheng W, Ambrose CS. The efficacy of live attenuated influenza vaccine against influenza-associated acute otitis media in children. *Pediatr Infect Dis J*. 2011;30(3):203-207. doi:10.1097/INF.0b013e3181faac7c
210. Rhorer J, Ambrose CS, Dickinson S, et al. Efficacy of live attenuated influenza vaccine in children: A meta-analysis of nine randomized clinical trials. *Vaccine*. 2009;27(7):1101-1110. doi:10.1016/j.vaccine.2008.11.093
211. Zangwill KM, Belshe RB. Safety and efficacy of trivalent inactivated influenza vaccine in young children: a summary for the new era of routine vaccination. *Pediatr Infect Dis J*. 2004;23(3):189-197. doi:10.1097/01.inf.0000116292.46143.d6
212. Tisa V, Barberis I, Faccio V, et al. Quadrivalent influenza vaccine: a new opportunity to reduce the influenza burden. *J Prev Med Hyg*. 2016;57(1):E28. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4910440/. Accessed October 29, 2018.
213. Carter NJ, Curran MP. Live attenuated influenza vaccine (FluMist®; Fluenz™): a review of its use in the prevention of seasonal influenza in children and adults. *Drugs*. 2011;71(12):1591-1622.

doi:10.2165/11206860-000000000-00000

214. Centers for Disease Control and Prevention (CDC). How Influenza (Flu) Vaccines Are Made. https://www.cdc.gov/flu/prevent/how-fluvaccine-made.htm. Published 2018. Accessed November 11, 2019.

215. Sekiya T, Mifsud EJ, Ohno M, et al. Inactivated whole virus particle vaccine with potent immunogenicity and limited IL-6 induction is ideal for influenza. *Vaccine*. 2019;37(15):2158-2166. doi:10.1016/j.vaccine.2019.02.057

216. Coffey F, Alabyev B, Manser T. Initial Clonal Expansion of Germinal Center B Cells Takes Place at the Perimeter of Follicles. *Immunity*. 2009;30(4):599-609. doi:10.1016/j.immuni.2009.01.011

217. Nakaya HI, Wrammert J, Lee EK, et al. Systems biology of vaccination for seasonal influenza in humans. *Nat Immunol*. 2011;12(8):786-795. doi:10.1038/ni.2067

218. Calame KL. Plasma cells: finding new light at the end of B cell development. *Nat Immunol*. 2001;2(12):1103-1108. doi:10.1038/ni1201-1103

219. Heinonen S, Silvennoinen H, Lehtinen P, Vainionpää R, Ziegler T, Heikkinen T. Effectiveness of inactivated influenza vaccine in children aged 9 months to 3 years: An observational cohort study. *Lancet Infect Dis*. 2011;11(1):23-29. doi:10.1016/S1473-3099(10)70255-3

220. Belshe RB, Mendelman PM, Treanor J, et al. The efficacy of live attenuated, cold-adapted, trivalent, intranasal influenzavirus vaccine in children. *N Engl J Med*. 1998;338(20):1405-1412. doi:10.1056/NEJM199805143382002

221. Lee MS, Mahmood K, Adhikary L, et al. Measuring antibody responses to

a live attenuated influenza vaccine in children. *Pediatr Infect Dis J*. 2004;23(9):852-856. doi:10.1097/01.inf.0000137566.87691.3b

222. Beyer WEP, Palache AM, de Jong JC, Osterhaus ADME. Cold-adapted live influenza vaccine versus inactivated vaccine: systemic vaccine reactions, local and systemic antibody response, and vaccine efficacy. A meta-analysis. *Vaccine*. 2002;20(9-10):1340-1353. http://www.ncbi.nlm.nih.gov/pubmed/11818152. Accessed July 30, 2018.
223. Hambidge SJ, Glanz JM, France EK, et al. Safety of trivalent inactivated influenza vaccine in children 6 to 23 months old. *J Am Med Assoc*. 2006;296(16):1990-1997. doi:10.1001/jama.296.16.1990
224. Manz RA, Arce S, Cassese G, Hauser AE, Hiepe F, Radbruch A. Humoral immunity and long-lived plasma cells. *Curr Opin Immunol*. 2002;14(4):517-521. doi:10.1016/s0952-7915(02)00356-4
225. Palanichamy A, Barnard J, Zheng B, et al. Novel human transitional B cell populations revealed by B cell depletion therapy. *J Immunol*. 2009;182(10):5982-5993. doi:10.4049/jimmunol.0801859
226. El-Madhun AS, Cox RJ, Søreide A, Olofsson J, Haaheim LR. Systemic and Mucosal Immune Responses in Young Children and Adults after Parenteral Influenza Vaccination. *J Infect Dis*. 178:933-939. doi:10.2307/30117161
227. Sasaki S, He XS, Holmes TH, et al. Influence of prior influenza vaccination on antibody and B-cell responses. *PLoS One*. 2008;3(8). doi:10.1371/journal.pone.0002975
228. Wrammert J, Smith K, Miller J, et al. Rapid cloning of high-affinity human monoclonal antibodies against influenza virus. *Nature*. 2008;453(7195):667-671. doi:10.1038/nature06890

229. Kunkel EJ, Butcher EC. Plasma-cell homing. *Nat Rev Immunol*. 2003;3(10):822-829. doi:10.1038/nri1203

230. Zuckerman M, Cox R, Taylor J, Wood J, Haaheim L, Oxford J. Rapid immune response to influenza vaccination. *Lancet (London, England)*. 1993;342(8879):1113. doi:10.1016/0140-6736(93)92094-a

231. Brokstad KA, Cox RJ, Olofsson J, Jonsson R, Haaheim LR. Parenteral influenza vaccination induces a rapid systemic and local immune response. *J Infect Dis*. 1995;171(1):198-203. doi:10.1093/infdis/171.1.198

232. Brokstad KA, Cox RJ, Major D, Wood JM, Haaheim LR. Cross-reaction but no avidity change of the serum antibody response after influenza vaccination. *Vaccine*. 1995;13(16):1522-1528. doi:10.1016/0264-410x(95)00095-i

233. Nutt SL, Tarlinton DM. Germinal center B and follicular helper T cells: siblings, cousins or just good friends? *Nat Immunol*. 2011;12(6):472-477. doi:10.1038/ni.2019

234. Lanzavecchia A. Antigen-specific interaction between T and B cells. *Nature*. 1985;314(6011):537-539. doi:10.1038/314537a0

235. Allen CDC, Okada T, Cyster JG. Germinal-center organization and cellular dynamics. *Immunity*. 2007;27(2):190-202. doi:10.1016/j.immuni.2007.07.009

236. Abramson JS. Intranasal, cold-adapted, live, attenuated influenza vaccine. *Pediatr Infect Dis J*. 1999;18(12):1103-1104. doi:10.1097/00006454-199912000-00017

237. De Filette M, Fiers W, Martens W, et al. Improved design and intranasal delivery of an M2e-based human influenza A vaccine. *Vaccine*.

