Exploring the interplay between innate and adaptive immune responses to live attenuated influenza vaccine in Gambian children.

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Abstract

Influenza vaccination remains a challenge in low and middle income countries despite high rates of mortality and morbidity in children. World Health Organization and Serum Institute of India developed Nasovac-S, a single dose influenza vaccine to increase vaccine accessibility in these regions. The vaccine was trialled in some African Countries but no immunogenicity data was collected.

This thesis aimed to characterize the innate and adaptive immune responses pre and post LAIV in Gambian children and explore the relationship between the two responses post vaccination.

This study was nested within a randomised controlled trial investigating LAIV–microbiome interactions (NCT02972957) in healthy children aged 24–59 months who receive one dose of the WHO prequalified Russian-backbone trivalent LAIV containing A/17/California/2009/38 (Cal09) in 2017 and A/17/New York/15/5364 (NY15) in 2018. Cellular and humoral immune responses and phenotypes of immune cells pre and post LAIV were assessed using multi-parameter flow cytometry. Follicular helper T (Tfh) cells, were also investigated using the activation induced marker assay.

Post LAIV, decreased frequency of classical monocytes and increased frequency of intermediate monocytes in Gambian children was noted. Increased frequency of CD4+T cells but not CD8+T cells was also noted. LAIV induced bulk and influenza specific Tfh cells associated with seroconversion.

Change in the H1N1 strain of the vaccine in 2018 led to differences in viral shedding, replication and greater immunogenicity post LAIV. Further investigations determined that differences in immune responses observed between the two study vaccines were a result of a reduced replicative capacity of the pH1N1 Cal09 virus compared to pH1N1 NY15.

A single dose of LAIV in Gambian children led to perturbation of innate and adaptive immune cells post LAIV. Seroconversion post LAIV was associated with increase in frequency of intermediate monocytes and Tfh cells. Immunogenicity is also dependent on strain-selection for inclusion in influeza vaccine.

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Table of Contents

Abstract i

Acknowledgement ii

Publications v

List of Figures xi

List of Tables xiii

Abbreviations xiv

1 CHAPTER 1: INTRODUCTION 1

1.1 Global Influenza burden 1

1.2 Influenza virus 1

1.3 Influenza viral transmission 3

1.4 Influenza Infection in humans 3

1.4.1 Antigenic Drift 3
1.4.2 Antigenic Shift 4

1.5 Influenza in Sub Saharan Africa 6

1.6 Influenza Vaccines 7

1.6.1 Inactivated influenza vaccine (IIV) 8
1.6.2 Live Attenuated Influenza Vaccine (LAIV) formulations 8
1.6.3 Russian derived-LAIV (Ultravac®, Microgen) 13
1.6.4 Russian derived-LAIV produced by the Serum Institute of India Pvt Ltd (SIIPL, Nasovac-S) 13
1.6.5 Recent concerns with poor LAIV effectiveness 14

1.7 Immune responses to Influenza Vaccination 15

1.7.1 Innate immune responses 16
1.7.2 Adaptive Immune responses 23

1.8 Literature review conclusion (rationale for studying immune response to LAIV in African settings) 34

1.9 PhD Research questions 35

2 CHAPTER 2: MATERIALS AND METHODS 36

2.1 Study site 36

2.2 Ethical approval 36

2.3 NASIMMUNE Study design, randomization and study participants 36

2.4 Vaccination 38

2.5 Sampling timepoints 41

2.6 Laboratory Methods 42

2.6.1 Intracellular cytokine staining (T cell panel) 42
2.6.2 T cell phenotyping panel 43
2.6.3 Follicular hepler T (Tfh) cell panel 43
2.6.4 Innate panel 44
2.6.5 Haemagluttinin inhibition assay (HAI) 46
2.6.6 Protein microarray to detect anti-influenza IgA antibodies 46

2.7 Statistics 47

3 CHAPTER 3: EFFECT OF LAIV ON MONOCYTE AND DENDRITIC CELL POPULATIONS 48

3.1 Aim 48

3.2 Introduction 49

3.3 Results 51

3.3.1 Effect of LAIV vaccination on monocyte subsets 51
3.3.2 Reduced HLA-DR expression at day 2 post LAIV 54
3.3.3 LAIV vaccination induced cytokine production in monocytes 56
3.3.4 Changes in Dendritic cell populations post LAIV vaccination 58
3.3.5 LAIV vaccination induced cytokine production in pDCs but not mDC 61
3.3.6 CD14+CD16+ monocytes are associated with antibody responses post LAIV 63

3.4 Discussion 71

3.4.1 Monocyte Perturbation 71
3.4.2 Monocyte maturation 72
3.4.3 Chemokine receptor expression in monocyte subsets 73
3.4.4 HLA-DR expression in monocyte subsets 74

3.4.5 Cytokine production in monocyte subsets ... 74
3.4.6 Effect of LAIV vaccination on DC populations and cytokine production ... 75
3.4.7 Role of monocytes and DCs in humoral immune response to LAIV vaccination ... 76

3.5 Conclusion ... 77

4 CHAPTER 4: CD4+ AND CD8+ T-CELL RESPONSES TO LIVE ATTENUATED INFLUENZA VACCINE ... 78

4.1 Aim ... 78

4.2 Declaration ... 78

4.3 Introduction ... 79

4.4 Results ... 80

4.4.1 LAIV vaccination induced increased frequencies of CD4+ IFN-γ+ and IL2+ T cells but not CD8+ IFN-γ+ and IL2+ T cells ... 82
4.4.2 The proportion of mono- and dual-functional CD4+ T-cell responses to influenza antigens tested from baseline to day 21 post-LAIV ... 85
4.4.3 CD4+ and CD8+ T cell phenotypes pre and post LAIV vaccination ... 86

4.5 Discussion ... 93

4.5.1 Greater increase in frequency of CD4+T cells after LAIV vaccination ... 93
4.5.2 Effect of LAIV vaccination on cytokine production ... 93
4.5.3 Phenotype of CD4+ and CD8+ T cells post LAIV vaccination ... 94

4.6 Conclusion ... 95

5 CHAPTER 5: FOLLICULAR HELPER T CELL RESPONSES TO LAIV VACCINATION ... 96

5.1 Aim ... 96

5.2 Introduction ... 97

5.3 Results ... 99

5.3.1 *Ex-vivo* Tfh changes post LAIV vaccination ... 99
5.3.2 Increase in frequencies of CXCR3+ ICOS+ and CXCR3+ICOS+PD1+ Tfh cells in seroconverters but not in non seroconverters ... 102
5.3.3 No difference in frequencies of CXCR3+ ICOS+ and CXCR3+ICOS+PD1+ Tfh cells in IgA responders and non-IgA responders ... 104

5.3.4 Correlation between Tfh cells and antibody responses post vaccination ... 105

5.4 Effect of LAIV vaccination on Tfh subsets ... 107

5.4.1 Frequency of ICOS+ cells in Tfh subsets in seroconverters and non seroconverters ... 109
5.4.2 Increased frequency of ICOS+ cells in Tfh subsets in IgA responders but not in nonresponders 111
5.4.3 Detection of LAIV induced Tfh cells by activation marker induction ... 113
5.4.4 Increase in antigen specific Tfh cells after LAIV vaccination ... 116
5.4.5 Increased activation of antigen specific Tfh cells in seroconverters but not in non seroconverters ... 118
5.4.6 Increased frequency of CD25+, CD25+PDL1+ and OX40+CD25+ antigen specific Tfh cells in IgA nonresponders at Day 21 post LAIV ... 120
5.4.7 Frequency of LAIV induced Tfh cells in children with or without pre-existing antibody responses within CD45R0+CXCR5+Tfh cells ... 122
5.4.8 Increase in frequency of OX40+CD25+ antigen specific Tfh-1 cells post LAIV ... 125
5.4.9 Increase in frequency of CD25+ antigen specific Tfh-1 cells in seroconverters but not in non seroconverters ... 127
5.4.10 Increased frequency of CD25+, CD25+PDL1+ and OX40+CD25+ antigen specific Tfh-1 cells in IgA nonresponders at Day 21 post LAIV ... 129
5.4.11 Detection of LAIV induced Tfh cells in children with or without pre-existing antibody responses within CD45R0+CXCR5+CXCR3+ Tfh cells ... 131

5.5 Discussion ... 136

5.6 Conclusion ... 139

6 GENERAL DISCUSSION ... 140

6.1 Influenza Burden ... 140

6.2 Influenza vaccines ... 140

6.3 Immune correlates of protection ... 140

6.4 Increase in frequency of intermediate monocytes and their role in the adaptive immune response post LAIV ... 141

6.5 Increase in frequency of CD4+T cells post LAIV in Gambian children ... 142

6.6 Increased frequency of follicular helper T cells post LAIV ... 143

6.7 Conclusion ... 145

7 CHAPTER 7: STUDY OUTCOME AND FUTURE WORK 147

7.1 Study Limitations 147

7.2 Implication of the research 148

7.3 Future work 148

7.4 References 150

Appendix 1: Scientific Coordinating Commitee 174

Appendix 2: Ethics Committee 176

Appendix 3: Participant Information sheet and consent form 177

Appendix 4: Eligibility of participants 199

Appendix 5: Screening and Enrolment Log Template 201

Appendix 6: Sensitization Log Template 202

Appendix 7: Table of median responses for Tfh and AIM panel 203

Appendix 8: Publication 213

List of Figures

Figure 1: Diagram of Influenza A virus structure showing the surface glycoproteins, the internal proteins and the 8 negative sense RNA segments (adapted from (Eichberg *et al.*, 2022)) 1
Figure 2: Illustration of antigenic drift and shift in Influenza A virus 5
Figure 3: Study profile 40
Figure 4:Sequential Gating strategy showing identification of total monocytes and the different monocyte subsets in blood 52
Figure 5:Effect of LAIV vaccination on monocyte subsets... 53
Figure 6: Reduced HLA-DR expression at day 2 post LAIV 55
Figure 7: LAIV vaccination induced cytokine production in monocytes 57
Figure 8: Gating strategy showing identification of myeloid and plasmacytoid dendritic cells (DCs) in blood. 59
Figure 9: Effect of LAIV Vaccination on dendritic cell subsets 60
Figure 10: LAIV vaccination induced cytokine production in dendritic cells. 62
Figure 11: Increase in intermediate monocytes at day2 post LAIV in seroconverters 64
Figure 12: Increase in intermediate monocytes at day 7 post LAIV in seroconverters 66
Figure 13:Changes in pDC in post LAIV... 67
Figure 14: Increase in intermediate monocytes in IgA responders at day 2 post LAIV 68
Figure 15: Increase in intermediate monocytes in IgA responders at day 7 post LAIV 69
Figure 16: Changes in DC population post LAIV 70
Figure 17: Gating strategy for detection of IFN- γ and IL-2 in CD4+ and CD8+ T-cells.................. 80
Figure 18: CD8+ and CD4+ T-cell responses (IFN- γ and IL-2) following stimulation with Staphylococcal Enterotoxin B (SEB), negative control (co-stimulatory antibodies anti-CD28 and anti-CD48 alone) and an overlapping peptide pool matched to H3 haemagglutinin protein (from A/H3N2 Hong Kong/4801/2014) at day 21 post LAIV 81
Figure 19: LAIV vaccination induced increased frequencies of CD4+IFN- γ + and IL2+ T cells but not CD8+ IFN- γ + and IL2+T cells 84
Figure 20: The proportion of mono- and dual-functional CD4+ T-cell responses to influenza antigens tested from baseline to day 21 post-LAIV 85
Figure 21: Gating strategy for detection of influenza specific T cells 87
Figure 22: Total CD8 and CD4 T cell phenotype in Gambian children aged 24-59 months at baseline.. 89
Figure 23: No change in phenotype of flu specific CD4+T cells in response to LAIV 91
Figure 24: No change in phenotype of flu-specific CD8+T cells in response to LAIV........................ 92
Figure 25 A-I: Gating strategy to identify circulating follicular helper T cells (Tfh) in blood and different Tfh subsets following ex-vivo staining of whole blood in LAIV vaccinated children 100
Figure 26: Live attenuated influenza (LAIV) vaccination induces Tfh cells in blood 102

Figure 27: Increase in frequencies of CXCR3+ICOS+ and CXCR3+ICOS+PD1+ Tfh cells in seroconverters but not in non seroconverters 103
Figure 28: Frequencies of CXCR3+ICOS+ and CXCR3+ICOS+PD1+ Tfh cells post LAIV vaccination in IgA responders and nonresponders. 104
Figure 29: Correlation between fold change in CXCR3+ICOS+ and CXCR3+ICOS+PD1+ Tfh cells and maximum GMFR post LAIV vaccination 106
Figure 30: Frequency of ICOS+ cells in circulating Tfh subsets 108
Figure 31:Frequency of ICOS+cells in Tfh subsets in seroconverters and nonseroconverters .. 110
Figure 32:Frequency of ICOS+ cells in Tfh subsets in IgA responders and nonresponders........ 112
Figure 33 A-J: Activation induced marker (AIM) gating strategy to identify antigen specific follicular helper T cells (Tfh) in blood. 114
Figure 34: Induction of antigen specific Tfh cells after stimulation with influenza antigens...... 117
Figure 35: Induction of antigen specific Tfh cells after stimulation with influenza antigens in seroconverters and non seroconverters........ 119
Figure 36: Induction of antigen specific Tfh cells after stimulation with influenza antigens in IgA responders and nonresponders 121
Figure 37: Detection of AIM markers on antigen specific Tfh cells after stimulation with influenza antigens in baseline responders and nonresponders 124
Figure 38: Induction of antigen specific CXCR3+Tfh cells after stimulation with influenza antigens 126
Figure 39: Induction of antigen specific CXCR3+Tfh cells after stimulation with influenza antigens in seroconverters and non seroconverters 128
Figure 40: Induction of antigen specific CXCR3+Tfh cells after stimulation with influenza antigens in IgA responders and nonresponders........ 130
Figure 41: Detection of AIM markers on antigen specific Tfh cells after stimulation with influenza antigens in baseline responders and nonresponders 132
Figure 42: Heatmap showing p values of the frequency of circulating Tfh cells at baseline and day 7 post LAIV vaccination 133
Figure 43: Heatmap showing p values of the percentage of activation marker expression on CD45R0+CXCR5+ and CD45R0+CXCR5+CXCR3+ Tfh cells at baseline and day 7 post LAIV vaccination after stimulation with different influenza antigens........ 134
Figure 44: Heatmap showing p values of the percentage of activation marker expression on CD45R0+ CXCR5+ and CD45R0+CXCR5+CXCR3+ Tfh cells at baseline and day 7 and 21 post LAIV vaccination after stimulation with different influenza antigens 135
Figure 45: Intermediate monocytes mediate antibody responses post LAIV in Gambian children. 146

List of Tables

Table 1: Influenza genomic segments and their function (Adapted from (Kapoor and Dhama, 2014)) 2
Table 2: Randomized controlled trials showing efficacy of LAIV vaccination in adults and children (Osterholm *et al.*, 2011) 10
Table 3: Reduced efficacy of LAIV against pandemic H1N1 showing estimates from individual studies as well as consolidated estimates(Caspard *et al.*, 2017). *Abbreviations: Centre for disease control (CDC), Department of defence (DoD), Sentinel practitioner surveillance network (SPSN)*. 12
Table 4: Human TLR receptors and their ligands adapted from (Duan *et al.*, 2022) 17
Table 5: Randomization of participants to different groups 37
Table 6: Demographic characteristics and baseline and post LAIV influnza serological data 39
Table 7: Sampling overview 41
Table 8: Reagents for flow cytometry 44
Table 9:Flow cytometry panel and antibodies used 45
Table 10: Median and 95%CI of CXCR3+ICOS+ and CXCR3+ICOS+PD1+Tfh cells pre and post LAIV 203
Table 11: Median and 95CI% of bulk Tfh and Tfh subsets pre and post LAIV 204
Table 12: Median and 95% CI of antigen specific CD45R0+CXCR5+ Tfh cells pre and post LAIV. 205
Table 13: Median and 95%CI of antigen specific CD45R0+CXCR5+ Tfh cells pre and post LAIV in seroconverters and nonseroconverters 206
Table 14: Median and 95%CI of antigen specific CD45R0+CXCR5+ Tfh cells pre and post LAIV in IgA responders and nonresponders 207
Table 15: Median and 95%CI of antigen specific CD45R0+CXCR5+ Tfh cells pre and post LAIV in children with or without pre-existing responses to H1, H3 and B HA influenza antigens 208
Table 16: Median and 95%CI of antigen specific CD45R0+CXCR5+ CXCR3+ Tfh cells pre and post LAIV 209
Table 17: Median and 95%CI of antigen specific CD45R0+CXCR5+ CXCR3+ Tfh cells pre and post LAIV in seroconverters and non seroconverters 210
Table 18: Median and 95%CI of antigen specific CD45R0+CXCR5+ CXCR3+ Tfh cells pre and post LAIV in IgA responders and nonresponders 211
Table 19: Median and 95%CI of antigen specific CD45R0+CXCR5+ CXCR3+ Tfh cells pre and post LAIV in baseline responders and nonresponders 212

Abbreviations

ACIP	Advisory committee on Immunization Practice
ADCC	Antibody-dependent cell-mediated cytotoxicity
AIIV	Adjuvanted Inactivated influenza vaccines
AIM	Activation induced marker
ANISE	African Network for Influenza Surveillance and Epidemiology
ANOVA	Analysis of Variance
AS03	Adjuvant system 03
ASC	antibody secreting cells
ATT	Attenuation
BCL6	B-cell lymphoma 6 protein
BD	Becton Dickinson
CA	cold adapted
CCR5	CC chemokine receptor 5
CCR6	CC chemokine receptor 6
CD38	Cluster of differentiation 38
CDC	Centre for Disease Control and Prevention
CI	Confidence Interval
CM1	Influenza C matrix 1
CM2	Influenza C matrix 2
CMV	Cytomegalovirus
CTL	Cytotoxic T lymphocyte
CXCL	C-X-C Motif Chemokine Ligand
CXCR	C-X-C motif chemokine receptor
DC	Dendritic cells
DEGs	Differentially expressed genes
DMSO	Dimethyl sulfoxide
DoD	Department of Defence
ECE	Embryonated chicken eggs
ELISA	Enzyme Linked Immunosorbent Assay
FC	Fragment, crystallizable
FDA	Food and drug administration
FMO	Fluorescence Minus One
FV	Flu Vaccine Effectiveness Network
GATA	GATA binding protein
GC	Germinal center
GMFR	Geometric mean fold rise
GMT	Geometric mean titres
HA	Haemagglutinin
HAI	Haemagglutination Inhibition assay
HEF	Haemagglutinin-estrase-fusion
HI	Hemagglutination inhibition
HIC	High income country

HLA-DR	Human Leukocyte Antigen – DR isotype
H1N1	Haemagglutinin type 1 and Neuraminidase type 1.
H3N2	Haemagglutinin type 3 and Neuraminidase type 2.
H5N1	Haemagglutinin type 5 and Neuraminidase type 1.
HIV	Human Immunodeficiency Virus
IAV	Influenza A virus
IBV	Influenza B virus
ICOS	Inducible T cell costimulator
ICS	Intracellular cytokine staining
IFN-γ	Interferon gamma
IFN-α	Interferon alpha
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IIV	Inactivated influenza vaccines
IL-21	Interleukin 21
IL-22	Interleukin 22
IL-12	Interleukin 12
IL-17	Interleukin 17
IL-1β	Interleukin 1 beta
IL-2	Interleukin 22
IL-6	Interleukin 22
ILI	Influenza like illness
IN	Innate
IQR	Interquartile range
LAIV	Live Attenuated Influenza Vaccine
LILR	Leukocyte immunoglobulin (Ig)-like receptor
LLC	Limited liability company
LOQ	Limit of Quantitation
LMIC	Low and middle-income countries
LPS	Lipopolysaccharides
M	Matrix
MA-ARI	Medically attended acute respiratory illness
MCP	Monocyte chemoattractant protein
mDC	Myeloid Dendritic cells
MDCK	Madin-Darby Canine Kidney
MDV	Master donor virus
MFI	Mean Fluorescence Intensity
MF59	Oil in water adjuvant
MHC	Major histocompatibility complex
MNP	Matrix and Nucleoprotein
NA	Neuraminidase
NALT	Nasopharynx-associated lymphoid tissue

NICD	National Institute for Communicable Diseases
NK	Natural killer cell
NP	Nucleoprotein
NS	Non-structural protein
P3	Polymerase 3
PA	Polymerase acid
PAMPS	Pathogen associated molecular patterns
PB1	Polymerase basic 1
PB2	polymerase basic 2
PBMC	Peripheral blood mononuclear cells
PD1	Programmed cell death protein 1
pDC	plasmacytoid dendritic cells
PRR	Pathogen recognition receptors
RBC	Red blood cells
RCT	Randomized controlled trial
RIG-I	Retinoic acid-inducible gene-I
RNA	Ribonucleic acid
RORγT	RAR-related orphan receptor gamma
RT-PCR	Reverse-transcriptase polymerase chain reaction
SEB	Staphylococcus enterotoxin b
SFU	Spot forming unit
SIIPL	Serum Institute of India Pvt Ltd
SPICE	Simulation Program with Integrated Circuit Emphasis
SPSN	Sentinel practitioner surveillance network
TB	Tuberculosis
Tfh	Follicular helper T Cells
TH1	T helper type 1
TLR	Toll-like receptor
UNICEF	United Nations International Children's Emergency Fund
UK	United Kingdom
URT	Upper respiratory tract
USA	United States of America
USSR	Union of Soviet Socialist Republics
WHO	World Health Organisation

The aims of this PhD project are:

Aim 1

1. To characterize the detailed phenotype of innate immune cells pre and post vaccination with LAIV.
2. To assess the relationship between innate responses and later humoral immune responses post vaccination with LAIV.

Aim 2

1. To assess the magnitude of CD8+ and CD4+ T-cell in response to LAIV vaccination.
2. To assess the phenotype of influenza-specific CD8+ and CD4+ T-cells in the blood at baseline and post vaccination with LAIV.

Aim 3

1. To characterize the detailed phenotype of peripheral Tfh cells pre and post vaccination with LAIV.
2. To investigate if vaccine-induced changes correlate with mucosal IgA, serum haemagglutination inhibition titres and systemic CD4+ and CD8+ T-cell responses.

1 Chapter 1: Introduction

1.1 Global Influenza burden

Globally, influenza results in a substantial burden on health care systems, with an estimated 3 to 5 million people infected with influenza every year, leading to a loss of up to 290,000 (95% confidence interval 250,00 to 500,000) lives annually (World Health Organization, 2018). This may well be an underestimate of the actual burden, due to gaps in influenza data capture (Iuliano *et al.*, 2018). Mortality in children under 5 years is estimated at between 28 000–111 500 deaths annually, with 99% of these occuring in low and middle-income countries (LMIC) (Nair *et al.*, 2011). Higher rates of hospitalization in this age group have also been reported in sub-Saharan Africa compared to other parts of the world (Cohen *et al.*, 2015; Lafond *et al.*, 2016). Influenza virus circulates everywhere and can affect anybody. The 2012 World Health Organisation (WHO) position paper recommends that countries should consider immunizing high-risk groups against influenza. These groups include pregnant women, young children aged 6–59 months, adults with specific chronic illnesses, persons >65 years, and health care workers (World Health Organization, 2012, 2018).

1.2 Influenza virus

Influenza is an enveloped, segmented, single stranded, negative sense ribonucleic acid (RNA) virus belonging to the family Orthomyxomiridae as shown in Figure 1 below. Influenza virus can either be spherical or filamentous in shape with spherical shaped virions measuring up to 120 nm in diameter and filamentous ones up to 300nm or more (Kapoor and Dhama, 2014).

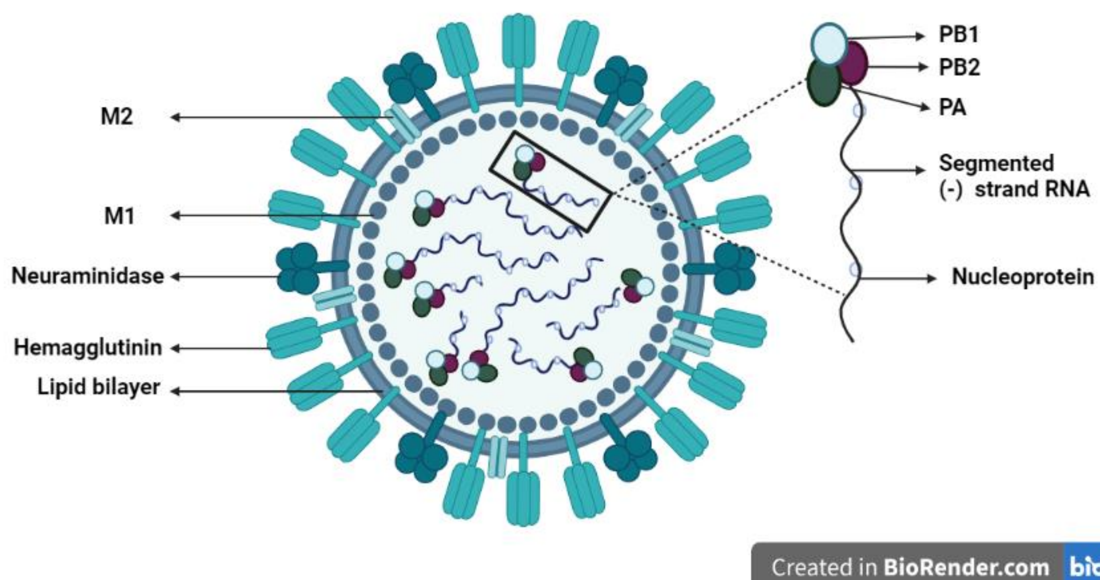


Figure 1: Diagram of Influenza A virus structure showing the surface glycoproteins, the internal proteins and the 8 negative sense RNA segments (adapted from (Eichberg *et al.*, 2022)**)**

There are 3 types of influenza viruses that affect humans. Influenza A, B and C, with differences in their genomic structure, epidemiology and pathogenicity (Kapoor and Dhama, 2014; Sridhar, Brokstad and Cox, 2015) as shown in Table 1 below.