2006;24(44-46):6597-6601. doi:10.1016/j.vaccine.2006.05.082

238. Vajdy M, Baudner B, Del Giudice G, O'Hagan D. A vaccination strategy to enhance mucosal and systemic antibody and T cell responses against influenza. *Clin Immunol*. 2007;123(2):166-175. doi:10.1016/j.clim.2007.01.009

239. Neutra MR, Kozlowski PA. Mucosal vaccines: the promise and the challenge. *Nat Rev Immunol*. 2006;6(2):148-158. doi:10.1038/nri1777

240. Clements ML, Murphy BR. Development and persistence of local and systemic antibody responses in adults given live attenuated or inactivated influenza A virus vaccine. *J Clin Microbiol*. 1986;23(1):66-72. http://www.ncbi.nlm.nih.gov/pubmed/3700610. Accessed October 31, 2019.

241. Beyer WEP, Palache AM, De Jong JC, Osterhaus ADME. Cold-adapted live influenza vaccine versus inactivated vaccine: Systemic vaccine reactions, local and systemic antibody response, and vaccine efficacy: A meta-analysis. *Vaccine*. 2002;20(9-10):1340-1353. doi:10.1016/S0264-410X(01)00471-6

242. Ambrose CS, Wu X, Belshe RB. The efficacy of live attenuated and inactivated influenza vaccines in children as a function of time postvaccination. *Pediatr Infect Dis J*. 2010;29(9):806-811. doi:10.1097/INF.0b013e3181e2872f

243. Ashkenazi S, Vertruyen A, Arístegui J, et al. Superior relative efficacy of live attenuated influenza vaccine compared with inactivated influenza vaccine in young children with recurrent respiratory tract infections. *Pediatr Infect Dis J*. 2006;25(10):870-879.

doi:10.1097/01.inf.0000237829.66310.85

244. Fleming DM, Crovari P, Wahn U, et al. Comparison of the efficacy and safety of live attenuated cold-adapted influenza vaccine, trivalent, with trivalent inactivated influenza virus vaccine in children and adolescents with asthma. *Pediatr Infect Dis J*. 2006;25(10):860-869. doi:10.1097/01.inf.0000237797.14283.cf

245. Belshe RB, Edwards KM, Vesikari T, et al. Live attenuated versus inactivated influenza vaccine in infants and young children. *N Engl J Med*. 2007;356(7):685-696. doi:10.1056/NEJMoa065368

246. Bergen R, Black S, Shinefield H, et al. Safety of cold-adapted live attenuated influenza vaccine in a large cohort of children and adolescents. *Pediatr Res*. 2004;23(2):138-144. doi:10.1097/01.inf.0000109392.96411.4f

247. Piedra PA, Gaglani MJ, Riggs M, et al. Live attenuated influenza vaccine, trivalent, is safe in healthy children 18 months to 4 years, 5 to 9 years, and 10 to 18 years of age in a community-based, nonrandomized, open-label trial. *Pediatrics*. 2005;116(3). doi:10.1542/peds.2004-2258

248. Clements ML, Betts RF, Tierney EL, Murphy BR. Serum and nasal wash antibodies associated with resistance to experimental challenge with influenza A wild-type virus. *J Clin Microbiol*. 1986;24(1):157-160.

249. Mohn KGI, Smith I, Sjursen H, Cox RJ. Immune responses after live attenuated influenza vaccination. *Hum Vaccines Immunother*. 2018;14(3):571-578. doi:10.1080/21645515.2017.1377376

250. Veguilla V, Hancock K, Schiffer J, et al. Sensitivity and specificity of serologic assays for detection of human infection with 2009 pandemic

H1N1 virus in U.S. populations. *J Clin Microbiol*. 2011;49(6):2210-2215. doi:10.1128/JCM.00229-11

251. Rynda-Apple A, Robinson KM, Alcorn JF. Influenza and bacterial superinfection: Illuminating the immunologic mechanisms of disease. *Infect Immun*. 2015;83(10):3764-3770. doi:10.1128/IAI.00298-15
252. Palacios G, Hornig M, Cisterna D, et al. Streptococcus pneumoniae coinfection is correlated with the severity of H1N1 pandemic influenza. *PLoS One*. 2009;4(12):e8540. doi:10.1371/journal.pone.0008540
253. Gill JR, Sheng Z-M, Ely SF, et al. Pulmonary pathologic findings of fatal 2009 pandemic influenza A/H1N1 viral infections. *Arch Pathol Lab Med*. 2010;134(2):235-243. doi:10.1043/1543-2165-134.2.235
254. Centers for Disease Control and Prevention (CDC). Bacterial coinfections in lung tissue specimens from fatal cases of 2009 pandemic influenza A (H1N1) - United States, May-August 2009. *MMWR Morb Mortal Wkly Rep*. 2009;58(38):1071-1074. http://www.ncbi.nlm.nih.gov/pubmed/19798021. Accessed October 16, 2019.
255. Shieh W-J, Blau DM, Denison AM, et al. 2009 pandemic influenza A (H1N1): pathology and pathogenesis of 100 fatal cases in the United States. *Am J Pathol*. 2010;177(1):166-175. doi:10.2353/ajpath.2010.100115
256. Mina MJ, Klugman KP. The role of influenza in the severity and transmission of respiratory bacterial disease. *Lancet Respir Med*. 2014;2(9):750-763. doi:10.1016/S2213-2600(14)70131-6
257. Campigotto A, Mubareka S. Influenza-associated bacterial pneumonia; managing and controlling infection on two fronts. *Expert Rev Anti Infect*

Ther. 2015;13(1):55-68. doi:10.1586/14787210.2015.981156

258. Martin-Loeches I, Van Someren Gréve F, Schultz MJ. Bacterial pneumonia as an influenza complication. *Curr Opin Infect Dis*. 2017;30(2):201-207. doi:10.1097/QCO.0000000000000347