Table 1: Influenza genomic segments and their function (Adapted from (Kapoor and Dhama, 2014))

Segments	Influenza A	Influenza B	Influenza C	Function
1	Polymerase basic 2 (PB2)	Polymerase basic 2 (PB2)	Polymerase basic 2 (PB2)	Viral replication
2	Polymerase basic 1 (PB1	Polymerase basic 1 (PB1	Polymerase basic 1 (PB1)	Viral replication
3	Polymerase acid (PA)	Polymerase acid (PA)	Polymerase acid (PA)	Viral replication
4	Haemagglutinin (HA)	Haemagglutinin (HA)	Haemaglutinnin-estrase-fusion (HEF)	Surface glycoprotein, attachment Subtype specific Neutralizing antibody target Vaccine target
5	Nucleoprotein (NP)	Nucleoprotein (NP)	Nucleoprotein (NP)	RNA transcription
6	Neuraminidase (NA)	Neuraminidase (NA)	Absent	Surface glycoprotein Viral release from host cells Vaccine target
7	Matrix (M)	Matrix (M)	Matrix (M)	Virus budding, Subtype specific
8	Nonstructural protein (NS 1 and NS 2)	Nonstructural protein (NS 1 and NS 2)	Nonstructural protein (NS 1 and NS 2)	Regulation of virus life cycle

1.3 Influenza viral transmission

Seasonal influenza virus is transmitted from human to human and three main ways have been described: aerosol, droplet and contact transmission following release of viral particles from the respiratory tract of an infected person upon coughing or sneezing (Cowling *et al.*, 2013). The aerosol route was recently shown to be an important means of viral transmission (Yan *et al.*, 2018). When these particles are inhaled, the virus binds to epithelial cells that line the upper and lower respiratory tracts. Human viruses tend to bind to epithelial cells expressing alpha 2,6-linked sialyloligosaccharide receptors in the upper respiratory tract, whilst avian viruses bind to cells in the lower respiratory tract expressing alpha 2,3-linked sialyloligosaccharide receptors (Shinya *et al.*, 2006). The virus can also be spread by direct contact with an infected person or non-porous surfaces as the virus can stay infectious for up to 48 hours (Paules and Subbarao, 2017; World Health Organization, 2018). Unlike influenza A and B which can cause illness during epidemics and pandemics, influenza C is usually associated with minor localized outbreaks and causes mild disease in adults (World Health Organization, 2018) and in children < 2 years old, although it can lead to respiratory infections requiring hospitalization (Matsuzaki *et al.*, 2006; Principi *et al.*, 2013). When influenza virus infection affects the lower respiratory tract, it causes primary viral pneumonia or secondary bacterial pneumonias which can often be fatal (Taubenberger and Morens, 2008).

1.4 Influenza Infection in humans

1.4.1 Antigenic Drift

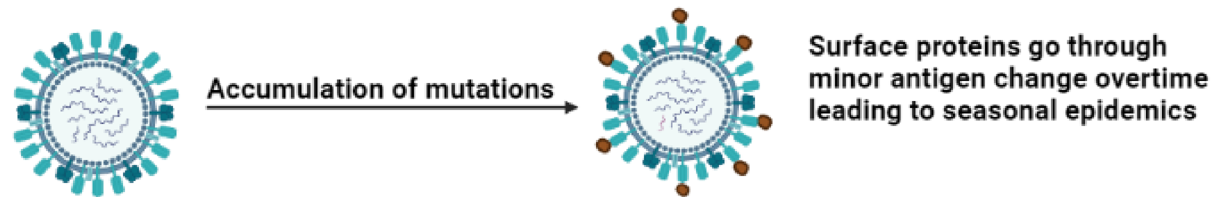
Influenza A viruses are further divided into subtypes according to the specific variety and combinations of the surface glycoproteins HA and NA. To date there are 18 HA and 11 NA identified. 16 HA and 9 NA were isolated from birds and the remaining 2 HA and NA from bats in two different regions of Central and South America (Tong *et al.*, 2013). Influenza B viruses are separated into two different lineages rather than subtypes: namely Yamagata and Victoria. Subtypes of influenza A and influenza B lineages are responsible for seasonal epidemics. Currently, influenza A/H1N1 and A/H3N2 and influenza B Victoria lineage and Yamagata lineages circulate during seasonal epidemics. Mutations occur on the HA and NA proteins leading to antigenic variation and allowing the virus to escape the immune system (antigenic drift). This occurs partly because the host humoral response selects for mutants with changes in HA and NA, resulting in strains that can avoid the neutralizing antibody response established by previous infection or vaccination. This allows the drifted viral strains to cause seasonal outbreaks of influenza (Kapoor and Dhama, 2014; Paules and Subbarao, 2017; CDC Newsroom, 2018; Rao, Nyquist and Stillwell, 2018) as shown in Figure 2.

Influenza B undergoes less antigenic drifting compared to influenza A (Chen and Holmes, 2008; Taubenberger and Morens, 2008). Influenza vaccination strategies are generally aimed at

inducing antibodies against the surface glycoproteins HA and NA, which are also the targets for many antiviral drugs (Wohlbold and Krammer, 2014). Antigenic drift is the reason why seasonal influenza vaccines are updated annually. Global surveillance data of influenza strains in the population is obtained from the WHO Global Influenza Surveillance and Response System and the dominant circulating strains identified. WHO collaborating centers then generate a vaccine seed strain through genetic re-assortment of a master strain and the field strains, which are then distributed to the vaccine producers for formulation of that season's vaccine. This process occurs twice every year, in time for the influenza season in northern and southern temperate regions. However there have been instances when the vaccine strain for that season does not match the virus circulating due to constant evolution of the virus and the time taken for vaccine manufacture (Chan *et al.*, 2018).

1.4.2 Antigenic Shift

Antigenic shift is an abrupt, major change in the influenza A viruses, resulting in new HA and/or NA proteins in influenza viruses that infect humans as shown in Figure 2. The diverse host range of influenza A, its ability to undergo genetic reassortment and its genetic and antigenic diversity are some of the factors responsible for periodic pandemics. Influenza A infects humans, mammals and birds, whilst influenza B and C predominantly infect humans. The wide range of hosts that influenza A infects is the major reason why it is responsible for pandemics, as these animals can serve as reservoirs for both human influenza viruses and those from other species, increasing the chance of genetic reassortment resulting in new strains. Recently, influenza B was isolated from seals (Bodewes *et al.*, 2013) and influenza C from pigs, horses and cattles (Zhang et al 2018). The pandemic H1N1 2009 influenza virus was as a result of genetic reassortment from three different species: one genetic segment from human influenza A H3N2, two segments from avian influenza A H1N1 and five segments from swine H1N1 (Al-muharrmi, 2010). Antigen shift results in a new influenza A subtype or a virus with HA and/or NA from an animal population with no similarity to the human subtype. As such most people do not have immunity to the new virus generated and because of this low level of pre-existing immunity to newly emerging influenza strains, influenza viruses may cause pandemics with severe illness and high mortality. Global mortality rates due to the 1918 pandemic "Spanish flu" was estimated at 50 million deaths. Three more pandemics have occurred since then, including the 1957 "Asian flu", 1968 "Hong Kong flu" and 2009 swine-origin pandemic H1N1 (pH1N1) with less mortality (World Health Organization, 2012, 2014; Paules and Subbarao, 2017). After a pandemic, the viruses can then start circulating during seasonal epidemics, as has happened with pH1N1, which replaced the previous seasonal H1N1 virus. Unlike the H1N1 and H3N2 viruses that cause seasonal epidemics, infection of humans with purely avian-origin influenza A viruses belonging to H2, H5, H6, H7, H9 and H10 HA subtypes can occur with the potential for causing influenza pandemics. However, to date consistent human-to-human transmissionability of these subtypes have not been reported.



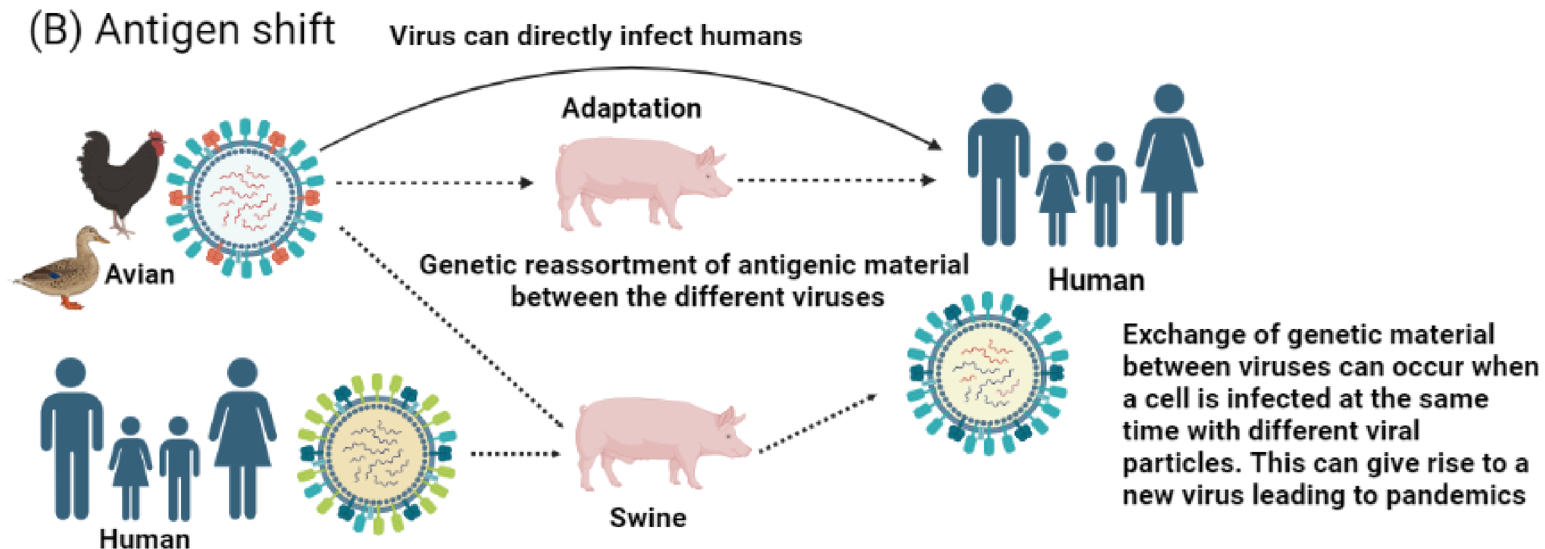


Figure 2: Illustration of antigenic drift and shift in Influenza A virus

(A) Antigenic drift occurs as a result of minor mutations that occur overtime given rise to a mutated virus that can cause seasonal epidemics. (B) Antigenic shift can be caused by direct infection of humans with Influenza A from Avian or Swine, by adaptation or by genetic reassortment of antigenic material between the different viruses. Created with BioRender.com adapted from (Yeo and Gan, 2021)

1.5 Influenza in Sub Saharan Africa

Data on influenza in Sub Saharan Africa is limited, with very few countries reporting influenza activity to the WHO compared to other parts of the world (Gessner, Shindo and Briand, 2011; Sanicas *et al.*, 2014). Despite this gap in data, a huge burden of morbidity and mortality is estimated to exist in this region (Nair *et al.*, 2011; Cohen *et al.*, 2015; Lafond *et al.*, 2016; Troeger *et al.*, 2017; Iuliano *et al.*, 2018). It is estimated that the rate of influenza-related hospitalization in children <5 years old is approximately 3-fold higher in Africa compared to Europe (150/100,000 children/year versus 48/100,000) (Lafond *et al.*, 2016). Influenza-related case fatality ratios in LMICs are also thought to be up to 15-fold higher than in high income countries (HIC) (Nair *et al.*, 2011). The high morbidity and mortality recorded in this region may be due to factors such as co-infection with tuberculosis (TB), Human Immunodeficiency Virus (HIV) and *Streptococcus pneumonia,* and challenges in health care management systems in Africa (Walaza, Cohen, *et al.*, 2015; Walaza, Tempia, *et al.*, 2015). With the emergence of the H5N1 avian inflenza and the 2009 pH1N1, the importance of influenza surveillance systems in Sub Saharan Africa was futher highlighted (Dawood *et al.*, 2012; Fischer *et al.*, 2014; Green, 2018). This prompted several international organizations to commit to influenza research and surveillance in Africa, including the WHO, and led to the the strengtening of influenza surveillance programs in a number of countries in Sub Saharan Africa. A major objective was to improve data sharing at both regional and global level through the WHO's FluNet and FluID data- bases. The United States Center for Disease Control and Prevention (CDC), in partnership with South Africa's National Institute for Communicable Diseases (NICD), also formed the African Network for Influenza Surveillance and Epidemiology (ANISE), which fulfilled a similar purpose (Amal *et al.*, 2011; Radin *et al.*, 2012; Steffen *et al.*, 2012; Schoub *et al.*, 2013; Polansky, Outin-Blenman and Moen, 2016; Iuliano *et al.*, 2018; Monto, 2018).

Techniques involved in influenza detection includes use of rapid test kits, viral culture, immunofluoresence assay as well as molecular techniques (Kim and Poudel, 2013). In recent years, capacity for detection on influenza has increased, with around 30 countries having existing influenza surveillance programmes in place with 20 of the contributing data towards global influenza surveillance (Green, 2018). Senegal and South Africa are among the African countries that have been regularly contributing towards global influenza surveillance data. In a surveillance study carried out in Senegal using samples collected from 9176 patients with infuenza-like illness between 1996 to 2009, 1233 (13%) tested positive for influenza by virus isolation and/or reverse-transciptase polymerase chain reaction (RT-PCR) (Niang *et al.*, 2012). The influenza A and B subtypes circulating in Senegal were antigenically similar to the ones circulating in other parts of the world at that time. Circulation of influenza A/H3N2 predominated during most years; 9 of the 14 years and the influenza season consistently peaked in July–September corresponding

with the rainy season (Niang *et al.*, 2012). In South Africa, a 25-year long surveillance study revealed a mean annual influenza detection rate of 28% (range, 23%–41%). As seen with the Senegal study, H3N2 influenza predominated in 14 of the 25 years and the influenza season generally starts before June, peaking in the first week of July and lasting for 6 to 18 weeks (mean of 10 weeks)(McAnerney *et al.*, 2012). An increase in surveillance sites in Africa was reported by Radin et al (Radin *et al.*, 2012). Unlike temperate countries, where there is usally one defined peak of influenza annually, often coinciding with colder weather, the seasonality of influenza in Africa is more complex. Countries in the north or south of the continent tend to have single influenza seasons coinciding with those in northern and southern temperate countries respectively. In some other parts of Africa, multiple peaks of influenza transmission occur, with co-circulation of both influenza A strains and influenza B (Cardoso *et al.*, 2012; Lutwama *et al.*, 2012; Mmbaga *et al.*, 2012; Nyatanyi *et al.*, 2012; Radin *et al.*, 2012). This poses a challenge for vaccination programmes in Africa as they are faced with the decision of whether to use the northern or southern hemisphere vaccine formulations (Radin *et al.*, 2012). Current vaccine manufacturing schedules are entirely based on requirements in northern and southern hemisphere temperate high income countries. Further research and a change of strategy will be required to increase access to influenza vaccines across Africa.

1.6 Influenza Vaccines

Vaccination is the most effective way of preventing infection with influenza virus. Protection mediated by influenza vaccination includes reduced risk against infection with the Influenza A and B viruses present in the vaccine and reduced disease severity and hospitalization rates especially in high risk group such as young children and older adults (Kissling *et al.*, 2023). Review of global influenza vaccination in 2016 showed that despite the very high burden of the disease in Africa, only 6 countries have national vaccinaton programmes for seasonal influenza and less than 1% of the population have access to seasonal influenza vaccines (Hirve *et al.*, 2016; Ortiz *et al.*, 2016). Licensed seasonal influenza vaccines are available as inactivated influenza vaccines (IIV) or live attenuated influenza virus (LAIV) vaccines. These can either be trivalent or quadrivalent. Trivalent vaccines contain influenza A/H1N1 and A/H3N2, together with the dominant lineage of influenza B (Victoria/Yamagata). Quadrivalent vaccines contain both influenza B lineages to increase protection against influenza B virus infections. A huge variability in vaccine efficacy occurs globally (with annual fluctuations depending on how good the match with circulating strains is), with an average of 50–60% estimated protection (Tewawong *et al.*, 2015). Efforts to develop newer more immunogenic vaccines are in place as reviewed by Sautto et al (Sautto, Kirchenbaum and Ross, 2018).

1.6.1 Inactivated influenza vaccine (IIV)

IIV is an injectable vaccine that is approved for use in persons 6 months and older, mostly administered as a single dose. Children aged 6 months to 8 years old that have not received influenza vaccine in the previous season should receive 2 doses, 4 weeks apart, instead of a single dose. IIV is safe for use in pregnant women and persons with chronic medical conditions.

Most commercially available influenza vaccines involve cultivation of vaccine strains in eggs. Inactivated vaccines contain inactivated, non-replicating virus. They can either be split virus, subunit vaccines or recombinant HA based vaccines. HA and NA are the main antigenic components in inactivated influenza vaccines. IIV vaccines generally have 15 μg HA per strain, although high dose vaccines with 60 μg HA per strain have been developed and trialled and proved to be safe and immunogenic. The high dose vaccines are especially ideal for the older adults who have lower responses to standard influenza vaccines. Adjuvants like oil-in-water (MF59 and AS03) increase the immunogenicity of the vaccine and are particularly used in the elderly and very young (Couch *et al.*, 2007; Sridhar, Brokstad and Cox, 2015). Of recent, Fluzone Intradermal, a trivalent recombinant haemagglutinin vaccine containing 135 μg of purified HA proteins (45 μg for each virus) was approved by the United States (U.S) Food and Drug Administration (FDA) for use in people 18 years to 64 during the 2012-2013 influenza season and this was updated to Flublok Quadrivalent in 2014 for the same age group.

1.6.2 Live Attenuated Influenza Vaccine (LAIV) formulations

LAIV is approved for use only in persons aged 2–49 years who do not have underlying medical conditions. LAIV should also not be administered to pregnant women. LAIV is given as a nasal spray. Children aged 2–8 years who have not received seasonal influenza vaccine during the previous influenza season should receive 2 doses, at least 4 weeks apart. Other individuals should receive one dose. LAIV is made from attenuated (weakened), viruses and does not cause influenza. They are based on Master Donor Viruses (MDVs) that have been rendered cold adapted (ca) , attenuated (att) and temperature sensitive (ts) to facilitate replication in the cooler upper respiratory tract (25°C) but not the warmer lungs. These phenotypes occur through serial passage of the master donor viruses for both the B/Ann Arbor/1/66 (MDV-B) and A/Ann Arbor/6/60 (H2N2) (MDV-A) viruses, (US derived MDVs) respectively, at progressively lower temperature. During the process of serial passage, mutations that led to the ca, ts, and att phenotypes were established in the polymerase complex (PB1, PB2, PA, and NP). For MDV-B , the ca, ts, were specific to PB2 (S630R), PA (V341M), NP (V114A, P410H, and A509T), and M1 (H159Q and M183V), and in MDV-A, they occur at PB2 (N265S), PB1 (K391E, D581G, and A661T), and NP (D34G) (21–29) (Maassab *et al.*, 1969; Santos, Jefferson *et al.*, 2017).

There are two different LAIV currently available for use: FluMist (or Fluenz in the United Kingdom, UK) manufactured by AstraZeneca/Medimmune based on the B/Ann Arbor/1/66

(MDV-B) and A/Ann Arbor/6/60 (H2N2) (MDV-A) strains and developed in the US. Another LAIV, developed in Russia, is based on the B/USSR/60/69 (MDV-B) and A/Leningrad/ 134/17/57(H2N2)(MDV-A) that was licensed in Russia in the 1970s. The live attenuated seed vaccine strains are constructed by re-assorting the HA and NA gene segments from circulating influenza strains with 6 gene segments from a master donor strain which is temperature sensitive (ts), cold-adapted (ca) and attenuated (att).

1.6.2.1 US-derived Ann-Arbor LAIV

In the United States (US) Ann-Arbor LAIV was first licensed as a trivalent vaccine (LAIV3, FluMist, MedImmune ,LLC) and approved for use in children and adults aged 5-49 years in 2003 and later extended for use in children 2 years old and over. It is not approved for use in children less than 2 years because of an increased risk of wheezing and hospitalisation reported in one study (Bergen *et al.*, 2004). LAIV has also been approved for use in eligible persons aged 2-49 years in countries such as South Korea, Israel, Hong Kong, Macau, Brazil, and the United Arab Emirates, whilst in Canada the age bracket is extended to 2–59 years of age. In the European Union, it is approved for use in eligible children 2–17 years of age (Baxter *et al.*, 2012). Safety and efficacy data on LAIV are mostly from USA, but a few studies were done in Asia, South Africa and some countries in Europe (Neto *et al.*, 2009).

1.6.2.2 Efficacy and effectiveness of LAIV in adults and children

LAIV has been trialled in both children and adults with data showing better efficacy against influenza infection in children compared to adults. Efficacy of LAIV in adults has shown varying results as highlighted in Table 2. In children, LAIV was the preferential vaccine to use in ages 2-8 years when available based on data showing that LAIV is moderately more effective than IIV in children aged 2 to 5 years old. The use of LAIV led to fewer cases of laboratory-confirmed influenza and otitis media compared to use of IIV (Ashkenazi *et al.*, 2006; Belshe *et al.*, 2007; Tanzi, 2014). The efficacy and effectiveness of influenza vaccines was reviewed by Osterholm et al as shown in Table 2 below (Osterholm *et al.*, 2011).

Table 2: Randomized controlled trials showing efficacy of LAIV vaccination in adults and children (Osterholm *et al.*, 2011).

	Population (dates)	Patients randomly allocated to receive LAIV and placebo	Vaccine efficacy (95% CI)	Reported antigenic match
Adults (≥60 years)				
De Villiers et al (2010)[37]	Community-dwelling ambulatory adults aged ≥60 years (2001–02)	3242	Overall 42% (21 to 57); 31% (–3 to 53) for patients aged 60–69 years; 57% (29 to 75) for patients aged ≥70 years	Type A: similar H3N2; type B: lineage match
Adults (18–49 years)				
Ohmit et al (2006)[24]	Healthy adults aged 18–46 years (2004–05)	725	48% (–7 to 74)	Type A: drifted H3N2; type B: mixed lineage
Ohmit et al (2008)[25]	Healthy adults aged 18–48 years (2005–06)	1191	8% (–194 to 67)	Type A: drifted H3N2; type B: lineage mismatch (1 isolate)
Monto et al (2009)[28]*	Healthy adults aged 18–49 years (2007–08)	1138	36% (0 to 59)	Type A: drifted H3N2; type B: lineage mismatch
Children (6 months–7 years)				
Belshe et al (1998)[32]	Healthy children aged 15–71 months (1996–97)	1602	93% (88 to 96)	Type A: similar H3N2; type B: lineage match
Belshe et al (2000)[33]	Healthy children aged 26–85 months (1997–98)	1358	87% (78 to 93)	Type A: drifted H3N2; type B: not reported (1 isolate)
Vesikari et al (2006)[34]	Healthy children aged 6–<36 months attending day care (2000–01)	1784	84% (74 to 90)	Type A: similar H3N2 and H1N1; type B: lineage match
Vesikari et al (2006)[34]	Healthy children aged 6–<36 months attending day care (2001–02)	1119	85% (78 to 90)	Type A: similar H3N2 and H1N1; type B: mixed lineage
Bracco Neto et al (2009)[38]	Healthy children aged 6–<36 months (2000–01)	1886	72% (62 to 80)	Majority of strains were similar (not reported by type)
Tam et al (2007)[35]	Healthy children aged 12–<36 months (2000–01)	3174	68% (59 to 75)	Type A: similar H3N2 and H1N1; type B: lineage match
Tam et al (2007)[35]	Healthy children aged 12–<36 months (2001–02)	2947	57% (30 to 74)	Type A: similar H3N2 and H1N1; type B: mixed lineage
Lum et al (2010)[36]	Healthy children aged 11–<24 months (2002–03)	1233	64% (40 to 79)	Type A: similar H1N1 and mixed H3N2; type B: mixed lineage

No studies were available for adults aged 50–59 years or children aged 8–17 years. *Authors reported culture, RT-PCR, and RT-PCR/culture; we report RT-PCR/culture results.

Table 3: Randomised controlled trials of live attenuated influenza vaccine (LAIV) meeting inclusion criteria

1.6.2.3 LAIV use in countries outside US

LAIV3 was trialled in countries outside the US and the vaccine was found to be safe and efficacious. Bracco Neto et al conducted a two year multisite study involving children aged 6 to 36 months in South Africa, Brazil and Argentina. Efficacy of 1 or 2 doses of LAIV or placebo against culture confirmed influenza was assessed. In Year 1, vaccine efficacy to the placebo group among recipients of 1 doses of LAIV was 57% (95%CI:44.7, 67.9) and for 2 doses it was 73.5%(95% CI: 63.6,81.0) against matched strains. In year 2, absolute efficacy of a single dose of LAIV was 65.2%, (95% CI:31.2,82.8) for those vaccinated with a single dose of LAIV in year 1, and for those that had two doses 73.6%(95% CI: 33.3,91.2). In year 2, efficacy was 57.0% (95% CI: 6.1,81.7) in subjects who received 2 doses of LAIV in year 1 and placebo in year 2. The efficacy of one dose of LAIV was 60.3% against matched strains and 59.4% against any strain, when those that received a placebo in year one were compared to those that received placebo or one dose of LAIV in year 2. The ability of two doses to confer protection without vaccination the subsequent year was demonstrated (Neto *et al.*, 2009).