259. Hanada S, Pirzadeh M, Carver KY, Deng JC. Respiratory viral infection-induced microbiome alterations and secondary bacterial pneumonia. *Front Immunol*. 2018;9(NOV). doi:10.3389/fimmu.2018.02640

260. Short KR, Diavatopoulos DA, Thornton R, et al. Influenza virus induces bacterial and nonbacterial otitis media. *J Infect Dis*. 2011;204(12):1857-1865. doi:10.1093/infdis/jir618

261. Vu HTT, Yoshida LM, Suzuki M, et al. Association between nasopharyngeal load of Streptococcus pneumoniae, viral coinfection, and radiologically confirmed pneumonia in Vietnamese children. *Pediatr Infect Dis J*. 2011;30(1):11-18. doi:10.1097/INF.0b013e3181f111a2

262. Li W, Moltedo B, Moran TM. Type I Interferon Induction during Influenza Virus Infection Increases Susceptibility to Secondary Streptococcus pneumoniae Infection by Negative Regulation of T Cells. *J Virol*. 2012;86(22):12304-12312. doi:10.1128/JVI.01269-12

263. Van Der Sluijs KF, Van Elden LJR, Nijhuis M, et al. Involvement of the platelet-activating factor receptor in host defense against Streptococcus pneumoniae during postinfluenza pneumonia. *Am J Physiol - Lung Cell Mol Physiol*. 2006;290(1). doi:10.1152/ajplung.00050.2005

264. McCullers JAA, Rehg JEE. Lethal synergism between influenza virus and Streptococcus pneumoniae: characterization of a mouse model and the role of platelet-activating factor receptor. *J Infect Dis*. 2002;186(3):341-350.

doi:10.1086/341462

265. Faure M, Rabourdin-Combe C. Innate immunity modulation in virus entry. *Curr Opin Virol*. 2011;1(1):6-12. doi:10.1016/j.coviro.2011.05.013

266. Wolter N, Tempia S, Cohen C, et al. High Nasopharyngeal Pneumococcal Density, Increased by Viral Coinfection, Is Associated With Invasive Pneumococcal Pneumonia. *J Infect Dis*. 2014;210:1-9. doi:10.1093/infdis/jiu326

267. Hussell T, Goulding J. Structured regulation of inflammation during respiratory viral infection. *Lancet Infect Dis*. 2010;10(5):360-366. doi:10.1016/S1473

268. Kudva A, Scheller E V., Robinson KM, et al. Influenza A Inhibits Th17-Mediated Host Defense against Bacterial Pneumonia in Mice. *J Immunol*. 2011;186(3):1666-1674. doi:10.1016/j.biotechadv.2011.08.021.Secreted

269. Metzger DW, Sun K. Immune dysfunction and bacterial coinfections following influenza. *J Immunol*. 2013;191:2047-2052. doi:10.4049/jimmunol.1301152

270. Wu Y, Tu W, Lam K, et al. Lethal co-infection of influenza virus and Streptococcus pneumoniae lowers antibody response to influenza virus in lung, and reduces germinal center B cells, T follicular helper cells and plasma cells in mediastinal lymph node. *J Virol*. 2015;89(4):2013-2023. doi:10.1128/JVI.02455-14

271. Richard AL, Siegel SJ, Erikson J, Weiser JN. TLR2 Signaling Decreases Transmission of Streptococcus pneumoniae by Limiting Bacterial Shedding in an Infant Mouse Influenza A Co-infection Model. *PLOS Pathog*. 2014;10(8):1-9. doi:10.1371/journal.ppat.1004339

272. Wolf AI, Strauman MC, Mozdzanowska K, et al. Pneumolysin expression by streptococcus pneumoniae protects colonized mice from influenza virus-induced disease. *Virology*. 2014;462-463:254-265. doi:10.1016/j.virol.2014.06.019

273. McCullers JA, McAuley JL, Browall S, Iverson AR, Boyd KL, Henriques Normark B. Influenza enhances susceptibility to natural acquisition of and disease due to Streptococcus pneumoniae in ferrets. *J Infect Dis*. 2010;202(8):1287-1295. doi:10.1086/656333

274. Mina MJ, Klugman KP, Rosch JW, McCullers JA. Live-attenuated influenza virus increases pneumococcal translocation and persistence within the middle ear. *J Infect Dis*. 2015;212(2):1-31. doi:10.1093/infdis/jiu804

275. Smith AM, Adler FR, Ribeiro RM, et al. Kinetics of Coinfection with Influenza A Virus and Streptococcus pneumoniae. *PLoS Pathog*. 2013;9(3). doi:10.1371/journal.ppat.1003238

276. Garcia CC, Russo RC, Guabiraba R, et al. Platelet-activating factor receptor plays a role in lung injury and death caused by Influenza A in mice. *PLoS Pathog*. 2010;6(11):e1001171. doi:10.1371/journal.ppat.1001171

277. Wolf AI, Strauman MC, Mozdzanowska K, et al. Co-infection with Streptococcus pneumoniae modulates the B cell response to influenza virus. *J Virol*. 2014;88(20):11995-12005. doi:10.1128/JVI.01833-14

278. Sakaguchi S, Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T. Regulatory T cells: how do they suppress immune responses? *Int Immunol*. 2009;21(10):1105-1111. doi:10.1093/intimm/dxp095

279. Low WT, Reid MM. Effect of virus infections on polymorph function in children. *Br Med J*. 1976;1(6025):1570. doi:10.1136/bmj.1.6025.1570

280. Abramson JS, Scott Giebink G, Mills EL, Quie PG. Polymorphonuclear leukocyte dysfunction during influenza virus infection in chinchillas. *J Infect Dis*. 1981;143(6):836-845. doi:10.1093/infdis/143.6.836

281. Short KR, Reading PC, Wang N, Diavatopoulos DA, Wijburg OL. Increased Nasopharyngeal Bacterial Titers and Local Inflammation Facilitate Transmission of Streptococcus pneumoniae. *MBio*. 2012;3(5):1-7. doi:10.1128/mBio.00255-12.Editor

282. Mina MJ, McCullers J a., Klugman KP. Live attenuated influenza vaccine enhances colonization of Streptococcus pneumoniae and Staphylococcus aureus in mice. *MBio*. 2014;5(1):1-10. doi:10.1128/mBio.01040-13

283. Thors V, Morales-Aza B, Oliver E, et al. Live attenuated intranasal vaccine does not increase rates of nasal pneumococcal colonisation in healthy children but may contribute to increased density. In: *Poster P-097. 9th Int. Symp. Pneumococci Pneumo- Coccal Dis., Hyderabad, India*. ; 2014.

284. Jochems SP, Marcon F, Carniel BF, et al. Inflammation induced by influenza virus impairs human innate immune control of pneumococcus. *Nat Immunol*. October 2018:1. doi:10.1038/s41590-018-0231-y

285. Coelingh KL, Belshe RB. No clinical association of live attenuated influenza virus with nasal carriage of bacteria or acute otitis media. *MBio*. 2014;5(3):e01145-14. doi:10.1128/mBio.01145-14

286. Heikkinen T, Block SL, Toback SL, Wu X, Ambrose CS. Effectiveness of intranasal live attenuated influenza vaccine against all-cause acute otitis media in children. *Pediatr Infect Dis J*. 2013;32(6):669-674. doi:10.1097/INF.0b013e3182840fe7

287. Wright AK a, Bangert M, Gritzfeld JF, et al. Experimental human

pneumococcal carriage augments IL-17A-dependent T-cell defence of the lung. *PLoS Pathog*. 2013;9(3):e1003274. doi:10.1371/journal.ppat.1003274

288. Wilson R, Cohen JM, Jose RJ, de Vogel C, Baxendale H, Brown JS. Protection against Streptococcus pneumoniae lung infection after nasopharyngeal colonization requires both humoral and cellular immune responses. *Mucosal Immunol*. 2015;8(3):627-639. doi:10.1038/mi.2014.95
289. Cremers AJH, Zomer AL, Gritzfeld JF, et al. The adult nasopharyngeal microbiome as a determinant of pneumococcal acquisition. *Microbiome*. 2014;2(44).
290. Thwaites RS, Jarvis HC, Singh N, et al. Absorption of nasal and bronchial fluids: Precision sampling of the human respiratory mucosa and laboratory processing of samples. *J Vis Exp*. 2018;2018(131). doi:10.3791/56413
291. O'BRIEN KL, NOHYNEK H, World Health Organization Pneumococcal Vaccine Trials Carriage Working Group. Report from a WHO Working Group: standard method for detecting upper respiratory carriage of Streptococcus pneumoniae. *Pediatr Infect Dis J*. 2003;22(2):e1-e11. doi:10.1097/01.inf.0000049347.42983.77
292. Baecher-Allan C, Wolf E, Hafler DA. MHC class II expression identifies functionally distinct human regulatory T cells. *J Immunol*. 2006;176(8):4622-4631. doi:10.4049/jimmunol.176.8.4622
293. Viallard J-F, Blanco P, André M, et al. CD8+HLA-DR+ T lymphocytes are increased in common variable immunodeficiency patients with impaired memory B-cell differentiation. *Clin Immunol*. 2006;119(1):51-58. doi:10.1016/j.clim.2005.11.011

294. Turner DL, Bickham KL, Thome JJT, et al. Lung niches for the generation and maintenance of tissue-resident memory T cells. *Mucosal Immunol*. 2014;7(3):501-510. doi:10.1038/mi.2013.67

295. Weiser JN, Ferreira DM, Paton JC. *Streptococcus Pneumoniae: Transmission, Colonization and Invasion*. Vol 16. Nature Publishing Group; 2018:355-367. doi:10.1038/s41579-018-0001-8

296. Fiore AE, Uyeki TM, Broder K, Finelli L, Euler GL, Singleton JA et al. Prevention and control of influenza with vaccines: recommendations of the Advisory Committee on Immunization Practices (ACIP), 2010. MMWR Recommendations and reports: Morbidity and mortality weekly report Recommendations and reports. *Centers Dis Control*. 2010:1-62.

297. Jahnsen FL, Farstad IN, Aanesen JP, Brandtzaeg P. Phenotypic distribution of T cells in human nasal mucosa differs from that in the gut. *Am J Respir Cell Mol Biol*. 1998;18(3):392-401. doi:10.1165/ajrcmb.18.3.2995

298. Fournier BM, Parkos CA. The role of neutrophils during intestinal inflammation. *Mucosal Immunol*. 2012;5(4):354-366. doi:10.1038/mi.2012.24

299. Müller L, Brighton LE, Carson JL, Fischer WA, Jaspers I. Culturing of human nasal epithelial cells at the air liquid interface. *J Vis Exp*. 2013;(80). doi:10.3791/50646

300. Proud D, Turner RB, Winther B, et al. Gene expression profiles during in vivo human rhinovirus infection: insights into the host response. *Am J Respir Crit Care Med*. 2008;178(9):962-968. doi:10.1164/rccm.200805-670OC

301. Følsgaard N V, Chawes BL, Rasmussen MA, et al. Neonatal cytokine profile in the airway mucosal lining fluid is skewed by maternal atopy. *Am J Respir Crit Care Med*. 2012;185(3):275-280. doi:10.1164/rccm.201108-1471OC

302. Leaker BR, Malkov VA, Mogg R, et al. The nasal mucosal late allergic reaction to grass pollen involves type 2 inflammation (IL-5 and IL-13), the inflammasome (IL-1β), and complement. *Mucosal Immunol*. 2017;10(2):408-420. doi:10.1038/mi.2016.74

303. Dhariwal J, Kitson J, Jones RE, et al. Nasal Lipopolysaccharide Challenge and Cytokine Measurement Reflects Innate Mucosal Immune Responsiveness. *PLoS One*. 2015;10(9):e0135363. doi:10.1371/journal.pone.0135363

304. Jackson DJ, Makrinioti H, Rana BMJ, et al. IL-33-dependent type 2 inflammation during rhinovirus-induced asthma exacerbations in vivo. *Am J Respir Crit Care Med*. 2014;190(12):1373-1382. doi:10.1164/rccm.201406-1039OC

305. Eggleston PA. Upper airway inflammatory diseases and bronchial hyperresponsiveness. *J Allergy Clin Immunol*. 1988;81(5 Pt 2):1036-1041. doi:10.1016/0091-6749(88)90176-5

306. Jochems SP, Ruiter K de, Solórzano C, et al. Innate and adaptive nasal mucosal immune responses following experimental human pneumococcal colonization. *J Clin Invest*. 2019;129(10). doi:10.1172/JCI128865

307. Holmgren J, Czerkinsky C. Mucosal immunity and vaccines. *Nat Med*. 2005;11(4 Suppl):S45-53. doi:10.1038/nm1213

308. Tacchi L, Musharrafieh R, Larragoite ET, et al. Nasal immunity is an ancient

arm of the mucosal immune system of vertebrates. *Nat Commun*. 2014;5. doi:10.1038/ncomms6205