1.6.2.4 LAIV use in Africa

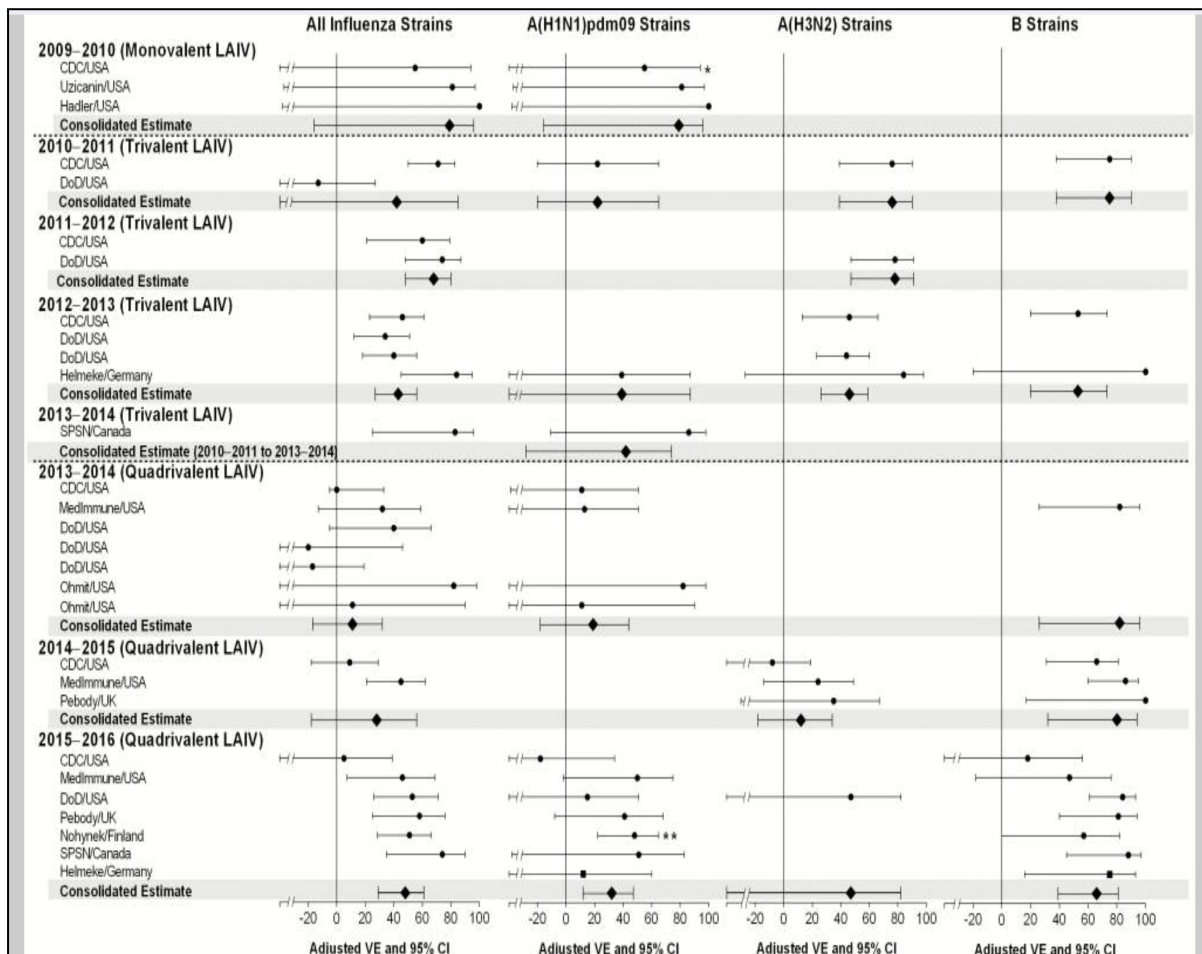
In Africa, very few countries have vaccination policies against influenza and there still remains much to be done in influenza research (Hirve, 2015; Ortiz *et al.*, 2016; Lindsey *et al.*, 2018). The burden of influenza in children under 5 years living in LMIC is high and there is an urgent need for availability of seasonal influenza vaccination in these settings. In Africa, South Africa is one of the few countries offering seasonal influenza vaccination. Compared to IIV, LAIV conferred better protection against severe disease and symptomatic infection in 3009 adults between the ages of 60 and 95 were enrolled in a prospective, randomized, open-label, multicenter trial (Forrest *et al.*, 2011). In another study done in South Africa where LAIV was compared to placebo, LAIV vaccine efficacy against influenza to vaccine-matched strains was estimated to be 42.3% (95% CI: 21.6%, 57.8%). Higher seroconversions rates were observed in LAIV recipients and seroconversion was highest in subjects seronegative at baseline (De Villiers *et al.*, 2009).

1.6.2.5 Change from LAIV3 to LAIV4 and the reduced efficacy of LAIV4

The Ann Arbor-based LAIV3 was subsequently replaced by a quadrivalent formulation (LAIV4, FluMist Quadrivalent, MedImmune), which was licensed in the United States in 2012 and became available during the 2013–14 influenza season. In that same season, A/H1N1 pdm09 circulation was the dominant influenza A virus in circulation and a reduced effectiveness of LAIV against this strain was observed in both 2013–14 and 2015–16 influenza seasons as reviewed by Caspard et al (Caspard *et al.*, 2017). Thermostability and vaccine handling issues were cited as reasons for this reduced efficacy with the A/H1N1pdm09 vaccine strain component (A/California/7/2009(H1N1)pdm09-like. This was later replaced with the more thermostable A/Bolivia/559/2013(H1N1) and used in the 2015–2016 LAIV quadrivalent vaccine (Grohskopf *et al.*, 2016; Pebody, McMenamin and Nohynek, 2018). Despite this change, there was no improvement in effectiveness of LAIV4 against A/H1N1 pdm09, especially in the US. During the 2015/16 influenza season, vaccine efficacy was reported by the CDC, as 3% (non-significant) and −21% (non-significant) against A/H1N1pdm09 compared with 63% (significant) and 65% (significant) for IIV. In children, 2–17 years of age, LAIV4 efficacy against A/H1N1 pdm09 infection was 15% (non-significant) compared with 68% (significant) for IIV in children (Pebody, McMenamin and Nohynek, 2018). It is worth noting that data from UK and Scotland showed efficacy of LAIV against influenza when used in children aged 2–6 years during this season. In England vaccine efficacy was 54.5% (95% CI, 31.5–68.4) for all influenza types combined, 48.3% (95% CI, 16.9–67.8) for A/H1N1 pdm09, and 70.6% (95% CI, 33.2–87.1) for B strains (Pebody, 2017) whilst in Scotland, vaccine effectiveness against all laboratory-confirmed influenza in 4- to 11-year-olds was 63% (95% CI, 50%–72%) and 68% (95% CI, 42%–83%) against clinically diagnosed influenza (NHS, 2015). This reduced effectiveness of LAIV against A/H1N1pdm09 led to Advisory committee on Immunization Practice (ACIP) making the interim recommendation

that LAIV4 should not be used in the U.S. for the 2016–2017 influenza season. Table 3 shows a result from a meta-analysis highlighting the reduced efficacy observed with LAIV vaccines since 2013.

Table 3: Reduced efficacy of LAIV against pandemic H1N1 showing estimates from individual studies as well as consolidated estimates(Caspard *et al.*, 2017). *Abbreviations: Centre for disease control (CDC), Department of defence (DoD), Sentinel practitioner surveillance network (SPSN)*



After not being recommended for use in 2016–17 and 2017–18 influenza season by ACIP, in February 2018, ACIP recommend that for the 2018–19 season, vaccination providers may choose to administer any licensed, age-appropriate influenza vaccinE including LAIV4 (Campbell and Grohskopf, 2018). This was based on data from the vaccine manufacturer showing increased shedding and seroconversion based on haemagluttinin inhibition assay titres (HAI) with a new A/Michigan/45/2015-like pH1N1 strain included in LAIV4, compared with the older formulations. This period of reduced efficacy of LAIV highlights the need for continued research on LAIV vaccines.

1.6.3 Russian derived-LAIV (Ultravac®, Microgen)

The Russian derived LAIV was licensed for use in Russia in 1970. The first evidence of safety and protective efficacy of recombinant LAIV was demonstrated in a large randomized control trial involving 30,000 children 3-15 years old using a bivalent vaccine that consisted of recombinants 47/25/1 (H1N1) and 47/7/2 (H3N2) of wild-type viruses A/Brazil/11/78 (H1N1) and A/Bangkok/1/79 (H3N2) with cold-adapted donor A/Leningrad/134/47/57 (H2N2). The vaccine was shown to be safe and < 1% of vaccines had symptoms of febrile illness for the five days post vaccination. Vaccine receipeints were 50% less likely to develop influenza illness compared to the control group(Alexandrova *et al.*, 1986).

A meta-analysis of 30 studies investigating the effectiveness and efficacy of influenza vaccines (LAIV, US and Russia strains and IIV) in reducing the incidence of influenza-like illness (ILI) or laboratory-confirmed influenza in children was conducted. LAIV vaccine efficacy for silmilar antigens against culture confirmed influenza was 83.4% (78.3%-88.8%) while IIV had an efficacy of 67.3% (58.2%-77.9%). The effectiveness of LAIV in preventing influenza like illness was 44.3% (42.6%-45.9%) compared to 42.6% (38.3%-47.5%) for IIV. LAIV was more efficacious in preventing influenza infection but a similar effectiveness against influenza like illness was observed (Lukšić *et al.*, 2013).

1.6.4 Russian derived-LAIV produced by the Serum Institute of India Pvt Ltd (SIIPL, Nasovac-S)

As part of the effort to increase access to influenza vaccines to developing countries, WHO's Global Pandemic Influenza Action Plan led to the transfer of the Russian-derived LAIV production technology to several vaccine manufacturers in LMIC including SIIPL. In 2009, SIIPL produced the pH1N1 monovalent vaccine (Nasovac™) which went through clinical trials for safety and efficacy. In 2010, it was licensed for use in India for persons aged 3 and over. SIIPL went on to develop a trivalent LAIV based on the same Russian derived-LAIV MDV (Nasovac-S), incorporating influenza A/pH1N1, A/H3N2 and influenza B strains. One dose of Nasovac-S for prevention of influenza in persons aged 2 and over was licensed and launched in India in July 2014 and prequalified by WHO (Rudenko *et al.*, 2016). Some of the efficacy and immunogenicity studies done using Nasovac-S are discussed below.

A phase 2, randomized, placebo-controlled trial in Bangladesh assesed immunological responses and viral shedding after LAIV vaccination. After vaccination, the percentage of children that had shedding was 45.3% for A/H3N2 and 67.3% for B vaccine strains but no shedding for A/H1N1 vaccine strains. For prevaccination HAI titres, 72% had antibodies to A/H3N2, 64% for A/H1N1 and 48% for B vaccine strains. The post vaccination HAI antibody titres induced post LAIV were 10.0% for A/H1N1, 32.7%, for A/H3N2 and 40.0% for B strains compared to 4% for A/H1N1,

6.0% for A/H3N2 and 9.3% for B the placebo group. Serum and mucosal antibody geometric mean titers (GMTs) for LAIV versus placebo groups for A/H3N2 and B were higher post vaccination while for A/H1N1, only the mucosal IgA GMT was significantly higher than placebo at day 21 (KDC, Lewis *et al.*, 2018).

Two phase 3 efficacy studies of Nasovac-S were subsequently conducted in Bangladesh (Brooks *et al.*, 2016) and Senegal (Victor *et al.*, 2016), with contradicting results. In the Bangladesh study, 1174 children aged 2-4 years were given LAIV, with 587 matched controls receiving placebo. Overall vaccine efficacy against vaccine-matched strains was 57·5% (95% CI 43·6–68·0), with 50·0% (9·2 to 72·5) efficacy against pH1N1 strains and 60·4% (44·8 to 71·6) against H3N2 strains. Vaccine efficacy against all circulating strains was 41.0% (95% CI: 28.0 to 51.6) (Brooks *et al.*, 2016).

In the Senegal study, 1173 LAIV vaccinated and 584 placebo control children were included in the per protocol study. A similar incidence (18%) of influenza illness was observed in both groups, giving a vaccine efficacy of 0·0% (95% CI −26·4 to 20·9). Efficacy against all vaccine matched strains was −6·1% (−50·0 to 25·0) and −9·7% (−62·6 to 26·1) for pH1N1. The only vaccine matched strain that circulated enough to assess efficacy was pH1N1 whilst H3N2 circulation was negligible. Viral shedding of any vaccine strain 2-4 days post vaccination was observed in 83% of LAIV vaccinees. For individual vaccine strain shedding, 52% shed A/H3N2, 66% shed B, and 22% shed A/H1N1. (Victor *et al.*, 2016).

1.6.5 Recent concerns with poor LAIV effectiveness

Effectiveness of LAIV vaccine against pH1N1 strains has been on the decline as shown by data from both HIC and LMIC (Victor *et al.*, 2016; Caspard *et al.*, 2017; KDC, Lewis *et al.*, 2018). In LMIC such as Bangladesh and Senegal, Nasovac-S was used with contrasting results. In Bangladesh, vaccine efficacy against pH1N1 was 50·0% (9·2 to 72·5)(Brooks *et al.*, 2016). No efficacy against pH1N1 was seen in the Senegal study (−9·7%, 95% CI −62·6 to 26·1) despite viral shedding : 22% of children shed A/H1N1 vaccine strain.

Around this same period, reduced efficacy of pH1N1 was noted in the US. Despite being produced from different MDVs, the same influenza A/H1N1 strain, (A/California/7/2009 (H1N1)-like) was used in FluMist and Nasovac-S (KDC, Lewis *et al.*, 2018) so it is possible that the issue of heat instability observed with A/California/7/2009 (H1N1) was also present in Nasovac-S (Cotter, Jin and Chen, 2014). A reduced replicative ability of A/California/7/2009 (H1N1) in a human alveolar cell line, reduced attachment of HA to α2,6-linked sialic acid receptors on epithlial cells of the upper respiratory tract was also noted (Ambrose, Bright and Mallory, 2016).

Pre-existing immunity has been shown to play a role in immune response to influenza vaccination (X. S. He *et al.*, 2008; Sridhar *et al.*, 2012; Sridhar, Saranya *et al.*, 2013; Hayward *et al.*, 2015). Prior to the study H1N1 viruses circulated widely in Bangladesh but circulation of this virus was limited in Senegal (Isakova-Sivak, 2016). In Bangladesh , all the children in the study were influenza vaccine naive. In the Senegal study, 512 (44%)of Nasovac-S vaccine recipients and 272 (46%) placebo recipients received trivalent influenza vaccine containing A/California/7/2009 [H1N1] like virus in mid 2010/2011. This shows that some of these children from both countries may have been exposed due to prior circulation of the virus in Bangladesh and vaccination in Senegal thus pre-existing immunity may not have been the reason for the lack of efficacy seen in the Senegal study (Brooks *et al.*, 2016; Victor *et al.*, 2016). The issue of single dose of Nasovac –S compared to double dose of LAIV was also highlighted but in the Bracco Neto et al study in South African children, a single dose of LAIV was shown to confer protection against influenza (Neto *et al.*, 2009). This study also provides evidence that LAIV does indeed work in African settings, albeit with a prior formulation that included pre-pandmic H1N1.

Production cost, mode of administration and a single dose of the vaccine, makes LAIV a great option to use as a seasonal vaccine for resource-limited countries. The negative result seen with LAIV in the Senegal study by Victor et al (Victor *et al.*, 2016) emphasizes the need for inclusion of an immunology arm in vaccine trials. Immunology data available on influenza vaccines were mostly from studies done in the developed world. These response may be different from responses in our setting as seen with the use of oral vaccines, reviewed by Levine et al (Levine, 2010). Therefore it is important to include immunogenicity studies within influenza vaccine trials in this settings to evaluate the benefits of using it. There is however a need for continued research on LAIV vaccines to ensure better protection especially in young children in LMIC were the burden of influenza infection is high.

1.7 Immune responses to Influenza Vaccination

The immune system comprises of innate and adaptive immunity, both of which play an important role in the elimination of abnormal cells and foreign pathogens. The innate immune system provides a swift but nonspecific immunological response to foreign organisms, identified via pattern recognition receptors whilst adaptive immune response is slow but highly specific to an antigen as the cells need to clonally expand in order to generate adequate populations of effector and memory cells (Biron, 2010). During primary exposure, components of the innate immune system, upon pathogen or antigen recognition, get activated and modulate the adaptive immune system leading to immunological memory. In turn, antigen-specific CD4+ T cells upon secondary exposure to a similar antigen or pathogen become activated and produce interleukin-2 (IL-2), which together with other cytokines produced by accessory cells such as macrophages and dendritic cells (DC) can activate natural killer (NK) cells leading to the release of interferon-

gamma (IFN-γ)(Horowitz *et al.*, 2010, 2012; Horowitz, Stegmann and Riley, 2011). The interplay between the two arms is crucial for a protective immune response to natural infection or upon administration of a vaccine.

1.7.1 Innate immune responses

Innate immune cells include, monocytes and macrophages, dendritic cells, basophils, eosinophils, neutrophils and NK cells. However for the purpose of this review, I will focus on the role of monocytes and dendritic cells in influenza infection and vaccination. Upon infection with influenza virus, viral conserved components called pathogen associated molecular patterns (PAMPs) are recognized by host pathogen recognition receptors (PRRs), such as retinoic acid-inducible gene-I protein (RIG-I) and toll-like receptor (TLR), leading to activation of innate immune signaling that induces the production of various cytokines and antiviral molecules. Table 4 below shows the known human TLRs that have been described, their cellular distribution and their ligands. TLR3, TLR7, and TLR8 are PRRs that are expressed on monocytes and dendritic cells. These cells are involved in sensing influenza virus components in cytoplasmic endosomes during viral replication (Chen et al., 2018).

Table 4: Human TLR receptors and their ligands adapted from (Duan *et al.*, 2022)

TLR	Agonist/Ligand	Cellular distribution	Pathogens recognized	Infectious agent
TLR 1 **TLR 1/ 2**	Bacterial lipoprotein, Triacyl lipopeptides Pam3CSK4	Surface	Bacteria	Mycobacteria
TLR 2	Bacterial lipoprotein	Surface	Bacteria	Staphylococcus aureus Listeria monocytogenes Dengue virus
TLR 3	Poly I:C, Poly – ICLC (Hiltonol), Double stranded Ribonucleic Acid (dsRNA)	Intracellular	Protozoa DNA viruses Retroviruses ssRNA	Neospora caninum HSV HIV Respiratory syncytial virus
TLR 4	Monophosphoryl lipid A, Lipopolysaccharide	Surface	Bacteria ssRNA	Staphylococcus aureus Mycobacteria Syncytial virus Rabies virus
TLR 5	Flagellin	Surface	Bacteria	Burkholderia pseudomallei
TLR 6 **TLR 2/6**	Cooperate with TLR2 against Diacyl lipopeptides	Surface	Bacteria ssRNA viruses	Legionella pneumophila Dengue virus
TLR 7	Single stranded Ribonucleic Acid (ssRNA)	Intracellular	Protozoa ssRNA viruses Retrovirus	Leishmania Influenza A HIV
TLR 8	Single stranded Ribonucleic Acid (ssRNA)	Intracellular	ssRNA viruses Retrovirus Bacteria	Influenza A HIV Staphylococcus aureus
TLR 9	CpG DNA	Intracellular	DNA viruses	HSV-1, HSV-2 (311) HPV (312) Adenovirus
TLR 10	Pam3Cys and FSL-1	Surface	Retrovirus	HIV

1.7.1.1 Monocytes

Human peripheral blood monocytes can be classified into three different subsets based on CD14 and CD16. The classical monocytes express high levels of the CD14 cell surface receptor ($CD14^{++}$ $CD16^{-}$ monocytes). Increased production of tumor necrosis factor alpha (TNF- α) and interleukin 6 (IL-6) and interleukin1 beta (IL-1β) in response to TLR agonists has also been noted in this subset. They are highly phagocytic and exhibit antimicrobial activity. Classical monocytes are among the first cells to be recruited to the site of infection and injury (Zigmond *et al.*, 2014) and this is in part mediated by their increased expression of chemokine receptors such as CCR1, CCR2,CCR5,CXCR1 and CXCR2 that allows them to travel to the site of infection (Weber *et al.*, 2000; Tsou *et al.*, 2007; Wong *et al.*, 2011). They also express very high levels of CD62L (L-selectin) and high levels of inflammatory molecules such as IL-6, IL-8, CCL2,CCL3 and CCL5 in the presence of LPS stimulation (Cros *et al.*, 2010). Classical monocytes were also found to be the main source of monocyte derived DCs with the ability to induce T cell proliferation and IFN-gamma production as opposed to the intermediate and nonclassical monocytes under the same conditions. These monocyte-derived dendritic cells lost their phagocytic capability, a primary function of classical monocytes through the increased expression of CD36 and CD136 markers (Boyette *et al.*, 2017). Upon release from the bone marrow, classical monocytes spend limited time in the blood and will usually die off after encounter with an antigen, develop into a more mature intermediate monocyte (Patel *et al.*, 2017) or leave the circulation through acquisition of DC like markers (Lee *et al.*, 2017), highlighting their plasticity.

The intermediate subset is characterized by high level expression of CD14 and low level expression of CD16 ($CD14^{++}CD16^{+}$ monocytes). They have enhanced pro-inflammatory function and upon stimulation with lipopolysaccharide (LPS) they produce a high amount of TNF- α as well as IL-1beta and IL-6 (Passos *et al.*, 2015; Jakubzick *et al.*, 2017). They are also refered to as inflammatory monocytes. High levels of HLA-ABC, HLA-DR, and CD40 was shown to be expressed by intermediate monocytes. (Weber *et al.*, 2000) as such they have been linked to playing a key role in antigen presentation and activation of T lymphocytes (Wong *et al.*, 2011). Intermediate monocytyes express higher levels of CCR5 and CX3CR1 They also express TLR 2, 4 and 5 receptors which are involved in proinflammatory responses (Wong *et al.*, 2012; Mukherjee *et al.*, 2015).

The non-classical subset express low levels of CD14 and additional co-express the CD16 receptor ($CD14^{+}CD16^{++}$ monocytes). They mostly secrete interferon-α (IFN-α) in response to intracellular TLR3 stimulation and produce lower levels of proinflammatory cytokines. Very high expression of CX3CR1 and low expression of CCR2 is noted in nonclassical monocytes. Nonclassical monocytes develop from the intermediate monocytes and spend the longest time in the blood (Patel *et al.*, 2017). Non classical monocytes are important in antigen presentation as evident by

the high expression of CD86 in this subset. They also express markers of inflammation including SLAN, CD115, siglec and TNFR2 (Wong *et al.*, 2011). They are otherwise refered to as patrolling monocyte because of their ability to patrol the vascular endothelium under both steady and inflammatory conditions (Yang *et al.*, 2014). In influenza A infection, nonclassical monocytes are present in the nasopharynx (Oshansky *et al.*, 2014) and have been shown to respond better to viral stimuli via TLR7 (Cros *et al.*, 2010) and important receptor for immunogenicity of influenza vaccines.

In humans, it is proposed that classical monocytes that originate from the bone marrow mature into intermediate monocytes and finally into nonclassical monocytes. Conversion of classical monocytes to intermediate monocytes in trauma patients was shown to be dependent on TGF-Beta and M-CSF (West *et al.*, 2012).

Monocytes and pDCs are the most infected among all cell types upon stimulation of peripheral blood mononuclear cells (PBMCs) with influenza virus. Within 18 hours of stimulation, monocytes differentiate into $CD16^{-}CD83^{+}$ mature dendritic cells that could rapidly activate T cells (Cao *et al.*, 2012). Irrespective of the subset, monocytes are readily infected with influenza A virus, with almost 50% of monocytes expressing HA after 8-16 hours post infection with Influenza H3N2 strain A/Udorn/72 (Udorn) (Hoeve *et al.*, 2012). Most of the data on monocyte infectivity with influenza virus is obtained from invitro studies. A rapid differentiation of monocytes to mDC has been shown upon infection of human PBMC with both influenza A and B viruses. No effect on the pDC was observed in this study. The authors went further to show that this expansion was dependent on the presence of live virus as UV- and heat-inactivated virus did not induce monocyte differentiation to mDC (Cao *et al.*, 2012).

Monocytes have the ability to present antigens to CD4+ and CD8+T cells through major histocompatibility (MHC) I and II molecules and initiate adaptive immune responses (Randolph, Jakubzick and Qu, 2008; Bio-Rad Laboratories, 2016).

Systems biology approach has been used to study the link between the early innate response to vaccination and the subsequent adaptive responses. Nakaya et al. compared early immune signatures after vaccination with LAIV or IIV and how this predicted post vaccination responses (Nakaya *et al.*, 2011). LAIV and IIV elicit different immune signatures. Leukocyte immunoglobulin (Ig)-like receptor (LILR) family genes are expressed by both innate and adaptive immune cells and is thought to be involved in regulating T cells and autoimmunity. A high expression of these LILR genes, as well as up-regulation of type 1 interferon related genes was noted. The highest number of differentially expressed genes (DEGs) was found in mDC of IIV vaccinees, while the pDC subset generated the highest number of differentially expressed genes in LAIV vaccinees (Nakaya *et al.*, 2011).