309. Mak TW, Saunders ME, Jett BD. Mucosal and Cutaneous Immunity. In: *Primer to the Immune Response*. Elsevier; 2014:269-292. doi:10.1016/B978-0-12-385245-8.00012-1
310. Melegaro A, Gay NJ, Medley GF. Estimating the transmission parameters of pneumococcal carriage in households. *Epidemiol Infect*. 2004;132(3):433-441. doi:10.1017/S0950268804001980
311. Simell B, Auranen K, Käyhty H, Goldblatt D, Dagan R, Brien KLO. The fundamental link between pneumococcal carriage and disease. *Expert Rev Vaccines*. 2012;11(7):841-855.
312. Gray BM, Converse GM, Dillon HC. Epidemiologic studies of Streptococcus pneumoniae in infants: acquisition, carriage, and infection during the first 24 months of life. *J Infect Dis*. 1980;142(6):923-933. doi:10.1093/infdis/142.6.923
313. Jochems SP, Weiser JN, Malley R, Ferreira DM. The immunological mechanisms that control pneumococcal carriage. 2017:1-14.
314. Albrich WC, Madhi SA, Adrian P V, et al. Pneumococcal colonisation density: a new marker for disease severity in HIV-infected adults with pneumonia. *BMJ Open*. 2014;4(8):e005953. doi:10.1136/bmjopen-2014-005953
315. Cauley LS, Vella AT. Why is coinfection with influenza virus and bacteria so difficult to control? *Discov Med*. 2015;19(102):33-40.
316. Smith AP, Lane LC, Van Opijnen T, et al. Dynamic pneumococcal genetic adaptations support bacterial growth and inflammation during coinfection

with influenza. doi:10.1101/659557

317. Jia L, Xie J, Zhao J, et al. Mechanisms of severe mortality-associated bacterial co-infections following influenza virus infection. *Front Cell Infect Microbiol*. 2017;7(AUG). doi:10.3389/fcimb.2017.00338

318. Thors V, Christensen H, Morales-Aza B, Vipond I, Muir P, Finn A. The Effects of Live Attenuated Influenza Vaccine on Nasopharyngeal Bacteria in Healthy 2 to 4 Year Olds. A Randomized Controlled Trial. *Am J Respir Crit Care Med*. 2016;193(12):1401-1409. doi:10.1164/rccm.201510-2000OC

319. Zhao L, Xu S, Fjaertoft G, Pauksen K, Håkansson L, Venge P. An enzyme-linked immunosorbent assay for human carcinoembryonic antigen-related cell adhesion molecule 8, a biological marker of granulocyte activities in vivo. *J Immunol Methods*. 2004;293(1-2):207-214. doi:10.1016/j.jim.2004.08.009

320. Kinhult J, Egesten A, Benson M, Uddman R, Cardell LO. Increased expression of surface activation markers on neutrophils following migration into the nasal lumen. *Clin Exp Allergy*. 2003;33(8):1141-1146. doi:10.1046/j.1365-2222.2003.01682.x

321. Segura E, Amigorena S. Identification of human inflammatory dendritic cells. *Oncoimmunology*. 2013;2(5). doi:10.4161/onci.23851

322. Dzionek A, Sohma Y, Nagafune J, et al. BDCA-2, a novel plasmacytoid dendritic cell-specific type II C-type lectin, mediates antigen capture and is a potent inhibitor of interferon α/β induction. *J Exp Med*. 2001;194(12):1823-1834. doi:10.1084/jem.194.12.1823

323. Lanthier PA, Huston GE, Moquin A, et al. Live attenuated influenza vaccine

(LAIV) impacts innate and adaptive immune responses. *Vaccine*. 2011;29(44):7849-7856. doi:10.1016/j.vaccine.2011.07.093

324. Li J, Arévalo MT, Chen Y, Chen S, Zeng M. T-cell-mediated cross-strain protective immunity elicited by prime–boost vaccination with a live attenuated influenza vaccine. *Int J Infect Dis*. 2014;27:37/43. doi:10.1016/j.ijid.2014.05.016
325. Matthias KA, Roche AM, Standish AJ, Shchepetov M, Weiser JN. Neutrophil-Toxin Interactions Promote Antigen Delivery and Mucosal Clearance of Streptococcus pneumoniae. *J Immunol*. 2008;180(9):6246-6254. doi:10.4049/JIMMUNOL.180.9.6246
326. Rijneveld AW, de Vos AF, Florquin S, Verbeek JS, van der Poll T. CD11b Limits Bacterial Outgrowth and Dissemination during Murine Pneumococcal Pneumonia. *J Infect Dis*. 2005;191(10):1755-1760. doi:10.1086/429633
327. Jochems SPSP, Piddock K, Rylance J, et al. Novel Analysis of Immune Cells from Nasal Microbiopsy Demonstrates Reliable, Reproducible Data for Immune Populations, and Superior Cytokine Detection Compared to Nasal Wash. *PLoS One*. 2017;12(1):e0169805. doi:10.1371/journal.pone.0169805
328. Rylance J, Piters W, Pojar S, et al. Effect of Live Attenuated Influenza Vaccine on Pneumococcal Carriage. *bioRxiv*. 2018;343319. doi:10.1101/343319
329. Siegel SJ, Tamashiro E, Weiser JN. Clearance of Pneumococcal Colonization in Infants Is Delayed through Altered Macrophage Trafficking. *PLoS Pathog*. 2015;11(6):e1005004. doi:10.1371/journal.ppat.1005004

330. Krone CL, Trzcin K, Zborowski T, Sanders EAM, Bogaert D. Impaired Innate Mucosal Immunity in Aged Mice Permits Prolonged Streptococcus pneumoniae Colonization. *Infect Immun*. 2013;81(12):4615-4625. doi:10.1128/IAI.00618-13

331. Gill MA, Long K, Kwon T, et al. Differential recruitment of dendritic cells and monocytes to respiratory mucosal sites in children with influenza virus or respiratory syncytial virus infection. *J Infect Dis*. 2008;198(11):1667-1676. doi:10.1086/593018

332. Standish AJ, Weiser JN. Human Neutrophils Kill Streptococcus pneumoniae via Serine Proteases . *J Immunol*. 2009;183(4):2602-2609. doi:10.4049/jimmunol.0900688

333. Ronchetti R, Villa MP, Martella S, et al. Nasal cellularity in 183 unselected schoolchildren aged 9 to 11 years. *Pediatrics*. 2002;110(6):1137-1142. doi:10.1542/peds.110.6.1137

334. Bogaert D, Thompson CM, Trzcinski K, Malley R, Lipsitch M. The role of complement in innate and adaptive immunity to pneumococcal colonization and sepsis in a murine model. *Vaccine*. 2010;28(3):681-685. doi:10.1016/j.vaccine.2009.10.085

335. Ambrose CS, Luke C, Coelingh K. Current status of live attenuated influenza vaccine in the United States for seasonal and pandemic influenza. *Influenza Other Respi Viruses*. 2008;2(6):193-202. doi:10.1111/j.1750-2659.2008.00056.x

336. Weiser JN. The pneumococcus: Why a commensal misbehaves. *J Mol Med*. 2010;88:97-102. doi:10.1007/s00109-009-0557-x

337. Lu Y-J, Gross J, Bogaert D, et al. Interleukin-17A mediates acquired

immunity to pneumococcal colonization. *PLoS Pathog*. 2008;4(9):e1000159. doi:10.1371/journal.ppat.1000159

338. Liu MT, Armstrong D, Hamilton TA, Lane TE. Expression of Mig (monokine induced by interferon-gamma) is important in T lymphocyte recruitment and host defense following viral infection of the central nervous system. *J Immunol*. 2001;166(3):1790-1795. doi:10.4049/jimmunol.166.3.1790
339. Mahalingam S, Farber JM, Karupiah G. The interferon-inducible chemokines MuMig and Crg-2 exhibit antiviral activity In vivo. *J Virol*. 1999;73(2):1479-1491. http://www.ncbi.nlm.nih.gov/pubmed/9882354. Accessed October 27, 2019.
340. Aggarwal NR, Tsushima K, Eto Y, et al. Immunological Priming Requires Regulatory T Cells and IL-10-Producing Macrophages To Accelerate Resolution from Severe Lung Inflammation. *J Immunol*. 2014;192(9):4453-4464. doi:10.4049/jimmunol.1400146
341. Cao J, Wang D, Xu F, et al. Activation of IL-27 signalling promotes development of postinfluenza pneumococcal pneumonia. *EMBO Mol Med*. 2014;6(1):120-140. doi:10.1002/emmm.201302890
342. Iuliano AD, Roguski KM, Chang HH, et al. Estimates of global seasonal influenza-associated respiratory mortality: a modelling study. *Lancet (London, England)*. 2018;391(10127):1285-1300. doi:10.1016/S0140-6736(17)33293-2
343. Pauly MD, Procario MC, Lauring AS. A novel twelve class fluctuation test reveals higher than expected mutation rates for influenza A viruses. *Elife*. 2017;6. doi:10.7554/eLife.26437
344. van de Sandt CE, Kreijtz JHCM, Rimmelzwaan GF. Evasion of influenza A

viruses from innate and adaptive immune responses. *Viruses*. 2012;4(9):1438-1476. doi:10.3390/v4091438

345. Sridhar S, Brokstad K a, Cox RJ. Influenza Vaccination Strategies: Comparing Inactivated and Live Attenuated Influenza Vaccines. *Vaccines*. 2015;3(2):373-389. doi:10.3390/vaccines3020373
346. Committee on Infectious Diseases, American Academy of Pediatrics. Recommendations for Prevention and Control of Influenza in Children, 2015-2016. *Pediatrics*. 2015;136(4):792-808. doi:10.1542/peds.2015-2920
347. Zens KD, Chen JK, Farber DL. Vaccine-generated lung tissue resident memory T cells provide heterosubtypic protection to influenza infection. *JCI Insight*. 2016;1(10):37-54. doi:10.1016/bs.mcb.2015.01.016.Observing
348. Hoft DF, Babusis E, Worku S, et al. Live and inactivated influenza vaccines induce similar humoral responses, but only live vaccines induce diverse T-cell responses in young children. *J Infect Dis*. 2011;204(6):845-853. doi:10.1093/infdis/jir436
349. Mohn KG-I, Smith I, Sjursen H, Cox R. Immune responses after live attenuated influenza vaccination. *Hum Vaccin Immunother*. 2017:00-00. doi:10.1080/21645515.2017.1377376
350. Schotsaert M, García-Sastre A. Inactivated influenza virus vaccines: the future of TIV and QIV. *Curr Opin Virol*. 2017;23:102-106. doi:10.1016/j.coviro.2017.04.005
351. World Health Organization (WHO). Influenza vaccine viruses and reagents. WHO. http://www.who.int/influenza/vaccines/virus/en/. Published 2018. Accessed November 6, 2018.
352. World Health Organization. *Evaluation of Influenza Vaccine Effectiveness:*

A Guide to the Design and Interpretation of Observational Studies. Geneva; 2017. http://apps.who.int/. Accessed November 7, 2018.

353. Centers for Disease Control and Prevention. Influenza (flu). https://www.cdc.gov/flu/. Accessed October 30, 2018.
354. Kelly HA, Sullivan SG, Grant KA, Fielding JE. Moderate influenza vaccine effectiveness with variable effectiveness by match between circulating and vaccine strains in Australian adults aged 20-64 years, 2007-2011. *Influenza Other Respi Viruses*. 2013;7(5):729-737. doi:10.1111/irv.12018
355. Salk HM, Simon WL, Lambert ND, et al. Taxa of the Nasal Microbiome Are Associated with Influenza-Specific IgA Response to Live Attenuated Influenza Vaccine. *PLoS One*. 2016;11(9):e0162803. doi:10.1371/journal.pone.0162803
356. Sekirov I, Russell SL, Antunes LCM, Finlay BB. Gut Microbiota in Health and Disease. *Physiol Rev*. 2010;90(3):859-904. doi:10.1152/physrev.00045.2009
357. Sabin AB, Ramos-Alvarez M, Alvarez-Amezquita J, et al. Effects of Rapid Mass Immunization on Population Under Conditions Of Massive Enteric Infection With Other Viruses. *J Am Med Assoc*. 1960;173(14):196-201. http://www.ama-assn.org/ama/pub/category/1736.html.
358. Valdez Y, Brown EM, Finlay BB. Influence of the microbiota on vaccine effectiveness. *Trends Immunol*. 2014;35(11):526-537. doi:10.1016/j.it.2014.07.003
359. Ferreira RBR, Antunes LCM, Brett Finlay B. Should the human microbiome be considered when developing vaccines? *PLoS Pathog*. 2010;6(11):1-2. doi:10.1371/journal.ppat.1001190