Mohanty et al assessed in vivo innate immune responses in monocyte populations in blood pre and post IIV vaccination, from 31 young (aged 21-30 years) and 36 older (aged > 65 years) adults (Mohanty *et al.*, 2014). Inflammatory monocytes were induced after vaccination in both young and older adults peaking at day 2 with a gradual decline to baseline levels at day 28. Classical monocytes were higher at baseline especially for the younger adults, reduced at day 2 post vaccination and eventually increased at day 7 and 28 post vaccination to baseline levels. In classical and inflammatory monocytes, production of cytokines such as TNF- α and IL-6 was induced at day 2 post vaccination and peaked at day 28. At all timepoints post vaccination, cytokine production was age dependent with high levels of TNF- α and IL-6 in the young adults and high levels of IL-10 in the older adults. This was observed for both classical and inflammatory monocytes. Cytokine production after vaccination was associated with antibody responses by HAI. In both the younger and older adults, seroconversion was associated with an increase in the levels of TNF-α and IL-6 at all time points post vaccination for both monocyte populations (Mohanty et al 2014).

In IAV infected patients, an influx of innate immune cells to the nasopharynx has been reported (Vangeti, 2019). The authors also reported a similar frequency of classical monocytes in blood in influenza infected patients compared to controls, however in the nasopharynx, a significant increase in classical monocytes was noted in IAV infected patients but not the healty controls. In a subsequent study, seroconversion post influenza infection was associated with a decrease in intermediate monocytes in blood and nasal wash during the acute phase of the infection (Wong *et al.*, 2021).

A pilot study of UK adults given LAIV showed an expansion of intermediate monocytes in blood at day 3 after vaccination (de Silva et al, unpublished), but the detailed functional properties of these cells and the downstream effects on adaptive immunity are still unknown. The exact role that monocytes play in helping to induce mucosal antibody and systemic T-cell effector mechanisms upon LAIV vaccination is unknown.

1.7.1.2 Dendritic Cells

During primary or secondary immune responses to viral infections or vaccinations, DCs play a part in processing viral antigens for presentation to T cells leading to the activation and proliferation of virus-specific T cells (He et al. 2006). Human DCs are broadly classified as myeloid DCs (mDCs) or plasmacytoid DCs (pDC) and are both derived from the bone marrow progenitor cells and lack the lineage markers CD3, CD19/20 and CD56 which identify T, B and NK cells respectively. The mDCs are also known as the classical DCs and are professional antigen presenting cells. They express markers such as CD11c, CD13, CD33 and CD11b which are markers

specific to myeloid cells. They also express major histocompatability complex II (MHC II) as well as co-stimulatory molecules. They contribute to viral control through phagocytosis and endocytosis. They produce high amounts of IL-12 that activates T cells. The mDC subset can be further divided into two subsets namely mDC1 and mDC2. Plasmacytoid DCs (pDCs) on the other hand express CD123, CD303 and CD30 (Collin, Mcgovern and Haniffa, 2013).

TLR expression differs among the two DC subsets with previous studies showing expression of TLR 7 and 9 mostly on pDCs and TLR 1-6 on the mDC subset (Hornung *et al.*, 2002; Seya *et al.*, 2005). Upon pathogen recognition and activation, these cells produce large amounts of type I IFN that is important in viral responses.

TLR responses are important in viral recognition, as such, Panda et al (Panda *et al.*, 2010) conducted a study in two age groups, the young adults, 21-30 years and the older adults, 65 years. In the young adults, TLR induced cytokine production in mDC population was lower in older adults compared to younger adults. These lower responses seen in mDCs of the older age group influenced the humoral responses. Eighty percent (80%) of young adults seroconverted post vaccination to H3N2 and B whilst in older adults, 8% seroconverted to H3N2 but none for B. TLR induced production of TNF, IL-6 and IL-12 p40 in mDC was seen to correlate with seroconversion in H3N2 and influenza B. IFN-α and TNF- α on pDCs also correlated with seroconversion. TLR-induced production of IL-12/p40 in mDCs was significantly associated with seroprotection to all three strains in the vaccine. Correlation was also seen with IL-12/p40 production in mDCs and TNF-α and IFN-α in pDCs and seroprotection. Seroprotection is a 1:64 increase in HAI titres post vaccination. Seroprotection was also less likely in the older adults. This study highlights how defects in activation of DCs can influence immune response to influenza infection (Panda *et al.*, 2010). It also provides evidence to support the use of adjuvanted vaccines in the elderly to enhance the immune response to seasonal influenza vaccination.

Studies have shown that in blood, a significant decrease in mDC subsets, but not the pDC or the monocyte population in the first few days following vaccination with IIV. This was observed 5 to 10 days post vaccination with the levels returning to baseline after a month. An inverse correlation was observed between this decrease in mDC frequency and HAI antibody responses from baseline to one month post vaccination (Kobie, Treanor and Ritchlin, 2014). However, in influenza infected patients, a decrease in both mDC populations and pDC in blood and an increase of these populations in the nasopharynx was noted in patients compared to healthy controls (Vangeti, 2019).

Nakaya et al used system biology approach to assess immune responses associated with vaccination with LAIV or IIV. 37 interferon- related genes induced by vaccination with either of the vaccines were differentially expressed in PBMC (Nakaya *et al.*, 2011). The highest number of differentially expressed genes (DEGs) was found in mDC of IIV vaccinees, while the pDC subset generated the highest number of differentially expressed genes in LAIV vaccinees (Nakaya *et al.*, 2011; H.I. *et al.*, 2015). The increase in DC populations after LAIV vaccination has been established, the exact role they play with respect to influenza-specific adaptive immune responses has not been studied.

1.7.2 Adaptive Immune responses

The adaptive immune system comprises of the cellular and humoral arms and both of these have been studied after LAIV vaccination, with contrasting results depending on age of the individual. The humoral and cellular immune responses post LAIV vaccination will be discussed separately below.

1.7.2.1 Humoral responses

Both mucosal and systemic humoral immunity is thought to be important in protection against influenza. Infection with influenza A or B viruses induces a protective immunity partly mediated by antibodies directed against the viral HA, which is the main immunogenic target in both natural infections and vaccination (Sridhar, Brokstad and Cox, 2015). Antibodies against NA are also increasingly being recognized as important in protection against influenza although their role is less well known (Wohlbold and Krammer, 2014). Mucosal immune responses, are thought to be one of the main correlates of protection following LAIV but variation in mucosal responses by age as well as difficulty in sampling and assaying mucosal samples has made it difficult to establish its use as a correlate of protection (Ambrose, Christopher S. *et al.*, 2012; Mohn *et al.*, 2018).

When the virus enters the body through the nasal openings and upper respiratory tract (URT), HA and NA specific antibodies control viral entry and replication at the initial stage of the infection. This is key in protection against influenza infection. This occurs primarily through secretory IgA, the major neutralizing antibodies present on the mucosa and IgM which is produced very early upon infection (Chen *et al.*, 2018). LAIV induces mucosal IgA which protects against illness and viral replication levels and this was shown to persist 6 months post vaccination (Rossen *et al.*, 1970; Clements and Murphy, 1986; Clements *et al.*, 1986).

The presence of these antibodies at the site of infection is key for effective viral clearance as demonstrated by Rossen et al. (Rossen *et al.*, 1970) when they showed high IgA levels in prevaccination nasal washings in adults limits influenza infection and thus reduce clinical illness. Brokstad et al looked at the baseline levels of antibody secreting cells (ASC) in blood, tonsils and nasal tissues among 19 adults that were neither exposed nor vaccinated against influenza one year prior to the study . They found the lowest numbers of antibody secreting cells and antibody titres in the tonsils and the blood. In the nasal mucosa however, there was a 10 to 100 fold increase in the antibody titres and the number of antibody secreting cells (Brokstad *et al.*, 2001).

Induction of both systemic and mucosal antibodies was observed in LAIV and IIV adult vaccinees after challenge with H1N1 and H3N2 wild type virus. LAIV was however better at inducing mucosal antibodies, whilst IIV was better at inducing systemic antibodies (Clements and Murphy, 1986). LAIV administration resulted in induction of serum IgA responses in 83% of vaccinees, and serum IgG in 72% of vaccinees. IIV resulted in serum IgA responses in 96% of vaccinees, and

serum IgG in 100% of vaccinees. In the nasal wash samples, IgG induction was seen in 59% of LAIV vaccinees compared to 94% in IIV vaccinees. Mucosal IgA induction was seen in 83% of LAIV vacinees and 38% of IIV vaccinees. Whilst the levels of mucosal IgA waned down rapidly, systemic IgA and IgG were maintained for up to 6 months in most vaccinees regardless of the type of vaccine administered (Clements and Murphy, 1986).

Longetivity of systemic immune responses was also demonstrated in children. Vaccination of children 3-17 years old, with either 1 or 2 doses of LAIV, led to an increase in influenza virus specific memory B cells in blood post vaccination as well as HAI titres (Mohn *et al.*, 2015). At baseline, HAI titres above protective level for H1N1 was found in 66% of children, post vaccination with two doses, titres were increased but the difference with baseline levels was not significant. For H3N2, 53% had protective titres at baseline, these were significantly increased at each time point post vaccination. These responses were maintained for up to 1 year with 78% of the children still having titres above the protective level for H1N1 and 96% for H3N2. Influenza specific IgM memory B cells were high at baseline, increased significantly post vaccination and maintained up to 1 year for H1N1, H3N2 and influenza B strains. IgA memory B cells were induced by all strains post vaccination. After the first dose a significant difference was only seen with influenza B and after the second dose for both influenza B and H1N1. The increase in IgA memory B cells for H3N2 was not significant at all time points post vaccination. IgG memory B cells were the least induced and a significant difference post vaccination was seen with H3N2 and influenza B. These influenza specific memory B cells were all maintained above baseline levels for up to 1 year (Mohn *et al.*, 2015).

The ability of antibodies induced upon LAIV vaccination to protect against infection was demonstrated in human challenge studies in both children and adults. In a placebo controlled trial in 15-71 months old, involving 126 children vaccinated with LAIV and 66 placebo, the authors found that prevaccination levels of HI determined viral shedding regardless of whether the participant received vaccine or placebo (Belshe *et al.*, 2000). Post challenge, only 2% of vaccinated children who were seropositive at baseline shed virus whilst those in the placebo group that were seropositive did not shed virus. Viral shedding was higher in the seronegative group for both vaccine recipients 9% and placebo recipients 37%. Nasal IgA titres were also protective against infection. In the children that had baseline IgA responses, 1% of vaccinated children and 12% of placebo recipients shed virus. In the children that had no IgA responses at baseline, 12% in the vaccine group and 36% in the placebo group had viral shedding. Therefore both serum antibody (HAI) and nasal IgA antibodies correlated with LAIV induced protection.
In human challenge studies, Gould et al showed that in the absence of serum antibody responses, IgA responses were correlated with protection from symptomatic infection (Gould *et al.*, 2017). In adults with low HAI titre to the challenge virus, HAI titres, IgG and IgA specific to HIN1 were

all increased post challenge. Post challenge there was also a correlation between IgG and IgA titres. IgG did not correlate with disease protection but both systemic and mucosal IgA correlated with protection. Protection was measured by viral shedding post challenge (Gould *et al.*, 2017).

To gain a better understanding of the role of nasal IgA in influenza infection, Ambrose et al (Ambrose *et al.*, 2012) used data from three different studies across two years and assessed nasal IgA responses. These were randomized studies comparing vaccination with LAIV or placebo in children 6–36 months of age. A 2-fold increase in IgA titres was seen in LAIV vaccines compared to placebo recipients post vaccination. Geometric mean fold rise of strain-specific nasal IgA to total nasal IgA pre and post vaccination was calculated for year 1 and 2. The ratios for LAIV vaccination in both years ranged from 1.2 to 6.2 whilst for placebo it was 0.55 to 2.2. GMFR for total IgA was also higher for year 1 and 2 together. LAIV recipients had between 1.1 and 2.4 whilst placebo recipients had 0.7 to 1.6. Haemagglutination inhibition titres were also assessed, and for all strains LAIV recipients had a significant increase in HAI titres compared to placebo recipients ($p < 0.001$ for all 3 strains). An association between HAI response and IgA response was observed. An increased HAI titre post vaccination corresponded to higher IgA response and this was seen for all strains. For H1N1, 48% HAI responders had IgA compared to 33% that had no increase in HAI titres. For H3N2, 57% HAI responders had IgA compared to 37% that had no increase in HAI titres. For influenza B, 65% HAI responders had IgA compared to 39% that had no increase in HAI titres. When all the data was pooled across all the different strains in year 1, total IgA ratio was 3 fold higher in subjects without influenza compared to those with influenza whilst in year 2 the ratio was lower, 2.1. When vaccine matched strains were analyzed, a 3 fold increase in IgA was seen in subjects with influenza compared to those without. In year 2 however, influenza illness recorded was low but despite this a 1.4 fold higher IgA level was seen in those without illness compared to those with illness although the difference was not significant . This study highlights the important role of IgA in LAIV induced protection but also the huge variability seen in nasal IgA levels (Ambrose *et al.*, 2012).

Antibody responses to LAIV can be strain specific, Gould et al (Gould *et al.*, 2017) also showed that in H3N2 specific responses, IgA titres increased post vaccination but the fold change increase was lower than that observed for H1N1. There was no increase in H3N2 HI titres post challenge and no correlation between IgA levels post vaccination and protection against infection (Gould *et al.*, 2017). This strain specific responses was again demonstrated by Weinberg et al (Weinberg *et al.*, 2016) in a study involving both HIV negative and HIV positive children. The authors showed that LAIV was safe in HIV positive children and their status did not affect their antibody responses. Responses to vaccine pH1N1-09 strain, the circulating H1N1-14 strain and B Yamagata were assessed post vaccination in young children. HAI titres for H1N1 strains were similar at baseline but higher than the titres for B Yamagata. These were all increased post

vaccination, but titres for B Yamagata were still lower than the pre vaccination titres of the H1N1 strains. For nasal IgA responses, baseline titres were similar for all strains and increased post vaccination but the fold increase for B Yamagata was higher than the H1N1 strains (Weinberg *et al.*, 2016).

Serum antibodies are quantified by haemagglutination inhibition assays. The assay is dependent on the hemagglutinin protein on the viral surface to bind to sialic acids on the surface of red blood cells (RBCs). Presence of specific anti-HA antibodies inhibits agglutination which will otherwise occur between the virus and the red blood cells. Turkey red blood cells or horse red blood cells are used in the assay, depending on the virus being tested. The highest dilution of antibody that prevents hemagglutination is called the haemagglutination inhibition (HI) titer. A HI titer threshold of 1:40 is generally considered to provide 50% reduction in the risk of influenza and about 80% of people develop this after natural exposure (Sridhar, Brokstad and Cox, 2015). This is based on a large human challenge study involving 1032 adult volunteers, challenged with different strains of influenza A and B conducted where levels of HI antibodies were determined prior to infection with influenza(Hobson *et al.*, 1972). While this threshold is considered a correlate of protection from influenza following IIV in healthy adults, the exact requirements in children or immunocompromised hosts is not known (Belshe *et al.*, 2000; Gould *et al.*, 2017). Furthermore, as LAIV does not induce significant serum antibodies yet protects from influenza infection, this correlate of protection is also less applicable (Sridhar, Brokstad and Cox, 2015).

1.7.2.2 Cellular responses

Adaptive cellular immune responses to influenza includes both CD4+ and CD8+ T cells. This requires activation of a naïve T cells by an antigen presenting cell such as DC. Three signals are needed for a T cell to be activated, they include binding of MHC-peptide complex to T cell receptor, binding of the B7 costimulatory molecule to CD28 on the T cell surface and inflammatory signal mediated by cytokines such as IL-12. MHC presentation can either be via MHC-I or MHC-II, based on peptide size. MHC Class I binds peptides with 8-10 residues whilst MHC-II binds longer peptides of 13-25 residues. CD4+T cells are activated via MHC Class II whilst CD8+T cells are activated via MHC Class I(Charles A Janeway *et al.*, 2001). Prior to binding of antigen to MHC, the antigens are processed via multiple ways. Exogenous antigens are obtained from outside the cell and can be from bacteria, viral, protozoal, parasitic and fungal infections. They are usually presented by APCs in the context of MHC Class II, leading to CD4+T cell activation. Endogenous antigens on the other hand are derived from proteins produced within the cells and usually arise from viral infections and tumor cells. These endogenous antigens are presented in the context of MHC Class II and activate CD8+T cells. Endogenous antigens can be processed and presented by any nucleated cell. Cross presentation is another process by which antigens can be processed and has been described in the context of influenza A infection. It describes the process by which exogenous antigens captured by phagocytic APCs such as DCs are processed and presented in the context of MHC-Class I to activate CD8+ T cells (Smed-Sörensen *et al.*, 2012). Upon activation, naïve CD4+T cells can differentiate into effector cells such as Th1, Th2, Th9, Th 17, Tfh and Tregs (Luckheeram *et al.*, 2012).

In human challenge studies of influenza infection, CD4+ T cells were shown to control influenza associated illness (McMichael *et al.*, 1983) whilst CD8+ T cells protect against infection (Lanthier *et al.*, 2011). LAIV has been shown to induce both cellular and humoral responses (Lanthier et al. 2011; Mohn et al. 2018; He et al. 2006) and shows higher efficacy and better immune responses in children compared to adults (Ambrose, Levin and Belshe, 2010). However, unlike IIV, no correlates of protection exist for LAIV.

1.7.2.2.1 CD8 T cell responses

CD8+T cells are important in killing virally infected cells through release of perforin and granzyme or through cytokine release. Upon vaccination with LAIV, cytokines and chemokines such as CCR5 and CXCL8 are produced, with this inducing activation of CD8+ T cells to contribute to viral clearance by enhancing cytolytic activity in them (Lanthier *et al.*, 2011). CD8+ T cell responses are mainly directed to the internal proteins nuclearprotein (NP) and to a lesser extent matrix protein 1 (M1) and polymerase basic protein 1 (PB1)(Chen *et al.*, 2014). Because of the conserved nature of these proteins, mutations occur at a lower rate compared to HA and NA. CD8+ T cells have the ability to confer cross protection against different influenza strains (Sridhar,

Saranya *et al.*, 2013). The 2009 H1N1 pandemic presented the opportunity to carry out this experiment. Forty three (43) participants lacking pH1N1-neutralizing antibodies through the pandemic were followed up to assess presence of crossreactive T cells and cellular responses to the internal nucleoprotein, matrix 1 and polymerase basic 1 and their association with severity of influenza illness. The 12 participants that developed illness without fever had a higher frequency of pre-existing crossreactive total cytokine-secreting T cells to live pH1N1 virus compared to the 13 that were ill and had fever. These cells were identified as CD8+IFN-γ+IL-2− crossreactive T cells. Seventy percent (70%) of them expressed CCR5,and 33% expressed CD107a/b in response to live pH1N1 virus. CCR5 is a chemokine that has been shown to facilitate recruitment of memory T cells to the lungs (Kohlmeier *et al.*, 2008) during respiratory viral infections and CD107a is a cytotoxic marker thus demonstrating the ability of these cells to kill virally infected cells and home to the lungs upon influenza infection. Presence of these cells correlated with less severe influenza illness and reduced viral shedding (Sridhar, Saranya *et al.*, 2013). Influenza specific CD8+ T cells have also been shown to confer cross protection against other strains of the influenza virus (McMichael *et al.*, 1983), McMicheal et al challenged volunteers with a high doses of a LAIV and monitored them for 7 days. Lower viral shedding was associated with the frequency of pre-existing MHC class 1 restricted CD8+T cells. This highlights the ability of CD8 T cells to confer cross protection (McMichael *et al.*, 1983). Studies have shown that conserved pre-existing cross reactive CD8+T that are generated during seasonal epidemics and pandemics are important in protection against severe illness and prevent deaths during subsequent pandemics as seen with the 1918 influenza pandemic (Guus F. Rimmelzwaan *et al.*, 2009; Quiñones-Parra *et al.*, 2014, 2016; Short, Kedzierska and van de Sandt, 2018). The level of pre-existing T cells needed for protection was determined in a study by Hayward et al (Hayward *et al.*, 2015). They monitored individuals infected with pH1N1 influenza and influenza specific T cells were quantified during seasonal and pandemic influenza seasons of 2006 to 2010. Forty three (43%) of the participants had baseline pre-existing T cells to different influenza peptides dominated by nucleoprotein, followed by matrix and polymerase basic 1, with a median total influenza specific T-cell response of 83 spot formimg units per million periheral blood mononuclear cells (83SFU/ PBMC). Cross reactivity between pre-existing influenza nucleoprotein and matrix specific T cells to pandemic H1N1 2009 (H1N1pdm2009) was observed. A pre-exposure NP- specific T-cell response greater than or equal to 20 SFU/PBMC was significantly associated with reduction in viral shedding and this effect was significant for both seasonal and pandemic influenza demonstrating heterotypic potential of pre-existing T cells (Hayward *et al.*, 2015).

In children, induction of pre-existing T cells was also described and were shown to last for over 6 months after LAIV vaccination (Mohn, Kristin G.I. *et al.*, 2017). Fourteen (14) children were vaccinated with trivalent LAIV to test for influenza-specific cross-reactive T cells in blood and

determine their specificity and durability post vaccination. To assess cross reactive responses, viral strains that the children could not have been exposed to were used ('heterologous strains'). Between 70 to 83% of children had pre-existing IFN- γ+ T cells to internal proteins of the heterologous strain, compared to 57 to 70% to the vaccine strain. These pre-existing T cell responses were above the suggested protective titre of 100 SFUs/10^6 PBMCs (Forrest *et al.*, 2008). Post vaccination, out of the 14 children, only 3 children had HA to the heterologous Switzerland/13(H3N2) strains after LAIV vaccination. When T cell responses to internal proteins were assesed, 6 children had a significant increase in IL-2+ and IFN-γ+IL-2+ secreting CD8+ T cells. This study highlights that in the absence of detectable haemagluttin antibodies, a detectable T cell response was noted and LAIV vaccination boosted pre-existing T cells (Mohn, Kristin G.I. *et al.*, 2017).

Phenotypic changes to CD8+ T cells in blood occur after vaccination and these changes are dependent on age as well as the type of vaccine used. At baseline, there is a higher number of IFN-γ+CD8+T cells in adults compared to children but expression of CD27 and perforin on CD8+ T cells is higher in the children. Upon vaccination with LAIV in adults, there was no increase in influenza specific IFN-γ+CD8+CD27+ T cells but a significant increase was seen with IIV vaccination. In children however, there was no increase in influenza specific CD8+CD27+ T cells with both LAIV and IIV vaccines. In children receiving IIV vaccines, IFN-γ+CD8+T cells producing perforin increased significantly 28 days post vaccination. No increase in IFN-γ+CD8+ producing perforin was seen for children receiving LAIV or adults receiving either IIV or LAIV vaccination. The exact role the CD8+T cells play in the immune response to LAIV remains unclear. Some studies show an induction of CD8+ T cells (Mohn, Kristin G.I. *et al.*, 2017) whilst other studies showed no induction after vaccination with LAIV (X. S. He *et al.*, 2008; Weinberg *et al.*, 2016).

1.7.2.2.2 CD4 T cell responses

CD4+T cells contribute to clearance of pathogens in multiple ways. These include cytokine production leading to the recruitment of other immune cells to the site of infection, cytotoxicity, B cell activation, differentiation, and subsequent antibody production and isotype switching . Antibodies were thought to be the main mediators of protection against influenza illness, however, it is increasingly becoming clear that CD4+T cells are also important. CD4+ T cell reponses are mainly directed at the internal MP1 and NP proteins (Chen *et al.*, 2014). Studies have shown that at day 6-7 post vaccination with LAIV, there is an upregulation of genes that drive T cells to become activated and differentiate into different T cell subsets. Cytokines such as IL-2 and IFN- γ which are important in T cell activation and differentiation also peaked at this point (Panapasa *et al.*, 2015).

In a human challenge study by Wilkinson et al, the role of T-cells in controlling influenza was assessed in blood (Wilkinson *et al.*, 2012). T cell responses to surface and internal proteins of influenza were monitored in healthy adult volunteers with no pre-existing antibodies to challenge virus H3N2 or H1N1. A significant increase in pre-existing IFN-γ+CD4+T cells with cytotoxic capabilities responding to peptides specific to matrix and nuclearprotein at day 7 post challenge was observed. A correlation between these CD4+T cells and a decrease in severity of infection and viral shedding was observed (Wilkinson *et al.*, 2012). In addition to this, Sridhar et al (Sridhar *et al.*, 2015) followed patients during the H1N1 2009 pandemic as they developed infection and showed that lower levels of pre-existing CD4+T cells that produce IL-2 and CD4+IL2+TNF-α-IFN-γ- induce strong antibody responses after influenza infection. These antibodies were maintained for up to 1.5 years post infection with titres above the protective level (Sridhar *et al.*, 2015).

LAIV has the ability to induce cellular immune responses and this makes it a better option compared to IIV. Mucosal IgA are among the first line of defence upon influenza infection, but when the virus evades the antibody responses it can then be targeted by T cells. In studies comparing immune responses to LAIV and IIV vaccination in blood , only LAIV induced significant T cell responses post vaccination (He *et al.*, 2006). Children were also better at inducing T cell responses compared to adults. At baseline, children had lower levels of IFN-γ producing CD4+ and CD8+T cells after stimulation with influenza virus, compared to adults. Post vaccination with LAIV, IFN-γ producing CD4+ and CD8+ T cells increased significantly in children but the only increase in adults was in CD8+T cells at day 10. In these children, the increase in IFN-γ producing CD4+ and CD8+T cells were significant at day 10 and day 28 post LAIV vaccination compared to baseline levels. For IIV, the only significant increase observed in children was in the CD4+T cells, day 10 post vaccination and in adults day 28 post vaccination. Fold change in IFN-γ responses at day 10 and 28 post vaccination was significantly higher in the children compared to the adults. LAIV vaccinees had higher fold change in IFN-γ production by CD4+T cells compared to IIV vaccinees and this was significant at all timepoints (He *et al.*, 2006). Contrary to these findings of low levels of T cell increase post IIV vaccination in adults, Chirkova and colleagues showed an increase in both CD4+ and CD8+T-memory-cell responses in peripheral blood of healthy young adult volunteers that received two doses of live attenuated influenza A (H5N2) vaccine (Chirkova *et al.*, 2011). Significant increase in T cells post vaccination was seen at day 63 post vaccination in the CD8+T subset. In the CD4+T cells however, significant increase was seen at day 42 and 63 post vaccination.