360. Oh JZ, Ravindran R, Chassaing B, et al. TLR5-mediated sensing of gut microbiota is necessary for antibody responses to seasonal influenza vaccination. *Immunity*. 2014;41(3):478-492. doi:10.1016/j.immuni.2014.08.009

361. Shetty AK, Maldonado YA. Current Trends in Streptococcus pneumoniae Infections and Their Treatment. *Curr Pediatr Rep*. 2013;1(3):158-169. doi:10.1007/s40124-013-0021-9

362. Cheng X, Zengel JR, Suguitan AL, et al. Evaluation of the Humoral and Cellular Immune Responses Elicited by the Live Attenuated and Inactivated Influenza Vaccines and Their Roles in Heterologous Protection in Ferrets. *J Infect Dis 2013;208594–602*. 2013;208(4):594-602. doi:10.1093/infdis/jit207

363. Ivanov S, Paget C, Trottein F. Role of Non-conventional T Lymphocytes in Respiratory Infections: The Case of the Pneumococcus. *PLoS Pathog*. 2014;10(10):1-11. doi:10.1371/journal.ppat.1004300

364. Steele CR, Oppenheim DE, Hayday AC. Gamma(delta) T cells: non-classical ligands for non-classical cells. *Curr Biol*. 2000;10(7):R282-5. http://www.ncbi.nlm.nih.gov/pubmed/10753741. Accessed July 30, 2018.

365. Keshavarz M, Mirzaei H, Salemi M, et al. Influenza vaccine: Where are we and where do we go? *Rev Med Virol*. 2019;29(1):e2014. doi:10.1002/rmv.2014

366. Block SL, Yogev R, Hayden FG, Ambrose CS, Zeng W, Walker RE. Shedding and immunogenicity of live attenuated influenza vaccine virus in subjects 5-49 years of age. *Vaccine*. 2008;26(38):4940-4946. doi:10.1016/j.vaccine.2008.07.013

367. Rodriguez Weber MA, Claeys C, Doniz CA, et al. Immunogenicity and Safety of Inactivated Quadrivalent and Trivalent Influenza Vaccines in Children 18–47 Months of Age. *Pediatr Infect Dis J*. 2014;33(12):1262-1269. doi:10.1097/INF.0000000000000463

368. Treanor JJ, Kotloff K, Betts RF, et al. Evaluation of trivalent, live, cold-adapted (CAIV-T) and inactivated (TIV) influenza vaccines in prevention of virus infection and illness following challenge of adults with wild-type influenza A (H1N1), A (H3N2), and B viruses. *Vaccine*. 1999;18(9-10):899-906. http://www.ncbi.nlm.nih.gov/pubmed/10580204. Accessed July 30, 2018.

369. REYNOLDS HY. Immunoglobulin G and Its Function in the Human Respiratory Tract. *Mayo Clin Proc*. 1988;63(2):161-174. doi:10.1016/S0025-6196(12)64949-0

370. Fukuyama Y, King J, Kobayashi R, et al. Mucosal S-IgA Abs is required for complete immunity to Streptococcus pneumoniae. *J Immunol*. 2010;184(1). http://www.jimmunol.org/content/184/1_Supplement/46.6. Accessed October 29, 2018.

371. Zens KD, Chen JK, Farber DL. Vaccine-generated lung tissue–resident memory T cells provide heterosubtypic protection to influenza infection. *JCI Insight*. 2016;1(10). doi:10.1172/jci.insight.85832

372. Wang Z, Kedzierski L, Nuessing S, et al. Establishment of memory CD8+ T cells with live attenuated influenza virus across different vaccination doses. *J Gen Virol*. 2016;97(12):3205-3214. doi:10.1099/jgv.0.000651

373. Treanor JJ, Talbot HK, Ohmit SE, et al. Effectiveness of Seasonal Influenza Vaccines in the United States During a Season With Circulation of All Three

Vaccine Strains. *Clin Infect Dis*. 2012;55(7):951-959. doi:10.1093/cid/cis574

374. Bogaert D, de Groot R, Hermans PWM, et al. Streptococcus pneumoniae colonisation: the key to pneumococcal disease. *Lancet Infect Dis*. 2004;4(3):144-154. doi:10.1016/S1473-3099(04)00938-7
375. Bogaert D, Keijser B, Huse S, et al. Variability and Diversity of Nasopharyngeal Microbiota in Children: A Metagenomic Analysis. Semple M, ed. *PLoS One*. 2011;6(2):e17035. doi:10.1371/journal.pone.0017035
376. Victor JC, Lewis KDC, Diallo A, et al. Efficacy of a Russian-backbone live attenuated influenza vaccine among children in Senegal: a randomised, double-blind, placebo-controlled trial. *Artic Lancet Glob Heal*. 2016;4:955-965. doi:10.1016/S2214-109X(16)30201-7
377. Adegbola RA, Deantonio R, Hill PC, et al. Carriage of Streptococcus pneumoniae and Other Respiratory Bacterial Pathogens in Low and Lower-Middle Income Countries: A Systematic Review and Meta-Analysis. *PLoS One*. 2014;9(8). doi:10.1371/journal.pone.0103293
378. Smith AM, McCullers JA. Secondary Bacterial Infections in Influenza Virus Infection Pathogenesis. In: *Current Topics in Microbiology and Immunology*. Vol 29. ; 2013:327-356. doi:10.1007/82
379. Lijek RS, Weiser JN. Co-infection subverts mucosal immunity in the upper respiratory tract. *Curr Opin Immunol*. 2013;24(4):417-423. doi:10.1016/j.coi.2012.05.005.Co-infection
380. Chiu C, Ellebedy AH, Wrammert J, Ahmed R. B Cell Responses to Influenza Infection and Vaccination. In: *Current Topics in Microbiology and Immunology*. Vol 386. ; 2014:381-398. doi:10.1007/82_2014_425

381. Chiu C, Wrammert J, Li G-M, McCausland M, Wilson PC, Ahmed R. Cross-reactive humoral responses to influenza and their implications for a universal vaccine. *Ann N Y Acad Sci*. 2013;1283(1):13-21. doi:10.1111/nyas.12012

382. Liang S, Mozdzanowska K, Palladino G, et al. Heterosubtypic immunity to influenza type A Heterosubtypic Immunity to Influenza Type A Virus in Mice Effector Mechanisms and Their longevity'. *J Immunol J Immunol by guest*. 1994;152:1653-1661. http://www.jimmunol.org/content/152/4/1653. Accessed February 1, 2018.

383. Thomas PG, Keating R, Hulse-Post DJ, Doherty PC. Cell-mediated Protection in Influenza Infection. *Emerg Infect Dis*. 2006;12:48-54.

384. Sridhar S. Heterosubtypic T-Cell Immunity to Influenza in Humans: Challenges for Universal T-Cell Influenza Vaccines. *Front Immunol*. 2016;7:195. doi:10.3389/fimmu.2016.00195

385. Laidlaw BJ, Craft JE, Kaech SM. The multifaceted role of CD4+ T cells in CD8+ T cell memory. *Nat Rev Immunol*. 2016;16(2):102-111. doi:10.1038/nri.2015.10

386. Teijaro JR, Turner D, Pham Q, Wherry EJ, Lefrancois L, Farber DL. Cutting Edge: Tissue-Retentive Lung Memory CD4 T Cells Mediate Optimal Protection to Respiratory Virus Infection. *J Immunol*. 2011;187(11):5510-5514. doi:10.4049/jimmunol.1102243

387. Wu T, Hu Y, Lee Y-T, et al. Lung-resident memory CD8 T cells (T_{RM}) are indispensable for optimal cross-protection against pulmonary virus infection. *J Leukoc Biol*. 2014;95(2):215-224. doi:10.1189/jlb.0313180

388. Zens KD, Farber DL. Memory CD4 T cells in Influenza. *Curr Top Microbiol*

Immunol. 2015;386:399–421. doi:10.1007/82

389. Demengeot J, Zelenay S, Moraes-Fontes MF, Caramalho Í, Coutinho A. Regulatory T cells in microbial infection. *Springer Semin Immunopathol*. 2006;28(1):41-50. doi:10.1007/s00281-006-0024-5

390. Workman CJ, Szymczak-Workman AL, Collison LW, Pillai MR, Vignali DAA. The development and function of regulatory T cells. *Cell Mol Life Sci*. 2009;66(16):2603-2622. doi:10.1007/s00018-009-0026-2

391. Cawcutt K, Kalil AC. Pneumonia with bacterial and viral coinfection. *Curr Opin Crit Care*. 2017;23(5):385-390. doi:10.1097/MCC.0000000000000435

392. Braciale TJ, Sun J, Kim TS. Regulating the adaptive immune response to respiratory virus infection. *Nat Rev Immunol*. 2012;12(4):295-305. doi:10.1038/nri3166.Regulating

393. Diavatopoulos D a, Short KR, Price JT, et al. Influenza A virus facilitates Streptococcus pneumoniae transmission and disease. *FASEB J*. 2010;24:1789-1798. doi:10.1096/fj.09-146779

394. He X-SX-S, Holmes TH, Zhang C, et al. Cellular Immune Responses in Children and Adults Receiving Inactivated or Live Attenuated Influenza Vaccines. *J Virol*. 2006;80(23):11756-11766. doi:10.1128/JVI.01460-06

395. Barría MI, Garrido JL, Stein C, et al. Localized mucosal response to intranasal live attenuated influenza vaccine in adults. *J Infect Dis*. 2013;207(1):115-124. doi:10.1093/infdis/jis641

396. Liu Y, Li S, Zhang G, et al. Genetic variants in IL1A and IL1B contribute to the susceptibility to 2009 pandemic H1N1 influenza A virus. *BMC Immunol*. 2013;14(1). doi:10.1186/1471-2172-14-37

397. Hogan RJ, Zhong W, Usherwood EJ, Cookenham T, Roberts AD, Woodland DL. Protection from respiratory virus infections can be mediated by antigen-specific CD4 + T cells that persist in the lungs. *J Exp Med*. 2001;193(8):981-986. doi:10.1084/jem.193.8.981

398. Peltola VT, McCullers JA. Respiratory viruses predisposing to bacterial infections: role of neuraminidase. *Pediatr Infect Dis J*. 2004;23(1 Suppl):S87-97. doi:10.1097/01.inf.0000108197.81270.35

399. Wu Y, Mao H, Ling M-T, et al. Successive influenza virus infection and Streptococcus pneumoniae stimulation alter human dendritic cell function. *BMC Infect Dis*. 2011;11(1):201. doi:10.1186/1471-2334-11-201

400. Deshmane SL, Kremlev S, Amini S, Sawaya BE. Monocyte chemoattractant protein-1 (MCP-1): An overview. *J Interf Cytokine Res*. 2009;29(6):313-325. doi:10.1089/jir.2008.0027

401. Newton AH, Cardani A, Braciale TJ. The host immune response in respiratory virus infection: balancing virus clearance and immunopathology. *Semin Immunopathol*. 2016;38(4):471-482. doi:10.1007/s00281-016-0558-0

402. Madera L. Mechanisms of immune response regulation by innate defense regulator peptides. 2006.

403. Bordon J, Aliberti S, Fernandez-Botran R, et al. Understanding the roles of cytokines and neutrophil activity and neutrophil apoptosis in the protective versus deleterious inflammatory response in pneumonia. *Int J Infect Dis*. 2013;17(2). doi:10.1016/j.ijid.2012.06.006

404. Appay V, Rowland-Jones SL. RANTES: A versatile and controversial chemokine. *Trends Immunol*. 2001;22(2):83-87. doi:10.1016/S1471-

4906(00)01812-3

405. Thwaites RS, Ito K, Chingono JMS, et al. Nasosorption is a minimally invasive diagnostic procedure for measurement of viral load and markers of mucosal inflammation in RSV bronchiolitis. *J Infect Dis*. 2017;215:2151240-2151244. doi:10.1093/infdis/jix150

406. Thwaites RS, Coates M, Ito K, et al. Reduced nasal viral load and IFN responses in infants with respiratory syncytial virus bronchiolitis and respiratory failure. *Am J Respir Crit Care Med*. 2018;198(8):1074-1084. doi:10.1164/rccm.201712-2567OC

407. Jha A, Dunning J, Tunstall T, et al. Patterns of systemic and local inflammation in patients with asthma hospitalised with influenza. *Eur Respir J*. 2019;54(4). doi:10.1183/13993003.00949-2019

408. Seok J, Warren HS, Cuenca AG, et al. Genomic responses in mouse models poorly mimic human inflammatory diseases. *Proc Natl Acad Sci U S A*. 2013;110(9):3507-3512. doi:10.1073/pnas.1222878110