As the search for correlates of protection to LAIV continues, He et al (He *et al.*, 2006) identified correlates of immune response post vaccination. These include age, type of vaccine and pre-existing immune response. These correlates were tested in samples from another dataset during the 2005 influenza season involving 56 participants, in which a different influenza A/H3N2 strain

was used in both the vaccines and the assays. An association was seen between the baseline percentage of influenza A specific CD4+ T-cells and post vaccination fold change in influenza specific CD4+ T-cells. An association was also seen between HAI response and baseline HAI titer. Fold change in HAI titre was associated with the type of vaccine used. A lower baseline immune response was associated with higher responses post vaccination (X. S. He *et al.*, 2008).

Cytokine prodution is among the mechanisms in which CD4+T cells contribute to immune response to infection. In the study by Mohn et al (Mohn, Kristin G.I. *et al.*, 2017), LAIV vaccination induced production of CD4+ T cells that produced single and multiple cytokines, including IFN-γ, IL-2, or TNF-α. The frequency of these multifunctional CD4+T cells was increased after receiving both dose 1 and dose 2. These cytokine responses were maintained till day 56 with significant increase in the cytokine levels compared to pre-vaccination levels. In previous studies cytokine production from CD4+ T cells have been shown to induce innate immune cells such as monocytes, DCs and natural killer cells thus enhancing the immune response upon infection or vaccination (Mohn *et al.*, 2018).

There has been concerns about lowering immune responses with repeated annual vaccinations (Castrucci, 2018), however, a 4 year follow up study on health care workers vaccinated repeatedly showed that this was not the case. Fourteen (14) health care workers were followed up from 2009 during the pandemic to 2013 and during this period the seasonal influenza vaccine used, contained H1N1pdm09. The authors reported that prevaccination H1N1pdm09-specific T cells, antibodies, and memory B cells were significantly increased after 3–4 repeated vaccinations and maintained at high levels throughout seasons 2012 and 2013. These cross-reactive IFN-γ-secreting CD4+T cells recognizing external or conserved internal epitopes were also maintained throughout 2012 and 2013. The authors concluded that annual seasonal vaccination improved the multifunctional memory CD4+ responses. IFN-γ+TNF-α+CD4+ T cells and HI antibodies were significantly increased after each vaccination (Trieu *et al.*, 2018).

To date, only few immunogenicity studies have been carried out, all in South Africa (Forrest *et al.*, 2011). Around 3009 adults aged 60-95 years were randomized to either receive a single dose of LAIV or IIV. Higher T cell responses to influenza A but not influenza B, was seen in LAIV recipients compared to IIV recipients, with IIV recipients having significantly greater responses to influenza B (Forrest *et al.*, 2011).

1.7.2.2.3 Follicular helper T cell responses

Follicular helper T (Tfh) cells are a type of CD4+T cells that provide help to B cells. These cells are important for germinal center formation, affinity maturation and the development of high affinity antibodies and memory B cells. Tfh cells express the chemokine receptor CXCR5, needed for migration into the B cell follicles, inducible costimulator (ICOS) which are highly expressed and

involved in development of Tfh cells, interleukin-21 (IL-21) and the transcription factor B cell lymphoma 6 (BCL6) which are important in the differentiation of Tfh cells (Bentebibel *et al.*, 2013; Aljurayyan *et al.*, 2018). Accessing and assessing Tfh cells from lymphoid sites in human studies is challenging but a functionally similar subset has been identified in the periphery as reviewed by Crotty et al 2014. A matured Tfh cell becomes a germinal center (GC) Tfh and will either a) enter a new GC, b) enter a new B cell follicle and undergo another round of somatic hypermutation within the same GC or exit the GC into the blood by downregulating BCL6 and expressing the memory marker, CD45R0+. These CD45R0+CXCR5+ Tfh cells also have the potential to provide help to B cells outside the GC or can be transitioning into a newly activated Tfh cell that has not entered the germinal centre (Crotty, 2014).

Blood Tfh cells can be divided into different subsets with distinct phenotypes and functions based on the expression of CXCR3 and CCR6, with 30 to 50% of blood memory Tfh cells expressing these two markers. The Th-1-like Tfh subset is identified as CXCR3+CCR6-: they express the transcription factor T-bet and mainly produces IFN-γ but lack the capacity to induce naive B cells. The Th-2-like Tfh subset is identified as CXCR3-CCR6-: they express the transcription factor GATA-3 and mainly produces IL-4, IL-5 and IL-13 and can induce naive B cells to produce IgG and IgE and to undergo isotype switching through IL-21 secretion. Finally, the Th-17-like Tfh subset is identified as CXCR3-CCR6+: they express transcription factor RORγT, produce IL-17A and IL-22 and induce IgA secretion (Schmitt, Bentebibel and Ueno, 2014).

In human peripheral blood, the Tfh subsets that predicted antibody responses and long term persistence of antibody titres was assessed in 42 healthy adults receiving a single dose of trivalent inactivated influenza vaccine, (IIV), MF59 adjuvanted trivalent inactivated influenza vaccine (AIIV), or saline placebo (Spensieri *et al.*, 2016). An increase in frequency of plasmablasts and antibodies to all 3 viral strains in the vaccine was observed with both vaccines compared to placebo. Antibodies peaked at day 28 and were maintained for up to 6 months. An expansion of Tfh cells that expressed ICOS and PD-1 increased in circulation day 7 post vaccination and reduced to baseline levels day 28 post vaccination. After stimulation with A/California/7/2009 (H1N1) subunit antigen, the numbers of total circulating CD4+ICOS+ Tfh cells and H1N1- specific CD4+IL-21+ICOS+CXCR5+ cells correlated with antibody responses post vaccination, independent of pre-existing antibody levels. In a subsequent study by Bentebibel et al, the authors assessed the contribution of these ICOS+PD1+ Tfh cells towards the quantity and the avidity of antibodies produced after IIV vaccination (Bentebibel *et al.*, 2016). An increase in the amount and the avidity of influenza-specific antibodies occurred the first 7 days after IIV vaccination and this strongly correlated with the increase of circulating ICOS+PD-1+CXCR3+ Tfh cells.

The antibody responses to influenza vaccination is poor in older adults (H.I. *et al.*, 2015), a high risk group for influenza infections. There is a need to understand the mechanism underlying generation of protective antibody responses in this age group. In older adults, a 35% decrease in the frequency of circulating Tfh cells seen in younger age groups was observed. These Tfh cells in older adults at baseline had a higher expression of ICOS and a decreased ability to give B cell help. Similar circulating levels of influenza-specific IgM and IgG antibodies were recorded for both groups but after vaccination. Compared to baseline levels, the total influenza-specific IgM and IgG responses in young adults at day 7, were 3.5-fold and 1.2-fold greater than that seen in older adults. The reduced Tfh responses in adults may explain why older adults respond poorly to influenza vaccination (Herati *et al.*, 2014).

Traditionally, licensed trivalent influenza vaccines contain 15 µg of the HA of each component (A/H3N2, A/H1N1, B) but with the need to produce vaccines that elicit better responses in the older age group, vaccines with 60 µg of the HA of each component (A/H3N2, A/H1N1, B) were produced. An increase in circulating Tfh cells expressing ICOS, CD38 or Ki67, as well as plasmablasts after vaccination with the high dose vaccine was observed (Couch *et al.*, 2007). LAIV induced Tfh proliferation, which correlated with anti-HA antibody production upon stimulation of human nasopharynx-associated lymphoid tissue (NALT) with live-attenuated influenza vaccine (LAIV). In the same study, an increase in Tfh-like ($CXCR5^+$ $ICOS^+$) $CD4^+$ T cells at day 7, as well as anti-HA IgG and IgM antibodies measured in the culture supernatants was seen upon PBMC stimulation with LAIV (Aljurayyan *et al.*, 2018).

To date, no studies have assessed Tfh responses in humans vaccinated with LAIV. The role of Tfh in LAIV-induced immune responses, both with regards to helping mucosal and systemic antibody responses as well as CD4+ and CD8+ T-cell responses is currently unknown.

1.8 Literature review conclusion (rationale for studying immune response to LAIV in African settings)

Although both the correlates of protection and immunological pathways that lead to robust adaptive responses are reasonably well established for IIV (Nakaya *et al.*, 2011; Bentebibel *et al.*, 2013, 2016; Mohanty *et al.*, 2014) neither of these are clear for LAIV. LAIV induces both cellular and humoral immunity, unlike IIV (He et al. 2006). These humoral and cellular responses can persist beyond 6 months and up to 1 year potentially protecting against infection prior to revaccination in the subsequent influenza season (Clements and Murphy, 1986; Mohn *et al.*, 2015). LAIV responses are better in children compared to adults (Rhorer *et al.*, 2009) and the ability of the vaccine to confer protective efficacy even with drifted strains of influenza (Osterholm *et al.*, 2011) makes it an ideal vaccine for consideration in the search for a universal influenza vaccine.

In order to better understand why LAIV works in some individuals and populations but not in others, a clearer account of the immunological mechanisms involved in LAIV-induced immunity is required. Immunogenicity data on LAIV was mostly generated from studies using the US derived LAIV. Little or no immunogenicity data exist for the Russian derived LAIV and the LAIV developed by SIIPL . There is hope that Nasovac-S will be rolled out for use in LMIC for seasonal influenza vaccination. However before this happens, it is important to generate data on immune responses to LAIV generated from our own setting. This data will provide information for generation of future LAIVs as well as its use in LMIC.

My PhD project is an exploratory study, and I aim to assess in detail the phenotype and function of the innate and adaptive immune cells involved in the response to LAIV. I will explore how the interplay between the different arms of the immune system impacts the adaptive immune responses that leads to protection from influenza infection and disease.

1.9 PhD Research questions

The PhD proposal will cover the following main aims and research areas:

Aim 1

1. To characterize the detailed phenotype of innate immune cells pre and post vaccination with LAIV.
2. To assess the relationship between innate responses and later humoral immune responses post vaccination with LAIV.

Aim 2

1. To assess the magnitude of CD8+ and CD4+ T-cell in response to LAIV vaccination.
2. To assess the phenotype of influenza-specific CD8+ and CD4+ T-cells in the blood at baseline and post vaccination with LAIV.

Aim 3

1. To characterize the detailed phenotype of peripheral Tfh cells pre and post vaccination with LAIV.
2. To see if the changes observed will correlate with mucosal IgA, serum haemagglutination inhibition titres and systemic CD4+ and CD8+ T-cell responses.

2 Chapter 2: Materials and methods

This PhD project was nested within the study titled, "A study of intranasal Live Attenuated Influenza Vaccine Immunogenicity and associations with the nasopharyngeal microbiome among children in the Gambia (NASIMMUNE)", (SCC1502). This was the first immunogenicity study of LAIV in Sub Saharan Africa undertaken in children. It was funded by a Wellcome Trust Intermediate Clinical Fellowship awarded to Professor Thushan de Silva (TIdS;110058/Z/15/Z).

2.1 Study site

The study was based in Sukuta, a peri-urban settlement in Kombo East, Western Region with an estimated population of 17,000 (2003 census). There is a main Government hospital within the community called the Sukuta Health Centre, within which MRC operates a smaller clinic run by a team of clinicians, nurses and field workers. The team has successfully conducted multiple clinical trials and vaccine studies since its establishment in 2001. All samples used in the study were collected at the clinic and delivered to the laboratory for processing.

2.2 Ethical approval

The Scientific Cordinating Committee of the MRC Gambia at LSHTM, The Gambia Government/MRC Joint Ethics Committee and the Medicines Control Agency of The Gambia gave approval for the study to be conducted. Parents provided consent, which was written or thumb printed for their children to be enrolled in the study. An impartial witness was present during the informed consent discussion if the parent was not English literate. The information was translated in a local language for easier understanding then the form signed by the impartial witness to confirm completeness of the consent provided.

2.3 NASIMMUNE Study design, randomization and study participants

The study was an open-label, phase 4 randomized controlled trial (RCT) (NCT02972957) carried out between June to October of 2017 and 2018. The study participants were randomized to receive LAIV on the day of enrolment (day 0) or delayed until day 21 (control Group C). The RCT design (children randomised 2:1 to receive LAIV or no vaccine) was based on the primary aims of exploring the interaction between the nasopharyngeal microbiome.

Prior to being enrolled into the study, informed consent was obtained from parent/guardian of the study participant and a unique subject identification number allocated, the details of which were recorded on the screening and enrollment log template, Appendix 6. A set of inclusion and exclusion criteria was used to assess the eligibilty of each participant to take part in the study after which they where randomized into one of three equally sized groups (Groups A, B and C).

Randomization was done using pre-prepared, sealed opaque envelopes to ensure allocation concealment. Block randomization stratified by gender was undertaken using a computer generated randomization sequence pre-prepared by an individual that was not part of the study or its analysis. To avoid allocation bias, members of the study team where blinded on the block size and randomization sequence. During the randomization process, envelopes where picked up in numerical sequence. The randomization list was then cross-checked by another staff member after which both staff will sign.The envelope is then opened by the staff to know the group to which a participant has been randomized. Three hundred and sixty five children aged 24–59 months were recruited into the main study based on inclusion and exclusion criteria as outlined in Appendix 4. Thirty- five children were recruited to Group D to receive azithromycin prior to LAIV, for exploratory aims investigating how modulating the microbiome may affect LAIV immunogenicity. They were recruited sequentially after recruitment in Groups A, B and C were completed, during the 2nd recruitment season (January – June 2018). The exact number of participants randomized to each group is shown below in Table 5.

Table 5: Randomization of participants to different groups

Year	Group A	Group B	Group C	Group D- Azithromycin	Gender
2017	**30 Intracellular cytokine staining Innate panel**	**31 Intracellular cytokine staining Innate panel Tfh panel**	**31**		**Male**
2018	**27 Intracellular cytokine staining T cell phenotyping**	**27 Intracellular cytokine staining Tfh panel T cell phenotyping**	**27**	**16 Tfh panel T cell phenotyping**	
2017	**28 Intracellular cytokine staining Innate panel**	**29 Intracellular cytokine staining Innate panel Tfh panel**	**29**		**Female**
2018	**20 Intracellular cytokine staining T cell phenotyping**	**20 Intracellular cytokine staining Tfh panel T cell phenotyping**	**21**	**16 Tfh panel T cell phenotyping**	

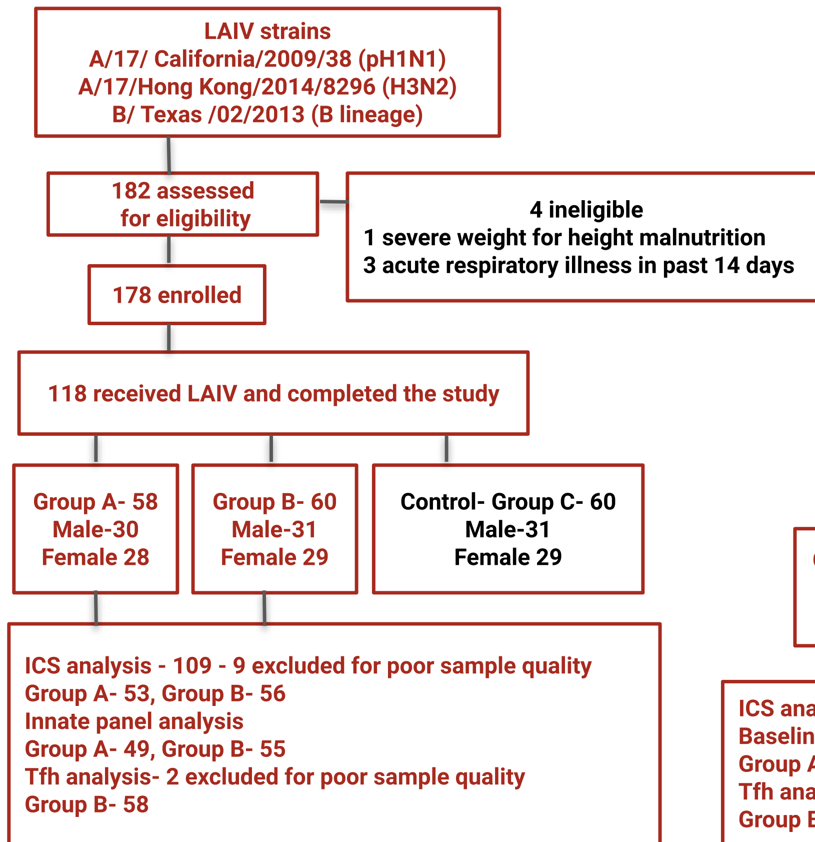
2.4 Vaccination

One 0.5 mL intranasal dose of Nasovac-S, a trivalent live-attenuated influenza vaccine (LAIV) was administered. This was formulated by the Serum Institute of India Pvt. Ltd, Pune, India and was based on the nothern hemisphere formulation according to current WHO recommendations. The 2017 vaccine formulation contained A/17/California/2009/38 (pH1N1), A/17/Hong Kong/2014/8296 (H3N2) and B/Texas/02/2013-CDC-LV8B (B-Victoria lineage). In 2017-18, the pH1N1 Cal09 strain (A/17/California/2009/38) was updated according to WHO recommendations to an (A/17/New York/15/5364, NY15). The 2018 vaccine formulation was A/17/New York/15/5364 (pH1N1), A/17/Hong Kong/2014/8296 (H3N2) and B/Texas/02/2013-CDC-LV8B (B-Victoria lineage). All strains were propagated in embryonated hen's eggs.

My PhD studies was done on samples from vaccinated children only, therefore represented an observational immunogenicity study. Group A and B differed only in their sampling schedule. Children in group A were bled at baseline, day 2 and day 21 post-LAIV, whilst children in Group B were bled at baseline, day 7 and day 21. For participants in Group D, their sampling schedule mirrored that of Group B and therefore I included children from this group in my project. The demographic characteristics, baseline and post vaccination influenza serological data is shown in Table 6 below and study profile is shown in Figure 3. Both are adapted from (Lindsey *et al.*, 2019).

Table 6: Demographic characteristics and baseline and post LAIV influnza serological data

	2016-17 LAIV (n=118)	2017-18 LAIV (n=126)	P value
Age (months)	35.1 (28.3 - 44.9)	35.3 (28.0 - 40.5)	p = 0.435
Sex			p = 0.608
Female	57 (48.3)	56 (44.4)	
Male	61 (51.7)	70 (55.6)	
Tribe	96 (81.4)	99 (78.5)	p = 0.265
	5 (4.2)	7 (5.5)	
	3 (2.5)	5 (4.0)	
	6 (5.1)	4 (3.2)	
	2 (1.7)	5 (4.0)	
	5 (4.2)	1 (0.8)	
	1 (0.9)	5 (4.0)	
Baseline seropositive (HI titre ≧1:10)	39 (33.1)	62 (49.2)	p = 0.013
	90 (76.3)	70 (55.6)	p < 0.001
	25 (21.2)	54 (42.9)	p < 0.001
Median HI titre in children seropositive at baseline (IQR)	160 (80 - 160)	226.3 (160 - 320)	p < 0.001
	160 (80 - 160)	160 (80 - 320)	p = 0.156
	160 (80 - 226.3)	226.3 (160 - 320)	p = 0.015
Seropositivity to each antigen post LAIV			
pH1N1	6	24	
H3N2	26	35	
B/Vic	40	30	
Total number of seropositive children post LAIV	72 (61.02)	89 (70.63)	



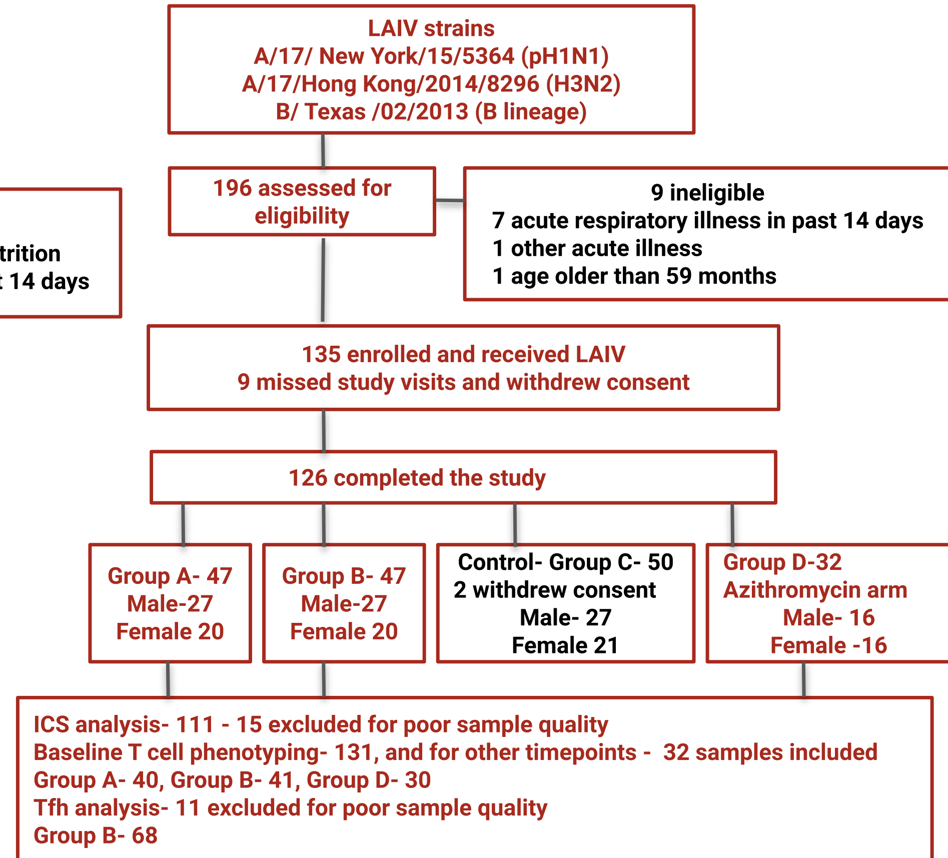


Figure 3: Study profile

Overview of participants who were enrolled into the study and randomized into the different study groups and participants that received the 2016-17 northern hemisphere (NH) Russian-backbone LAIV formulation and (B) and 2017-18 NH formulation (B). In 2016-17 all the participants completed the study. Abbreviations: Intracellular cytokine stainiing (ICS), T follicular helper T cells (Tfh). From the 131 participants included in the T cell phenotyping panel, blood samples were collected at baseline (V0) and screened for a pre-existing CD8+ T-cell IFN- γ response, defined as above the threshold from 2017 data of 0.15% (this was based on the distribution of negative values after background subtraction). 32 participants had pre-existing CD8 +T cell reponses and a further blood sample was analysed at 7 days and 21 days after vaccination. Samples with less than 100 events in the monocyte/DC/T cell gates were excluded during the flow analysis.

2.5 Sampling timepoints

I used samples from Group A, B and D as shown on Table 7 below. Innate responses were only assessed in 2017 and T cell phenotyping only in 2018.

Table 7: Sampling overview

Day	Oral swab	Group A Blood	Group B Blood	Group D Blood (2018 only)
0	Protein microarray	Innate panel (2017) ICS panel T cell phenotyping panel (2018) Haemagglutination assay	Innate panel (2017) ICS panel Tfh panel T cell phenotyping panel (2018) Haemagglutination assay	ICS panel Tfh panel T cell phenotyping panel (2018) Haemagglutination assay
2		Innate panel (2017) ICS panel T cell phenotyping panel (2018)		
7			Innate panel (2017) ICS panel Tfh panel T cell phenotyping panel (2018)	ICS panel Tfh panel T cell phenotyping panel (2018)
21	Protein microarray	ICS panel T cell phenotyping panel (2018) Haemagglutination assay	ICS panel Tfh panel T cell phenotyping panel (2018) Haemagglutination assay	ICS panel Tfh panel T cell phenotyping panel (2018) Haemagglutination assay

2.6 Laboratory Methods

All flow cytometry assays were optimized in the laboratory before the start of the study. This included antibody titrations and optimization of voltages to use on the flow cytometer for each of the panels. When all the volume of antibodies to use were obtained, a full panel test was done to ensure that the assay worked. Compensation controls were used for each experiment.

2.6.1 Intracellular cytokine staining (T cell panel)

CD4+ and CD8+ T-cell responses were quantified by stimulating 200µl whole blood with overlapping 15-18-mer peptide pools (2µg/ml) covering vaccine-matched Matrix and Nucleoprotein (MNP; 47 and 68 peptides respectively), haemagglutinin 1 (HA1; 74 peptides) and haemagglutinin 3 (HA3; 74 peptides) for 18 hours, in the presence of co-stimulatory antibodies anti-CD28 and anti-CD49 (BD Biosciences). The negative control tube contained 75% dimethyl sulfoxide (DMSO) to ensure that any DMSO-specific inhibitory effect was accounted for in the results. After 2 hours of incubation, Brefeldin A (BD Golgiplug) was added to all the tubes and reincubated for a further 16 hours. After the overnight incubation, live dead staining was done using Zombie Aqua (Biolegend) to discriminate between live and dead cells followed by surface staining with CD4 PerCPCy5.5 and CD8 FITC antibodies. Cells were then lysed with 2mls of 1 x BD FACS lysing solution to each tube and incubating at room temperature for 10 minutes in the dark. The cells were topped up with FACS buffer and centrifuged for 5 minutes at 1800rpm. 1mL of 1x Perm/Wash buffer was added to the cells to permeabilize them and tubes incubated at room temperature for 15 minutes in the dark. The tubes were centrifuged for 5 minutes at 1800rpm and supernatant poured off. Cells were resuspended in the residual perm buffer and IL2-PE and IFN- γ APC used to perform Intracellular cytokine staining. Cells were then incubated at room temperature for 30 minutes in the dark. Cells were washed by adding 2mL of Perm/Wash Buffer and centrifuged for 5 minutes at 1800rpm and the wash step repeated with 2mls of FACS buffer. The supernatant was poured off and tubes blotted to remove excess facs buffer. Cells were resuspended in 250µl of FACS buffer and vortexted. Tubes wre covered in foil and stored at 4°C in dark until acquisition. Cells were analyzed with an LSR Fortessa flow cytometer. A total of 200,000 events was recorded for each condition. Responses in the negative (anti-CD28/anti-CD49) controls were subtracted from peptide-stimulated conditions prior to further analysis. Negative values were set to zero. To avoid systematic bias in doing so, a threshold was set below which all positive values were also considered a non-response, as described previously (De Silva *et al.*, 2013).

2.6.2 T cell phenotyping panel

The assay was done using methods described for T cell ICS in section 2.6.1. However 150 µl of whole blood was used in the assay. The fluoresence minus one (FMO) controls were HLA-DR APC-Cy7, Ki67 BV510, CD69 AF700 and CD103 PE. For the surface staining I used CD4 PerCp Cy5.5, CD8 FITC, CD27 PE Cy7, CD45R0 bv605, HLA-DR APC-Cy7, CD69 AF700 and CD103 PE, whilst for intracellular cytokine staining I used Ki67 BV510 and IFN- γ APC.

2.6.3 Follicular hepler T (Tfh) cell panel

2.6.3.1 Ex vivo panel

200 µl of whole blood was used for the staining. Additional tubes for unstained and FMO controls for ICOS, PD-1, CXCR3, CCR6 and CXCR5 were included. CXCR5 bv421 was added to the required tubes and incubated for 15 minutes at 37°C and 5% CO_2. Surface staining was done using ICOS, PD-1, CXCR3, CCR6 and the tubes incubated at room temperature for a further 15 minutes in the dark. Cells were lysed by adding 2mls of 1 x BD FACS lysing solution to each tube and incubating at room temperature for 10 minutes in the dark. The cells were topped up with FACS buffer and centrifuged for 5 minutes at 1800rpm. Cells were resuspended in 250µl facs buffer and tubes covered in foil and stored at 4°C in dark until acquisition. Cells were analyzed with an LSR Fortessa flow cytometer.

2.6.3.2 Activation induced marker (AIM) assay panel

150 µl of whole blood was stimulated with influenza overlapping peptide of pooled haemagglutinin 3 (HA3; 74 peptides), haemagglutinin 1 (HA1; 74 peptides) Influenza B haemagglutinin (BHA; 80 peptides) for 2 hours at 37°C and 5% CO_2. 75% dimethyl sulfoxide (DMSO) was used as a negative control for MNP and HA3 to ensure that any DMSO-specific inhibitory effect was accounted for in the results. Additional tubes for FMO controls for OX40/CD25 and ICOS and PDL1 were set up. The required antigens and controls were added to each tube, CXCR5 AF700 was then added and tubes incubated for 18 hours 37°C and 5% CO_2.. A 15 minutes live dead staining was done using Zombie Violet (Biolegend) to discriminate between live and dead cells. CXCR5 AF700 was added into all tubes and incubated for 15 minutes at 37°C and 5% CO_2. This was followed by surface staining with CD4 PerCp Cy5.5 and CD45R0 bv605 in all tubes and ICOS PE, CD25 PE CY7, OX40 APC, PD-L1 FITC to the required tubes for a further 15 minutes. Cells were lysed by adding 2mls of 1 x BD FACS lysing solution to each tube and incubating at room temperature for 10 minutes in the dark. The cells were topped up with FACS buffer and centrifuged for 5 minutes at 1800rpm. Cells were resuspended in 250**µ**l facs buffer and tubes covered in foil and stored at 4°C in dark until acquisition. Cells were analyzed with an LSR Fortessa flow cytometer.

2.6.4 Innate panel

200**µ**l of whole blood was used for the staining. Additional tubes for unstained and FMO controls for TNF- α PerCPCy5.5, IL-10 PE, HLA-DR APC CY7, CD14 PB and CD16 PB were included. Surface staining was done using a cocktail of antibodies that included CD11c APC, CD66b PB, CD56 PB, CD3 PB and CD19 PB in all tubes and CD16 AF488, HLA-DR APC-Cy7 and CD14 bv605 only in the sample tubes. Tubes were incubated at room temperature for 30 minutes in the dark. Cells were lysed by adding 2mls of 1 x BD FACS lysing solution to each tube and incubated at room temperature for 10 minutes in the dark. This was followed by a wash step with 2mLs of FACS buffer to each tube and centrifuged for 5 minutes at 1800rpm. 1mL of 1x Perm/Wash buffer was added to the cells to permeabilize them and tubes incubated at room temperature for 15 minutes in the dark. The tubes were centrifuged for 5 minutes at 1800rpm and supernatant poured off. Cells were resuspended in the residual perm buffer and intracellular cytokine cocktail containing TNF- α PerCPCy5.5 and IL-10 PE was added to all sample tubes and FMOs that required it. Cells were then incubated at room temperature for 30 minutes in the dark. Cells were washed by adding 2mL of Perm/Wash Buffer for 5 minutes at 1800rpm and the wash step repeated with 2mls of FACS buffer. The supernatant was poured off and tubes blotted to remove excess facs buffer. Cells were resuspended in 250µl of FACS buffer and vortexted. Tubes wre covered in foil and stored at 4°C in dark until acquisition. Reagents and antibodies used for the different flow cytometry panels is shown below in Table 8 and 9 below.

Table 8: Reagents for flow cytometry

Reagents	Product code	Supplier
Lysing solution 10x concentrate	349202	BD Biosciences
Perm/wash buffer	554723	BD Biosciences
Purified mouse anti-human CD49d	340976	BD Biosciences
Purified mouse anti-human CD28	340975	BD Biosciences
Anti-Mouse Ig, negative control compensation particles set	552843	BD Biosciences
Anti-Rat Ig, negative control compensation particles set	552844	BD Biosciences
ArC Amine reactive compensation bead kit	A10628	ThermoFisher Scientific

Table 9:Flow cytometry panel and antibodies used

Antibody	Fluorochrome	Clone	Product code	Supplier	Isotype	Panel	Volume used (μl)
CD66b	Pacific Blue	G10FS	305112	Biolegend	Mouse IgM	IN	2
CD56	Pacific Blue	5.1H11	362520	Biolegend	Mouse IgG1	IN	2
CD3	Pacific Blue	SK7	344824	Biolegend	Mouse IgG1	IN	2
CD19	Pacific Blue	H1B19	302232	Biolegend	Mouse IgG1	IN	1
CD14	**BV605**	M5E2	301834	Biolegend	Mouse IgG2a	IN	1
IL-10	**PE-Cyanine7**	JES3-9D7	501420	Biolegend	Rat IgG1	IN	3
CD11c	**APC**	3.9	301614	Biolegend	Mouse IgG1	IN	2
CD123	**PE**	6H6	306005	Biolegend	Mouse IgG1	IN	1
CD16	**AF488**	3G8	302019	Biolegend	Mouse IgG1	IN	1
TNF-α	**PerCPCy5.5**	MAb11	502926	Biolegend	Mouse IgG1	IN	3
IL-2	**PE**	MQ1-17H12	500307	Biolegend	Rat IgG2a	ICS	3
IFN-Y	**APC**	B27	506510	Biolegend	Mouse IgG1	ICS/TP	3
Live dead (Zombie violet)		NA	423114	Biolegend		TP	1
CD4	**PerCPCy5.5**	RPA-T4 OKT4	300530 317428	Biolegend	Mouse IgG1 Mouse IgG2b	ICS/TFH/ TP	3
CD8	**FITC**	RPA-T8	301050	Biolegend	Mouse IgG1	ICS/TP	5
CD45RO	**bv605**	UCHL1	304238	Biolegend	Mouse IgG2a	TFH/ TP	1
CXCR3	**APC Cy7**	G025H7	353722	Biolegend	Mouse IgG1	TFH	2
CXCR5	**bv421**	J252D4	356920	Biolegend	Mouse IgG1	TFH	2
CD27-	**PE/Cy7**	M-T271	356412	Biolegend	Mouse IgG1	TP	2
OX40	**APC**	Ber ACT35	350008	Biolegend	Mouse IgG1	TFH	3
CD25	**PE Cy7**	M-A251	356108	Biolegend	Mouse IgG1	TFH	3
HLA-DR	**APC/Cy7**	L243	307618	Biolegend	Mouse IgG2a	TP/IN	1
PD1	**PE Cy 7**	EH12.2H 7	329918	Biolegend	Mouse IgG1	TFH	1
CD69	**AF700**	FN50	310922	Biolegend	Mouse IgG1	TP	1
CD103	**PE**	Ber ACT8	350206	Biolegend	Mouse IgG1	TP	2
Ki67	**BV510**	Ki-67	350518	Biolegend	Mouse IgG1	TP	3
CD278(ICOS)	**PE**	ISA-3	12-9948-42	Ebioscience	Mouse / IgG1	TFH	2
CD274 (PDL1)-	**FITC**	MIH1	558065	BD Biosciences	Mouse BALB/c IgG1	TFH	6

2.6.5 Haemagluttinin inhibition assay (HAI)

Serum samples were separated from whole blood samples in the laboratory and aliquoted into sarstedt tubes. All samples were stored in a -70^{0}C freezer at Biobank until the end of the study period. Samples were then sent to Public Health England were the HAI assay was performed according to standard methods, using vaccine HA and NA matched viruses (Ellis and Zambon, 1997). Seroconversion was defined as a ≥4-fold titre increase (to ≥1:40) from D0 to D21.

2.6.6 Protein microarray to detect anti-influenza IgA antibodies

Oracol+ swabs (Malvern Medical Development Ltd.) were used to collect buccal cavity oral fluid (OF) at day 0 and day 21 post vaccination. All samples were stored in a -70^{0}C freezer at Biobank until the end of the study period. Samples were then sent to RIVM, the Dutch National Institute for Public Health and the Environment. These samples were used to detect mucosal influenza-specific IgA responses at baseline and D21 post-LAIV using a protein microarray as previously described (Koopmans *et al.*, 2012; de Silva *et al.*, 2017). In brief nitrocellulose slides (ONCYTE® AVID, Grace Bio-Labs, USA) were coated with recombinant HA1 protein from Influenza A/17/California/2009/38 (pH1N1), A/17/Hong Kong/2014/8296 (H3N2) and B/Texas/02/2013 by applying two drops of 333ul each of the protein diluted in protein arraying buffer (Whatman, Maidstone, Kent, UK). This was followed by a 1 hour blocking step at 37^{0}C using the BLOTTO blocking buffer(Thermo Fisher Scoentific, USA). The slides were then washed three times with the protein array wash buffer (Whatman Bredford, USA). Diluted samples were then transferred to the slides and incubated for I hour at 37^{0}C. Another wash step is done as in above followed by a 1 hour incubation at 37^{0}C with goat anti-human IgG (Fc-fragment specific) conjugated with Dylight649-fluorescent dye (Jackson Immuno Research, West Grove, PA, USA). A final wash step is done and the slides were allowed to dry. Quantification of signals was done using the PowerScanner™ microarray scanner (Tecan Trading AG, Switzerland) and determination of the mean spot fluorescence foreground was done by using ScanArray® Express software (PerkinElmer, USA).

For the data analysis from the flow cytometry and antibody data, the negative values were set to zero and to avoid systematic bias in adjusting for negative values alone, based on the distribution of the negative values alone, a 90th percentile of the distribution was calculated and this was used as a threhold and also applied to the positive values. Below this threshold, all positive values were also considered a non-response, as described previously (Roederer, Nozzi and Nason, 2011). Null values were assigned a value halfway between zero and this threshold. To calculate the fold change, ratio between the responses from baseline to day 21 was calculated in R. The log fold change was also

calculated in R. A twofold increase after LAIV was considered a significant response as previously described (Lindsey *et al.*, 2019).

2.7 Statistics

The data were analysed using RStudio, Version 1.4.1103 (PBC, Boston, MA) and GraphPad Prism version 9 (GraphPad Software, San Diego, CA, USA) softwares. All statistical analysis were carried out using Wilcoxon signed-rank test, to compare responses before and after vaccination. For the ICS and T cell phenotyping panel, the proportion of mono- and dual-functional T-cell responses were estimated using Boolean gating on FlowJo 10.4 and statistical significance between timepoints tested with the Permutation test in SPICE V6.0. All tests were two-sided at 5% significance level and were Bonferroni-adjusted for multiple comparisons within each set of analyses. Correlations were analysed using Spearman's non-parametric correlation coefficients.

3 Chapter 3: Effect of LAIV on monocyte and dendritic cell populations

3.1 Aim

1. To characterize the monocyte and dendritic cell changes following live attenuated influenza vaccine (LAIV) and assess the relationship with later influenza-specific humoral immune responses. I specifically assessed.

2. Changes in monocyte subsets following LAIV, cytokine secretion and activation within each subset, and correlation with mucosal and serum antibody responses and peripheral CD4+ and CD8+ T-cell responses.

3. Changes in plasmacytoid dendritic cells (pDCs) and myeloid dendritic cells (mDCs) subsets following LAIV, cytokine secretion and activation within each subset, and correlation with mucosal and serum antibody responses and peripheral CD4+ and CD8+ T-cell responses.

3.2 Introduction

Innate immune cells play a vital role in providing a quick but efficient means of limiting viral infections, but crucially they also initiate the adaptive arm of the immune system. These include monocytes and dendritic-cells (DCs) carrying out three main functions in the immune system, namely, phagocytosis, antigen presentation, and production of cytokines. Interaction of their pattern recognition receptors (PRRs) and the pathogen associated molecular patterns (PAMPS) on the virus induces these above-mentioned responses leading to production of cytokines such as IL-6 and TNF-α to activate an effector function. Previous studies have shown that upon influenza virus infection, circulating monocytes rapidly differentiate into DCs with antiviral properties and release chemokines such as MCP-1 that can attract more monocytes to the site of infection (Cao *et al.*, 2012). I hypothesized that these functions are likely to be important in the interplay between innate and adaptive immune responses to a live attenuated mucosal vaccine such as LAIV.

In humans, monocytes can be classified into three different subsets based on CD14 and CD16: the classical monocytes, characterized by high level expression of the CD14 cell surface receptor ($CD14^{++}$ $CD16^{-}$ monocytes), the non-classical subset with low level expression of CD14 and additional co-expression of the CD16 receptor ($CD14^{+}CD16^{++}$ monocyte) and the intermediate subset with high level expression of CD14 and low-level expression of CD16 ($CD14^{++}CD16^{+}$ monocytes). Mohanty and colleagues demonstrated that these monocytes produce cytokines such as TNF- α and IL-6 following inactivated influenza vaccine (IIV), which correlated with induction of serum antibody responses (Mohanty et al 2014). The exact role that monocytes play in helping to induce mucosal antibody and systemic T-cell effector mechanisms upon LAIV vaccination is unknown. A pilot study of UK adults given LAIV showed an expansion of intermediate monocytes at day 3 after vaccination (de Silva et al, unpublished) but the detailed functional properties of these cells and the downstream effects on adaptive immunity are unknown.

Several types of DCs can be found in different tissue compartments of the body as well as in blood, but in this study, I focused on myeloid (CD11c+) and plasmacytoid (CD123+) DCs within the CD3/CD19/CD14/CD16- negative and HLA-DR+ cells. These are the two main types of DCs described in human blood, although with the advent of more advanced immunophenotyping techniques other subtypes have been described (Collin and Bigley 2018).

Myeloid DCs (mDCs), produce large amounts of IL-12 and induce strong T helper type 1 (Th1) and cytotoxic T lymphocyte (CTL) responses, whilst plasmacytoid DCs (pDCs) produce large amounts of type I interferons (IFN) in response to viral and bacterial stimuli. During the primary or secondary immune response to viral infections or vaccinations, DCs play a part in processing viral antigens for presentation to T cells, leading to the activation and proliferation of virus-specific T cells. Studies have shown a decrease in mDCs but not pDCs in the first few days following vaccination with IIV (Kobie et al 2015). Panda et al reported that seroconversion to the H3N2 and B strains in the 2007–2008 IIV was significantly associated with TLR-induced production of inflammatory cytokines by DCs. In this study, cytokine production post stimulation with TLRs was lower in older compared to younger adults. This observed decrease in cytokine production in older adults subsequently affected antibody responses to IIV, suggesting that immunosenescence may play a role in the poor response to IIV vaccination seen in adults (Panda et al 2010). The role of DCs following LAIV administration is not well elucidated. Thus, this chapter seeks to characterize and assess the functionality of different monocyte and DC subsets induced by LAIV vaccination in Gambian children.

To address this question, I used the serum and mucosal antibody data generated within the NASIMMUNE study (SCC1502). Serum antibody responses were measured at baseline and on day 21 post-LAIV using the HAI assay and as previously reported by collaborators at Public Health England, UK, a ≥ 4-fold increase in HAI titer from baseline was defined as seroconversion. Mucosal influenza-specific IgA was measured in oral fluid samples at baseline and day 21 post-LAIV using a protein microarray by collaborators at RIVM, The Netherlands. Mucosal influenza-specific IgA responders were defined as those with at least a two- fold change in antibody concentration from baseline to day 21. The detailed phenotype of monocytes and dendritic cells in children aged 24-59 were characterised at baseline and either day 2 (n=49, Group A) or day 7 (n= 55, Group B) after a single dose of LAIV (Nasovac-S) in 2017. This grouping was done to reduce the number of times that the children were bled. *Ex-vivo* cytokine staining was used as described in Section 2- Materials and methods.

3.3 Results

3.3.1 Effect of LAIV vaccination on monocyte subsets

The three monocyte populations in blood were identified based on expression of CD14 and CD16 as shown in the gating strategy below (Figure 4). The classical monocytes were identified as $CD14^{++}CD16^{-}$ cells, the intermediate monocytes as $CD14^{++}CD16^{+}$ and the nonclassical monocytes as $CD14^{+}CD16^{++}$. I evaluated the frequency of monocyte subsets in blood and found that classical monocytes were the most abundant, followed by nonclassical monocytes, with intermediate monocytes being lowest in frequency. This is in line with other studies where classical monocytes were shown to dominated in the blood in healthy individuals (Wong et al. 2021; Vangeti 2019).

A change in composition of these profiles, with a decrease in classical monocytes was observed following vaccination, but this was only significant at day 7 (Day 0 (Median (IQR) = 72.20% (63.60% and 80.30%), day 7 (Median (IQR) = 67.60% (60.00% and 77.10%), p=0.007) and not day 2 (Day 0 (Median (IQR)= 75.60% (66.40% and 81.25%), day 2 (Median (IQR)= 70.90% (63.50% and 77.05%), p=0.09 for day 2. An increase in intermediate monocytes was seen at both day 2 (Day 0 (Median (IQR)= 7.19% (4.19% and 11.85%), day 2 (Median (IQR)= 10.10% (8.09% and 15.70%), (p=0.0006) and day 7 (Day 0 (Median (IQR)= 12.30% (7.97% and 18.70%), day 7 (Median (IQR)= 15.70% (8.43% and 21.80%), (p=0.008) post vaccination. There was no change in the frequency of circulating non-classical monocytes post vaccination at both timepoints as shown below (Figure 5C).

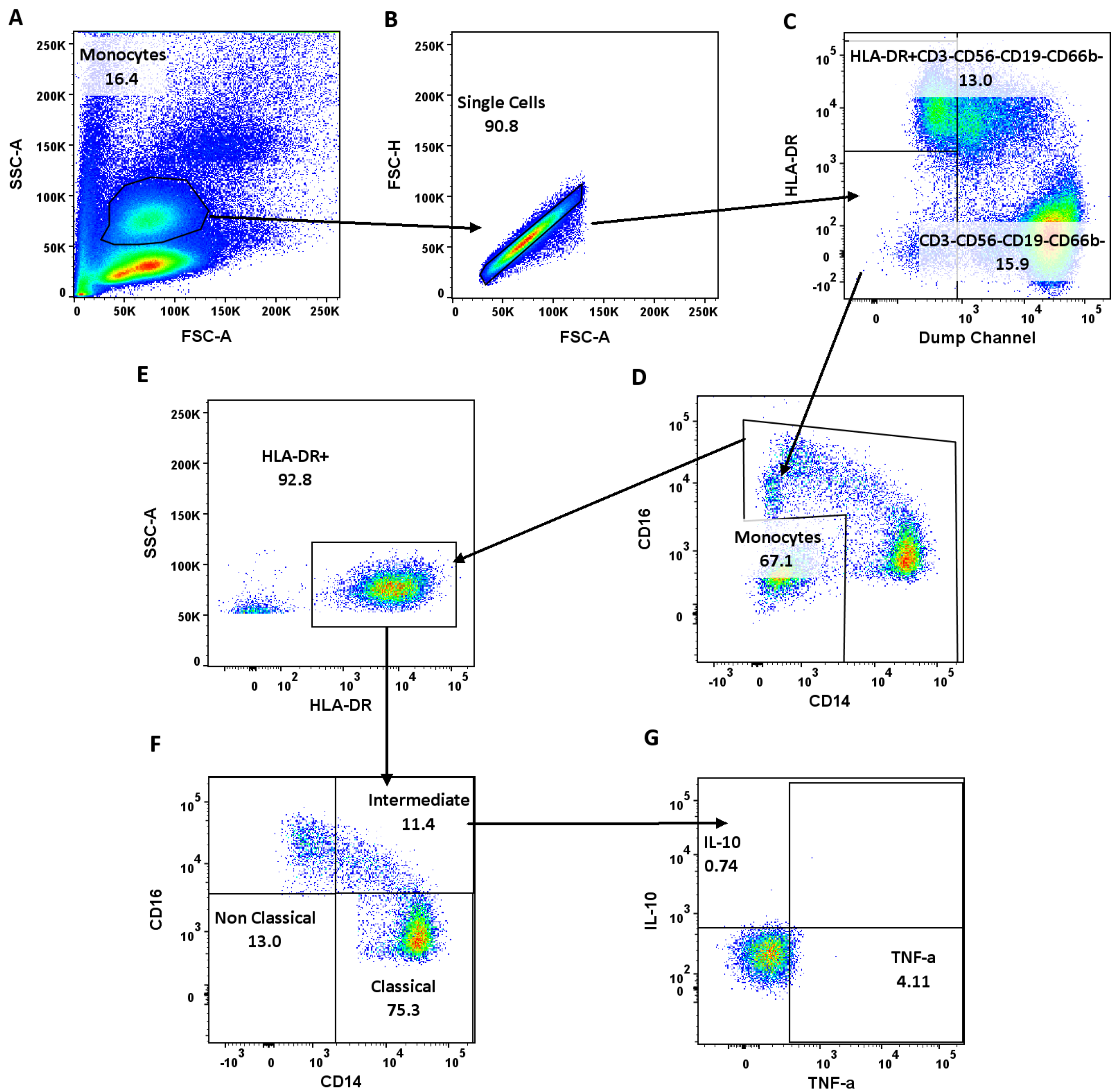


Figure 4:Sequential Gating strategy showing identification of total monocytes and the different monocyte subsets in blood

Monocytes were identified based on A) forward scatter (FSC) versus size scatter (SC). B) Single cells were selected based forward scatter area (FSC-A) and forward scatter height (FSC-H) C) CD3-CD56-CD19-CD66B- cells were then gated or exclude lymphoid cells. D) Monocytes were then gated on using CD14 and CD16. E) HLADR+ cells were then gated on CD14 and CD16 was used to identify the three human monocyte subsets. G) Within each subset I gated on IL-10 and TNI to identify cytokine producing cells. Plot G shows gating from intermediate monocytes at day 7 post LAIV.

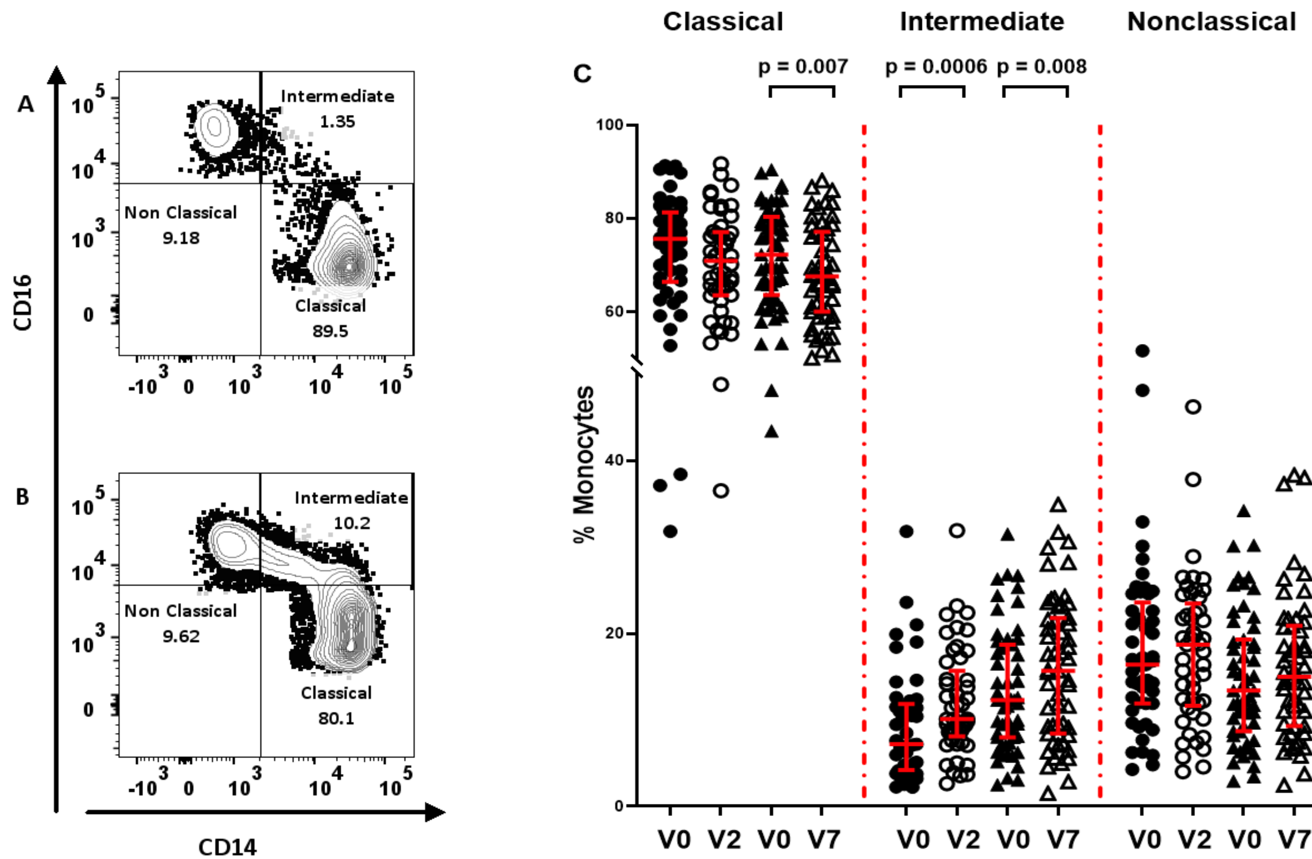


Figure 5:Effect of LAIV vaccination on monocyte subsets

Monocytes were identified based on expression of CD14 and CD16 and grouped into the three different subsets as shown in A) Day 0 and B) Day 7. The classical monocytes were identified as CD14++CD16- cells, the intermediate as CD14++CD16+ and the nonclassical as CD14+CD16++. C) The changes in the frequency of all 3 subsets was assessed in two groups of vaccines Group A (V0, pre vaccination and V2, day 2 post vaccination) and Group B (V0, pre vaccination and V7, day 7 post vaccination). Horizontal lines denote median and error bars denote interquartile range (IQR). For each monocyte population, closed circles represent day 0 for Group A and open circles day 2 whilst closed triangles represent day 0 for Group B and open triangles day 7. Wilcoxon matched pairs signed rank test was used to compare between the timepoints in each group. n=49, Group A, and n=55 for Group B. Due to multiple comparisons (n = 2), significance level was adjusted using Bonferroni's correction and P values of < 0.025 were considered statistically significant.

3.3.2 Reduced HLA-DR expression at day 2 post LAIV

HLA-DR is an MHC class II molecule constitutively expressed on human monocytes and which plays a role in antigen processing and presentation, largely for extracellular pathogen-derived peptides. Having observed changes in the distribution of monocyte subsets, I subsequently measured the expression of HLA-DR to see if this reflected the changes in differentiation observed post LAIV. As the presence HLA-DR was used to define all monocyte subsets, the median fluorescence intensity (MFI) was used to evaluate the relative expression of HLA-DR. I observed a significant decrease in the HLA-DR MFI on the total monocyte population at day 2 (Day 0 (Median (IQR)= 7340 (5716 and 10995), day 2 (Median (IQR)= 6123 (5072 and 8392), p=0.01) following vaccination, but no significant difference was seen at day 7 (Day 0 (Median (IQR)= 8187 (5507 and 11573), day 7 (Median (IQR)= 8045 (5862 and 11102), p=0.93) (Figure 6A). I therefore looked at HLADR expression in the monocyte subsets at baseline and post vaccination. At baseline, HLA-DR expression was highest in the intermediate monocytes compared to the classical and nonclassical subsets (Figure 6B). The decrease in HLA-DR in the total monocyte population at day 2 following vaccination was observed in both the classical (Day 0 (Median (IQR)= 4033 (3021 and 5186), day 2 (Median (IQR)= 2614 (1954 and 3991), p<0.0001) and intermediate (Day 0 (Median (IQR)= 6004 (3901 and 10445), day 2 (Median (IQR)= 5415 (3632and 8266), p=0.03) monocyte subsets, whilst expression levels remained unchanged in the non-classical subset (Day 0 (Median (IQR)= 4523 (3193 and 6273), day 2 (Median (IQR)= 4395 (3389 and 5814),p=0.88) Figure 6C). At day 7 post vaccination, no significant difference in HLA-DR expression was noted compared to baseline levels in all 3 subsets (Figure 6D).

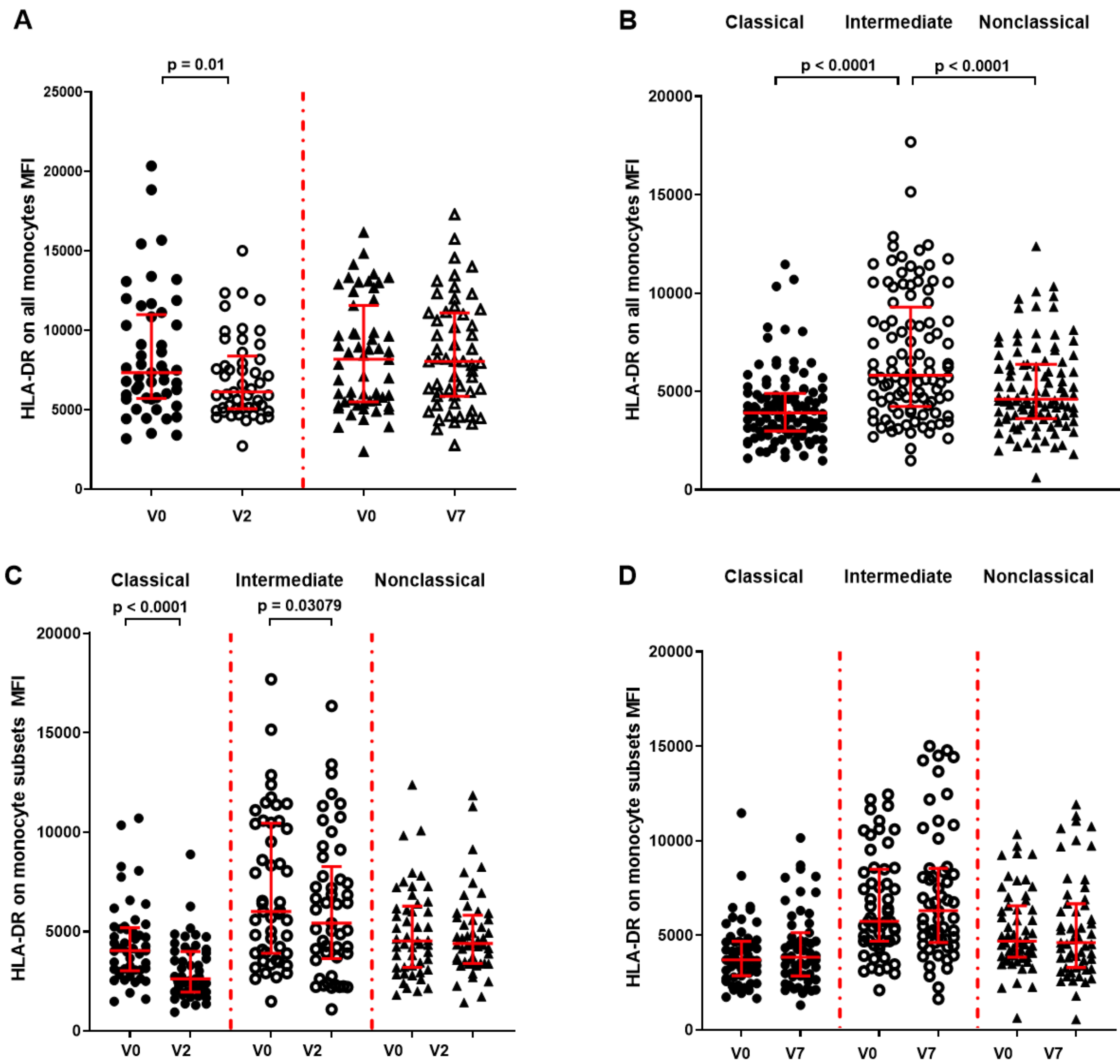


Figure 6: Reduced HLA-DR expression at day 2 post LAIV

The graph shows A) Median fluorescence intensity (MFI) of HLA-DR expression in total monocytes at baseline and day 2 (Group A) or Day 7 (Group B) post LAIV. B) MFI of HLA-DR expression on monocyte subsets at baseline. C) MFI of HLA-DR expression in monocyte subsets pre and day 2 post vaccination. D) MFI of HLA-DR expression in monocyte subsets pre and day 7 post vaccination. Horizontal lines denote median and error bars denote interquartile range (IQR). Closed circles represent classical monocytes, intermediate by open circles and non-classical monocytes by closed triangles. Wilcoxon matched pairs signed rank test was used to compare between timepoints in each group**.** n=49, Group A, and n=55 for Group B. Due to multiple comparisons (n = 2), significance level was adjusted using Bonferroni's correction and P values of < 0.025 were considered statistically significant.

3.3.3 LAIV vaccination induced cytokine production in monocytes

Monocytes produce cytokines to regulate other immune cells. I therefore assessed the production of TNF-α and IL-10 in monocytes pre and post vaccination using intracellular cytokine staining to assess whether differentiation of monocytes observed was associated with cytokine production. In all monocyte subsets, there was no increase in TNF- α production at day 2 post vaccination (Figure 7E), but a significant increase was seen at day 7 (Figure 7F). An increase in IL-10+cells at day 2 and 7 (Figure 7G and 7H) post LAIV vaccination was noted in all monocyte subsets. No difference in frequency of IL-10+ cells between the monocyte subsets pre and post vaccination was noted, but I found a higher frequency of TNF-α+cells in the intermediate and nonclassical monocyte subsets compared to the classical monocytes.

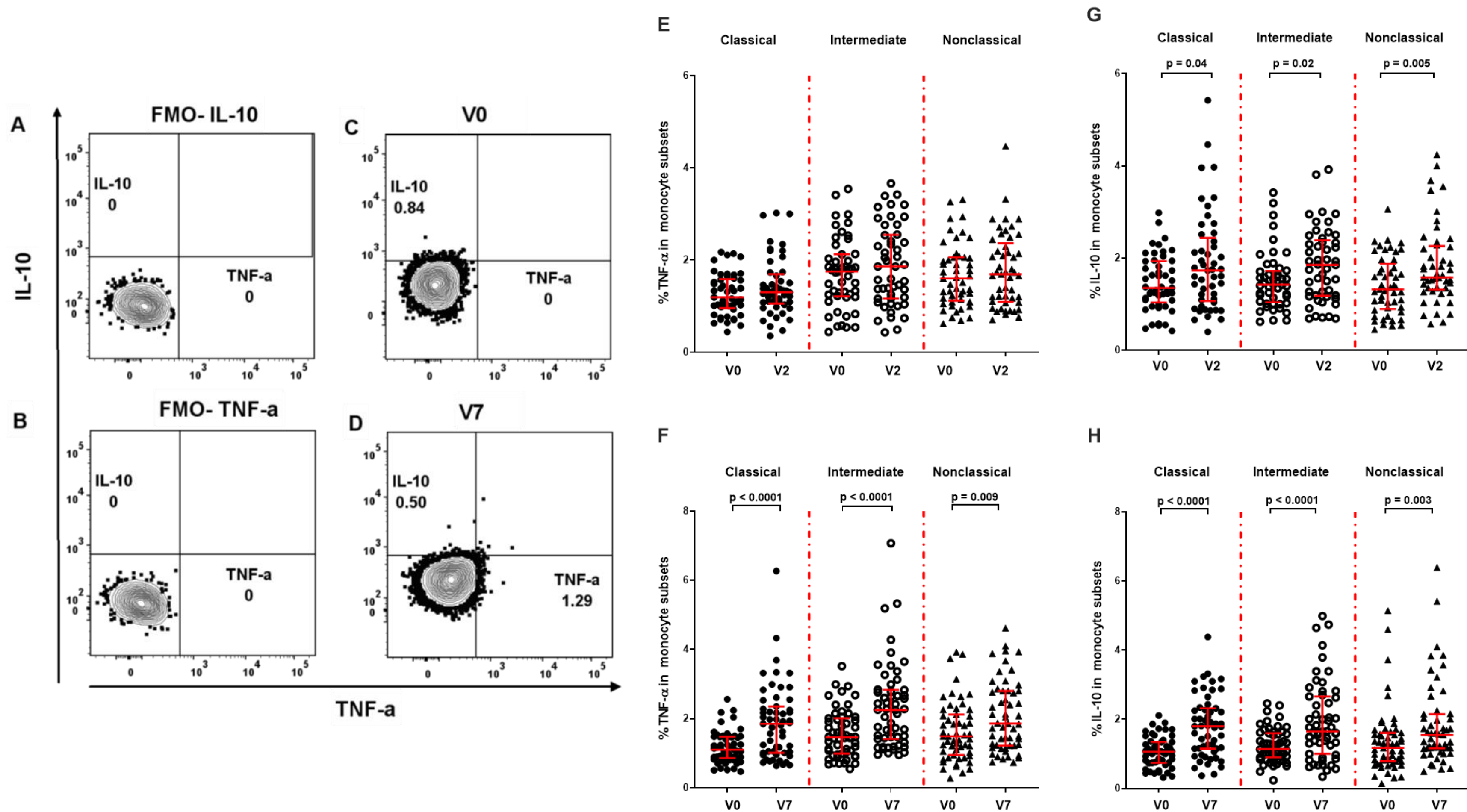


Figure 7: LAIV vaccination induced cytokine production in monocytes

Upon vaccination an increase in cytokine production in monocytes was noted (flow plot from representative donor showing cytokine production in classical monocytes in FMOs for A) IL-10 B) TNF-a and at C) Day 0 and D) Day 7 post LAIV. E) percentage of TNF- α producing cells in all monocyte subsets in Group A. D) F)Percentage of TNF- α producing cells in all monocyte subsets in Group B. G) Percentage of IL-10 producing cells in all monocyte subsets in Group A. H) and Group B. Horizontal lines denote median and error bars denote interquartile range. Classical monocytes are represented by closed circles, intermediate by open circles and non-classical by closed triangles. Wilcoxon matched pairs signed rank test was used to compare between timepoints in each group n-49, Group A, and n=55 for Group B. Due to multiple comparisons (n = 2), significance level was adjusted using Bonferroni's correction and P values of < 0.025 were considered statistically significant.

3.3.4 Changes in Dendritic cell populations post LAIV vaccination

Dendritic cells (DC) were identified based on the gating strategy shown below (Figure 8). I focused on two DC populations, the myeloid DCs identified as CD11c+ and the plasmacytoid DCs identified as CD123+ cells. No significant change was observed in the frequency of mDC at Day 2 (Group A) whereas a significant decrease was observed at Day 7 compared to baseline (p=0.02), (Group B), (Figure 9B). The frequency of pDC cells at day 2 post vaccination did not change (Group A, p=0.57), but an increase was seen from baseline to day 7 (Group B, p = 0.03) post LAIV (Figure 9C).

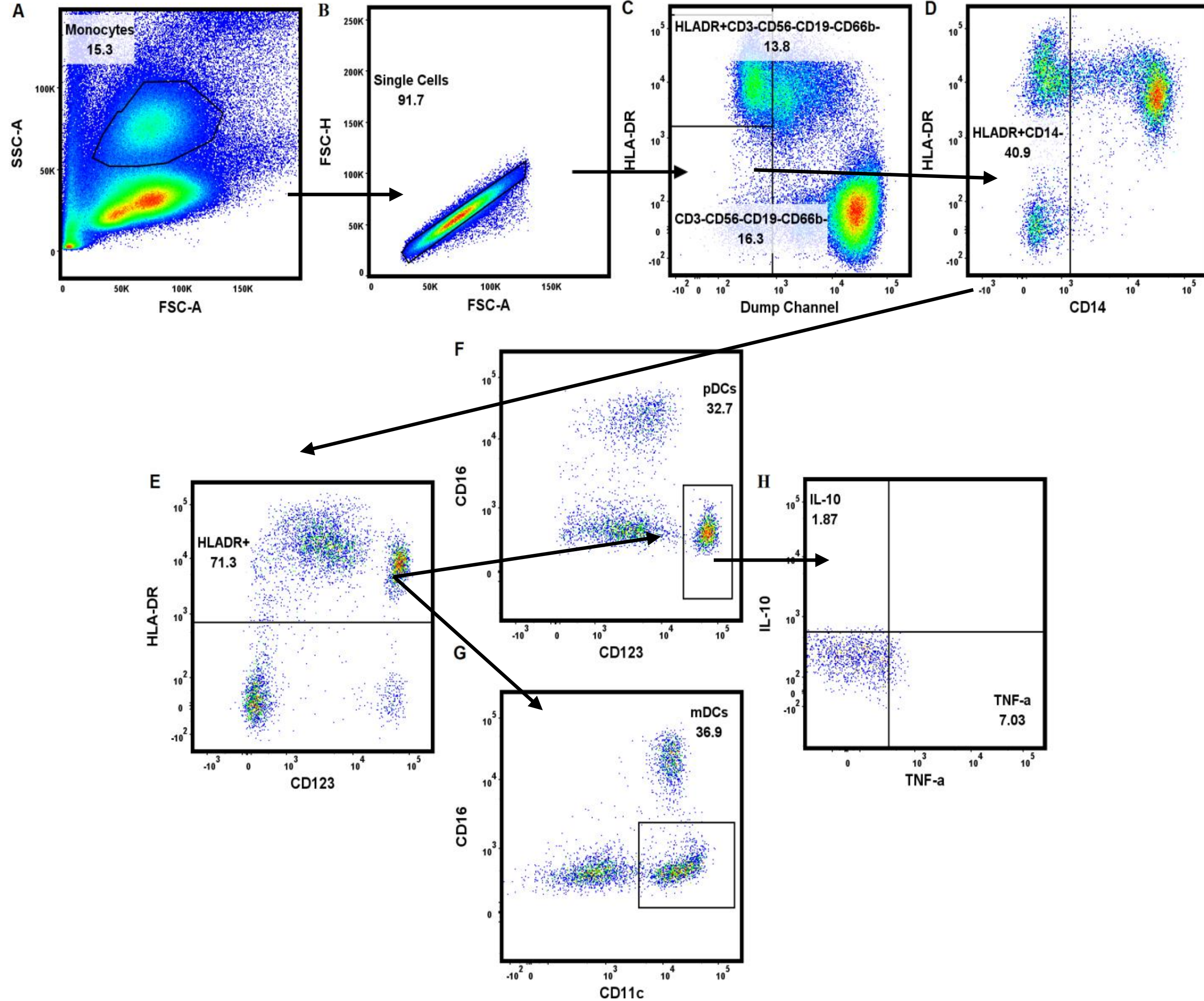


Figure 8: Gating strategy showing identification of myeloid and plasmacytoid dendritic cells (DCs) in blood.

Dendritic cells were identified based on A) forward scatter area (FSC-A) versus size scatter area (SSC-A). B) Single cells were identified based on forward scatter area (FSC-A) and forward scatter height (FSC-H) C) CD3-CD56-CD19-CD66B- cells were then gated on to exclude lymphoid cells. D)HLA-DR+ CD14- cells were then gated on E) HLADR+ cells were then gated on. F) Plasmacytoid DCs were identified based on CD16 and CD123 expression and G) Myeloid DCs were identified based on CD16 and CD11c expression. H) Within each DC subset I gated on IL-10 and TNF- α producing cells. Plot shows cytokine production in pDC at day 7 post LAIV.

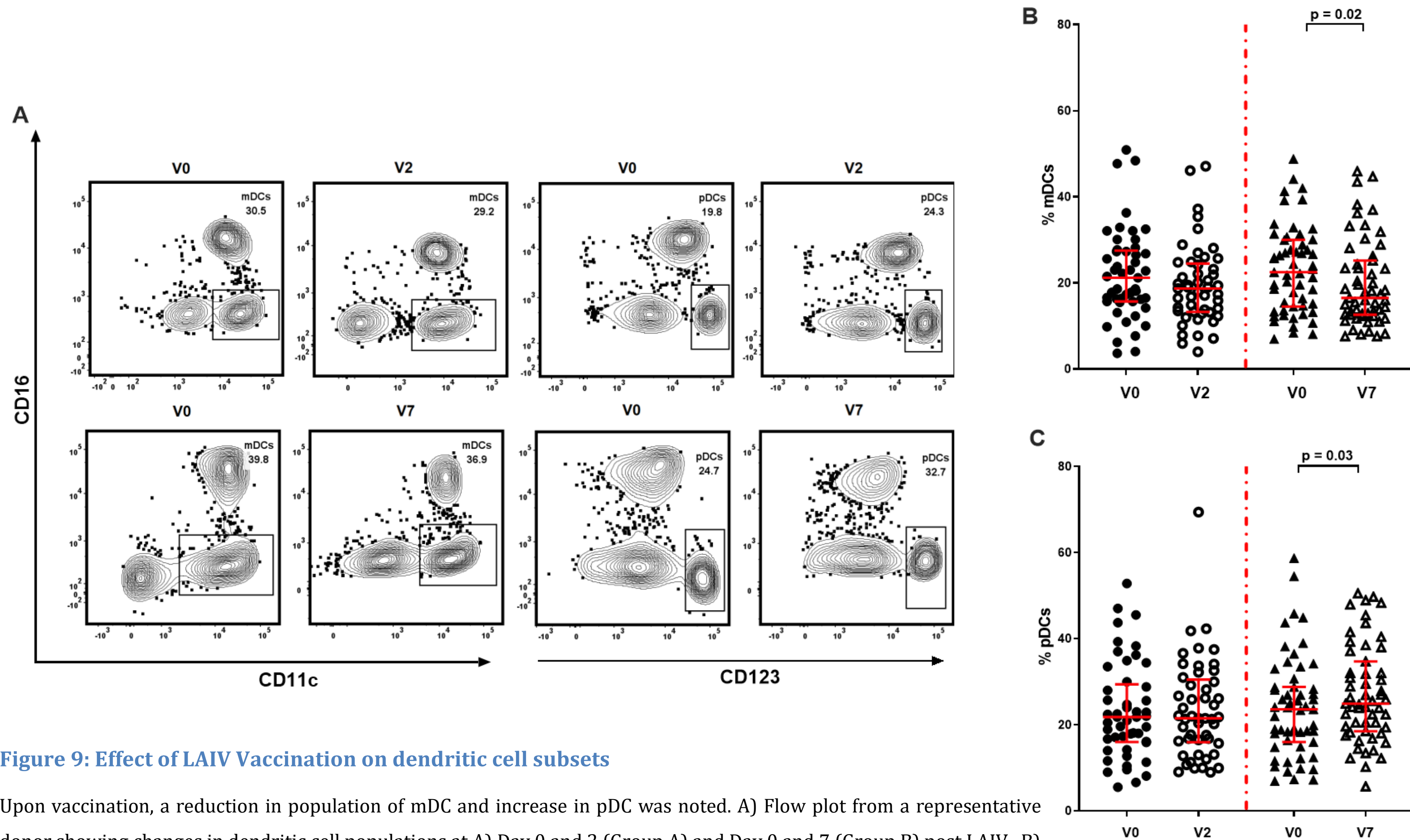


Figure 9: Effect of LAIV Vaccination on dendritic cell subsets

Upon vaccination, a reduction in population of mDC and increase in pDC was noted. A) Flow plot from a representative donor showing changes in dendritic cell populations at A) Day 0 and 2 (Group A) and Day 0 and 7 (Group B) post LAIV. B) Frequency of myeloid dendritic cells and C) plasmacytoid dendritic cells post LAIV in Group A and B. Horizontal lines denote median and error bars denote interquartile range. Wilcoxon matched pairs signed rank test was used to compare between the timepoints in each group. For Group A, n=49 and n=55 for Group B. Due to multiple comparisons (n = 2), significance level was adjusted using Bonferroni's correction and P values of < 0.025 were considered statistically significant.

3.3.5 LAIV vaccination induced cytokine production in pDCs but not mDC

Having observed an increased frequency to pDC 7 days after vaccination, I then examined whether this was reflected functionally in production of cytokines. IL-10 and TNF- α production was assessed in the pDCs and mDCs pre and post vaccination. I saw no difference in the frequency of IL-10 and TNF- α producing mDCs pre and at either 2 of 7 days post LAIV vaccination (Figure 10C and 10D). For the pDC population, at day 2 post vaccination, there was no difference in the frequency of IL-10 and TNF- α producing cells compared to baseline but an increase in the frequency of cytokine producing pDCs was seen between baseline and day 7 for both IL-10 (Group B, $p=0.0005$) and TNF- α (Group B, $p=0.0002$), (Figure 10E and 10F).

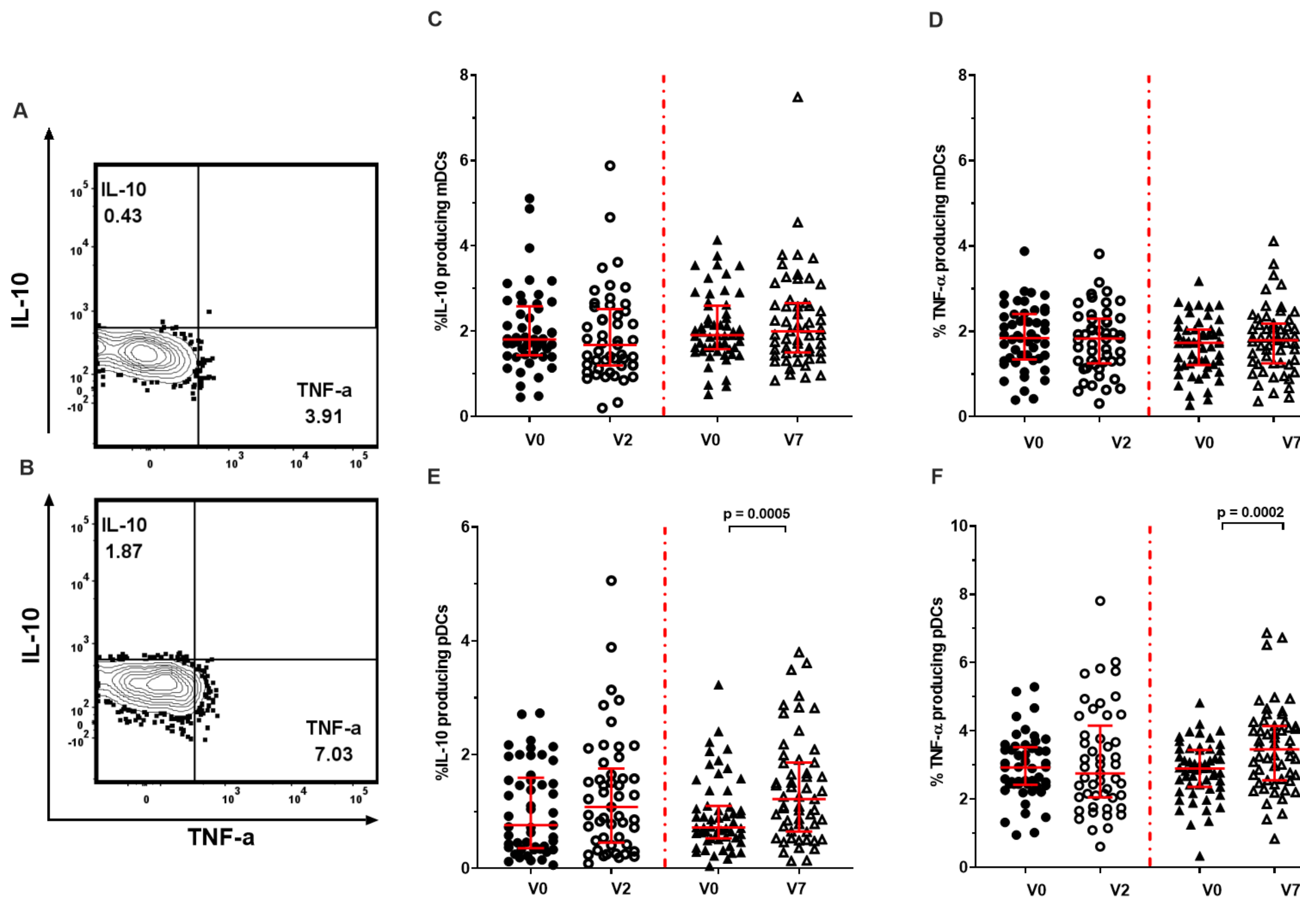


Figure 10: LAIV vaccination induced cytokine production in dendritic cells.

A representative flow plot showing increase in cytokine producing pDCs, A) Day 0 and B) Day 7. Percentage of cytokine producing cells within the mDCs in Group A and B. C) IL-10 and D) TNF- α. Percentage of cytokine producing cells within the pDCs in Group A and B. E) IL-10 and F) TNF- α.Horizontal lines denote median and error bars denote interquartile range. Wilcoxon matched pairs signed ranked test was used to compare between the timepoints in each group. For Group A, n= 49 and n=55 for Group B. Due to multiple comparisons (n = 2), significance level was adjusted using Bonferroni's correction and P values of < 0.025 were considered statistically significant.

3.3.6 CD14+CD16+ monocytes are associated with antibody responses post LAIV

The association of innate immune cells with antibody responses to influenza infection has been previously described (Wong et al). With respect to this recent finding, the effect of monocyte differentiation and dendritic cell activation/function on the serum and mucosal immune response post LAIV was analysed by using the serum and mucosal antibody data generated in the study. I compared the frequency of the different subset of innate cells in a sub-population of children who seroconverted to any of the three influenza strains included in the vaccine with those who did not seroconvert to any strain. HAI assays were performed on vaccine strain haemagglutinin-matched and neuraminidase-matched viruses. Seroconversion to LAIV was defined as a four-fold or greater increase in HAI titers (to ≥1:40) from baseline (day 0, D0) to day 21 (D21). There was a significant increase in the frequency of intermediate monocytes at day 2 post LAIV in both the seroconverters (Day 0 (Median (IQR)= 10.45% (4.13% and 12.95%), day 2 (Median (IQR)= 12.25% (9.25% and 18.98%), p=0.007) and the non-seroconverters (Day 0 (Median (IQR)= 6.94% (4.14% and 11.40%), day 2 (Median (IQR)= 9.18% (5.04% and 13.80%), p=0.04), Figure 11.

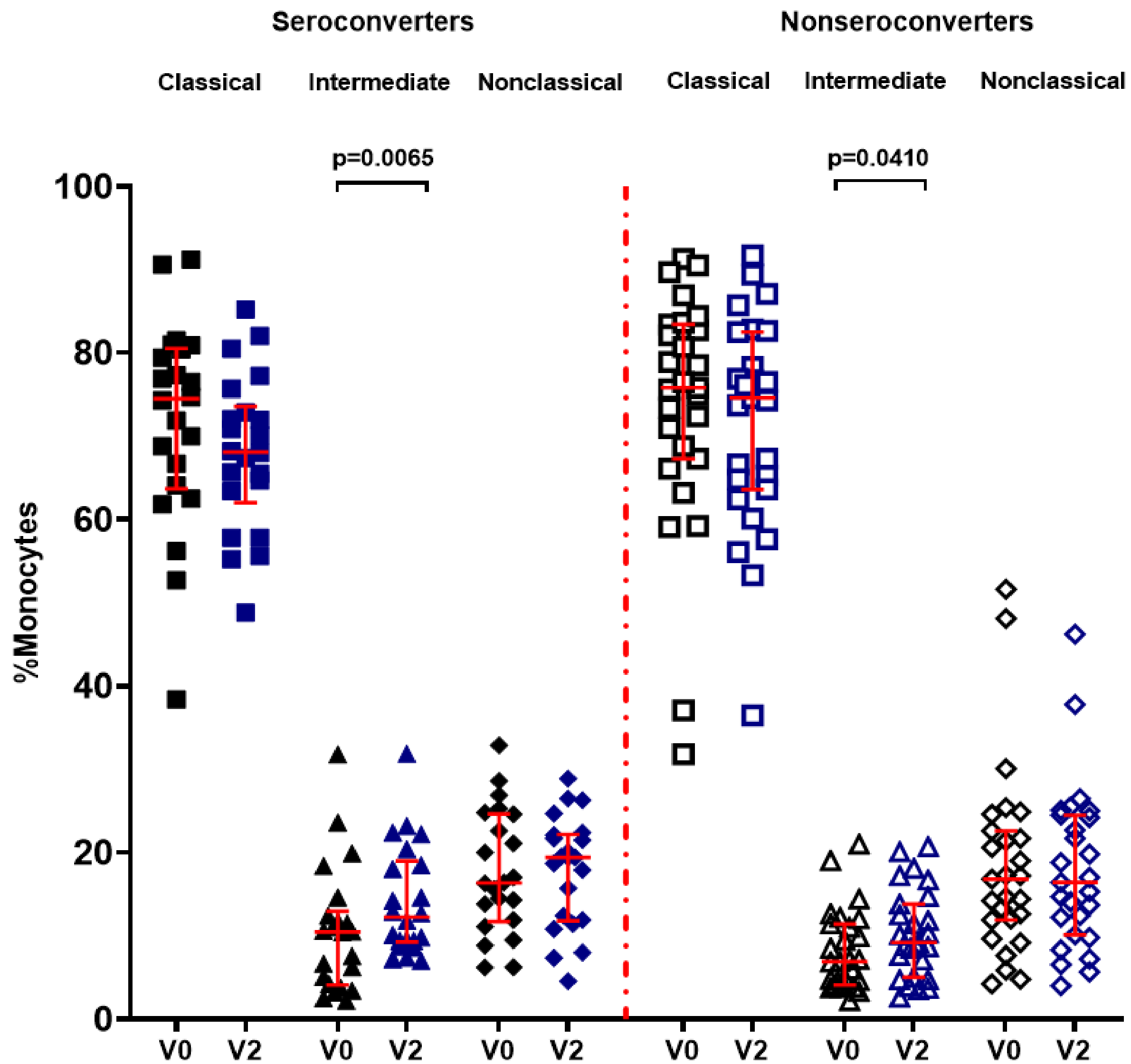


Figure 11: Increase in intermediate monocytes at day2 post LAIV in seroconverters

Changes in the frequency of monocyte subsets pre and day 2 post LAIV vaccination. Each symbol represents percentage of monocyte subset. Open symbols denote nonseroconverters and close symbol indicate seroconverters. Square (classical) triangle (intermediate) and diamond (nonclassical) monocytes. Squares represent classical monocytes, triangles represent intermediate monocytes and diamonds the nonclassical monocytes. Horizontal lines denote median and IQR. Wilcoxon matched pairs signed rank test was used to compare between the timepoints in each group, n=49. Seroconverters V0-V2- n=22, nonseroconverters V0-V2-n= 27. Due to multiple comparisons (n = 2), significance level was adjusted using Bonferroni's correction and P values of < 0.025 were considered statistically significant.

No significant difference in cell frequency was observed between seroconverters and non seroconverters for the classical and non-classical subsets. At day 7 post LAIV vaccination, a significant decrease in the frequency of classical monocytes (Day 0 (Median (IQR)= 76.40% (64.70% and 83.45%), day 2 (Median (IQR)= 68.10% (59.40% and 78.80%), p=0.0386) and an increase in frequency of intermediate monocytes (Day 0 (Median (IQR)= 11.60% (6.68% and 15.50%), day 7 (Median (IQR)= 14.20% (8.42% and 23.25%), p=0.0129) were noted in the seroconverters but no significant changes in these subsets were observed in the nonseroconverters (classical monocytes, Day 0 (Median (IQR)= 69.15% (62.08% and 78.08%), day 2 (Median (IQR)= 66.15% (60.40% and 70.85%), p=0.0386) and intermediate monocytes , Day 0 (Median (IQR)= 14.30% (8.09% and 19.53%), day 7 (Median (IQR)= 16.45% (8.77% and 21.43%), p=0.0129),(Figure 12).

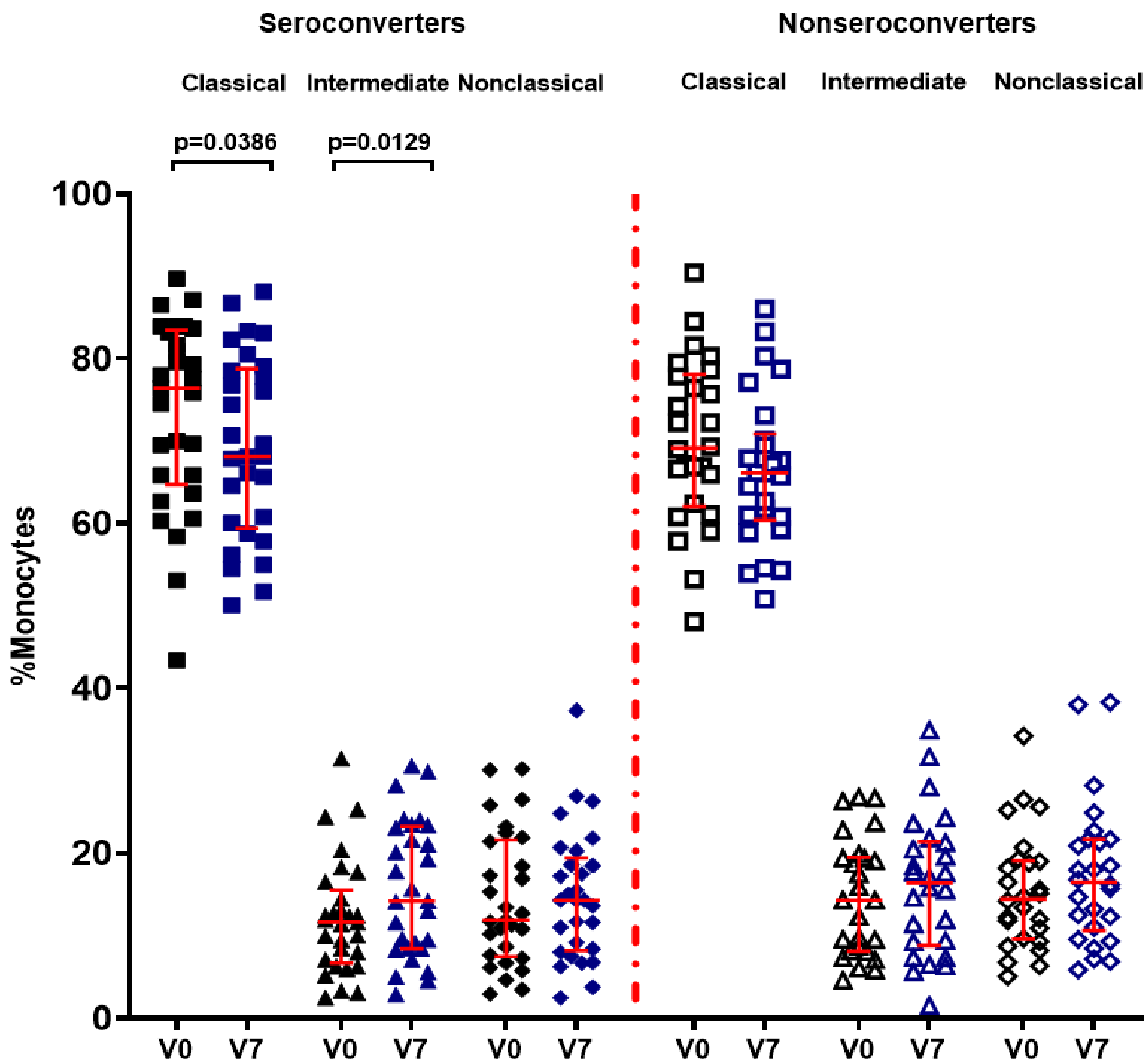


Figure 12: Increase in intermediate monocytes at day 7 post LAIV in seroconverters

Changes in the frequency of monocyte subsets pre and day 7 post LAIV vaccination. Each symbol represents percentage of monocyte subset. Open symbols denote nonseroconverters and close symbol indicate seroconverters. Square (classical) triangle (intermediate) and diamond (nonclassical) monocytes. Squares represent the classical monocytes, triangles represent intermediate monocytes and diamonds the nonclassical monocytes. Horizontal lines denote median and IQR. Wilcoxon matched pairs signed rank test was used to compare between the timepoints in each group. n=55. Seroconverters V0-V7 n=29, nonseroconverters V0-V7-n=26. Due to multiple comparisons (n = 2), significance level was adjusted using Bonferroni's correction and P values of < 0.025 were considered statistically significant.

When I compared the frequencies of dendritic cell populations between seroconverters and non-seroconverters, significant changes were noted in the pDC subset, with an increase observed at day 7 post vaccination in the seroconverters (Day 0 (Median (IQR)= 22.70% (14.20% and 28.40%), day 7 (Median (IQR)= 24.40% (17.33% and 30.53%), p=0.0399) but not in the nonseroconverters (Figure 13).

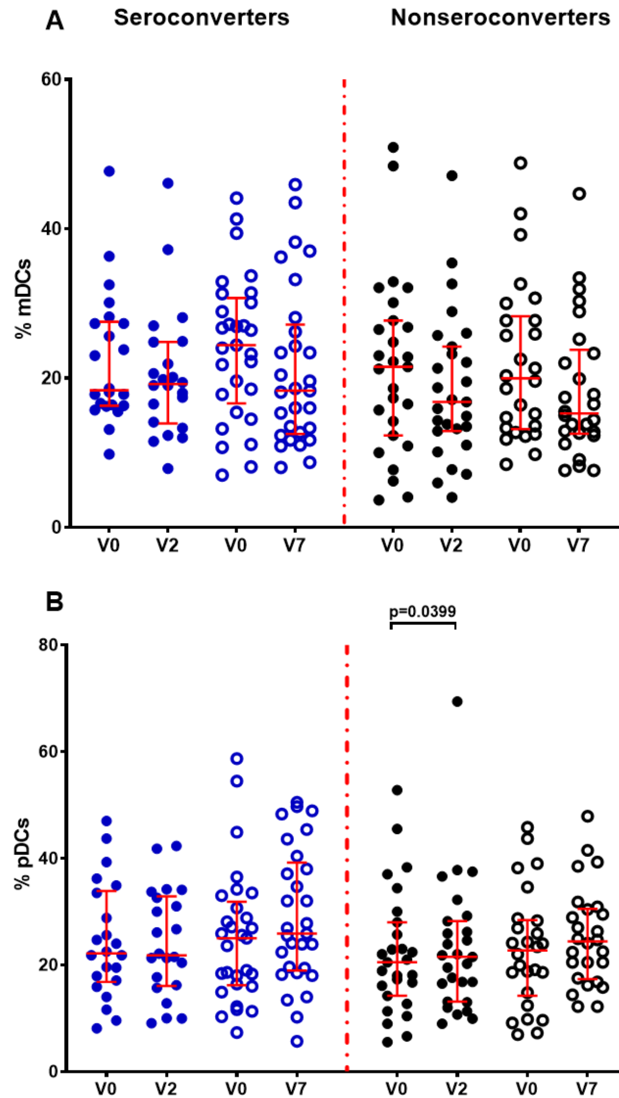


Figure 13:Changes in pDC in post LAIV

Changes in the frequency of dendritic cell populations pre and post LAIV vaccination at A) Baseline and day 2 (Group A) and B) Baseline and day 7 for (Group B) respectively Each symbol represents percentage of monocyte subset. Open symbols denote nonseroconverters and close symbol indicate seroconverters. Square (classical) triangle (intermediate) and diamond (nonclassical) monocytes. Squares represent the classical monocytes, triangles represent intermediate monocytes and diamonds the nonclassical monocytes. Horizontal lines denote median and IQR. Wilcoxon matched pairs signed rank test was used to compare between the timepoints in each group. Seroconverters V0-V2- n=22, V0-V7 n=29, nonseroconverters V0-V2-n= 27, V0-V7-n=26. Due to multiple comparisons (n = 2), significance level was adjusted using Bonferroni's correction and P values of < 0.025 were considered statistically significant.

Both seroconverters and non seroconverters demonstrated no significant change in the proportion of pDC were observed at day 2 post vaccination, or in the frequencies of mDC at both day 2 and 7 post vaccination.

With the recent finding that mucosal antibody responses correlated with innate immune cell activation post infection (Wong *et al.*, 2021), I also looked at the relationship between the IgA responses in the nasal wash and innate immune parameters. There was a significant increase in frequency of intermediate monocytes at day 2 post LAIV in the IgA responders (Day 0 (Median (IQR)= 6.61% (3.80% and 12.38%), day 2 (Median (IQR)= 10.75% (8.65% and 19.70%), p=0.0032) but not the non-responders (Day 0 (Median (IQR)= 10.40% (5.07% and 12.10%), day 2 (Median (IQR)= 9.82% (8.61% and 15.65%), p=0.0903) (Figure 14).

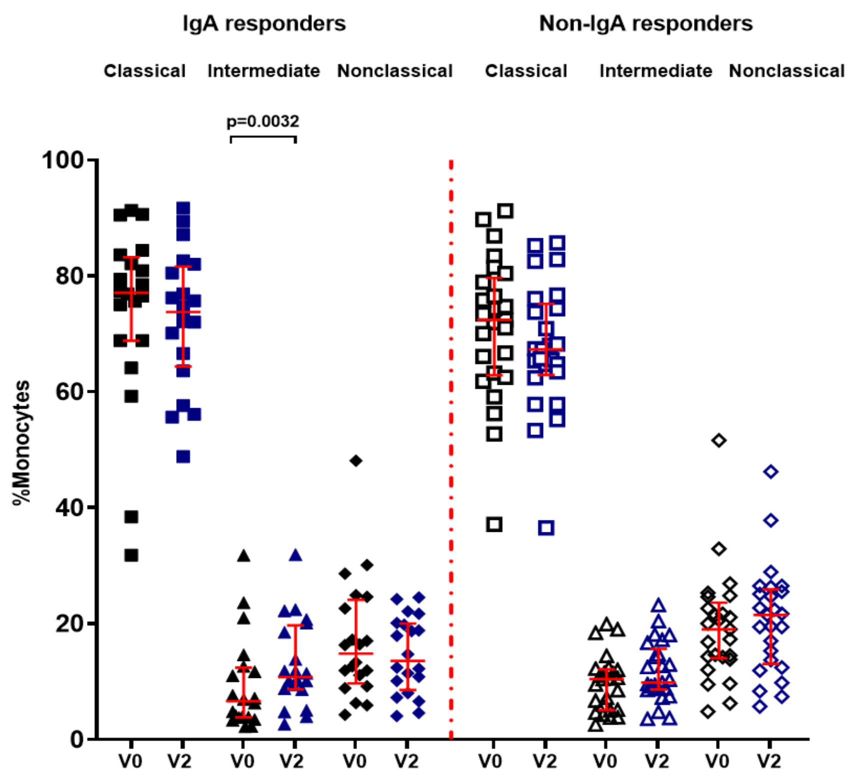


Figure 14: Increase in intermediate monocytes in IgA responders at day 2 post LAIV

Changes in the frequency of monocyte subsets pre and day 2 post LAIV vaccination. Each symbol represents percentage of monocyte subset. Open symbols denote non-IgA responders and close symbol indicate IgA responders. Square (classical) triangle (intermediate) and diamond (nonclassical) monocytes. Squares represent the classical monocytes, triangles represent intermediate monocytes and diamonds the nonclassical monocytes. Horizontal lines denote median and IQR. Wilcoxon matched pairs signed rank test was used to compare between the timepoints in each group. n=49. IgA responder V0-V2- n=20 , IgA nonresponder V0-V2-n= 25, Due to multiple comparisons (n = 2), significance level was adjusted using Bonferroni's correction and P values of < 0.025 were considered statistically significant.

No significant difference in frequency of cells was observed in IgA responders and nonresponders for the classical and non-classical subsets. At day 7 post LAIV vaccination, a significant decrease in frequency of classical monocytes was noted in both the IgA responders (Day 0 (Median (IQR)= 72.20% (65.85% and 79.95%), day 7 (Median (IQR)= 67.60% (60.80% and 73.05%), p=0.0239) and nonresponders (Day 0 (Median (IQR)= 70.90% (61.43% and 79.13%), day 7 (Median (IQR)= 66.65% (58.15% and 77.48%), p=0.0286). An increase in frequency of intermediate monocytes (Day 0 (Median (IQR)= 12.30% (9.11% and 14.40%), day 7 (Median (IQR)= 14.10% (9.32% and 20.35%), p=0.0195) was only noted in the responders but not the nonresponders (Day 0 (Median (IQR)= 13.90% (8.04% and 21.95%), day 7 (Median (IQR)= 15.75% (7.41% and 23.15%), p=0.2897) (Figure 15).

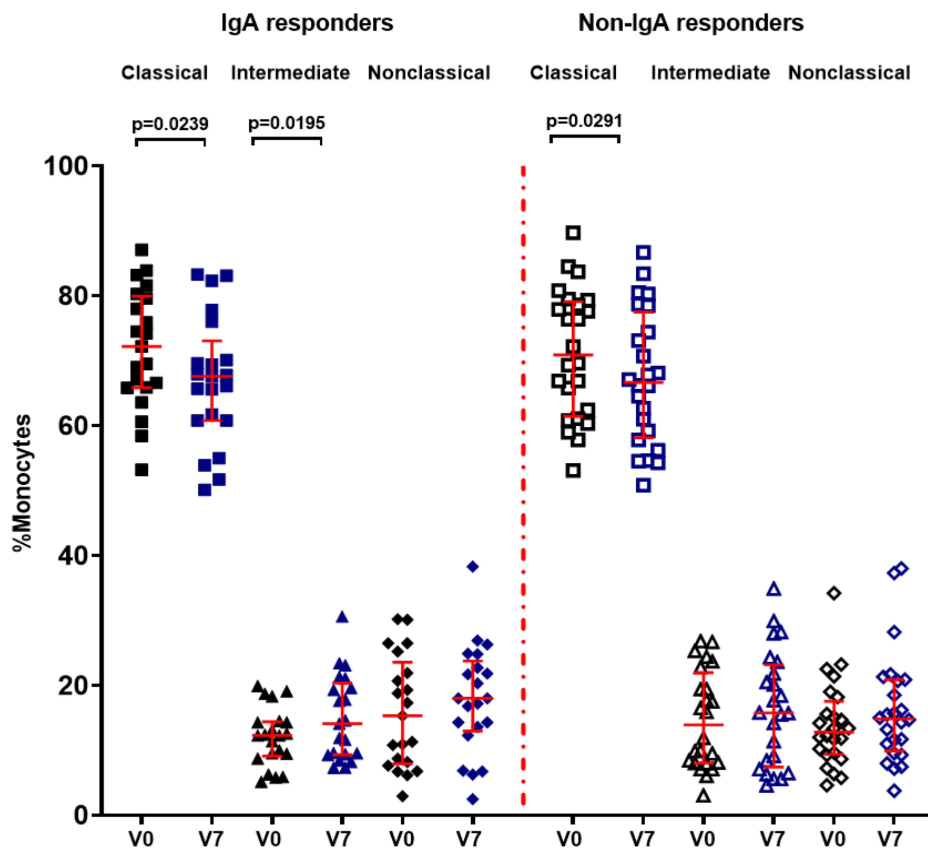


Figure 15: Increase in intermediate monocytes in IgA responders at day 7 post LAIV

Changes in the frequency of monocyte subsets pre and day 7 post LAIV vaccination. Open symbols denote non-IgA responders and close symbol indicate IgA responders. Square (classical) triangle (intermediate) and diamond (nonclassical) monocytes. Squares represent the classical monocytes, triangles represent intermediate monocytes and diamonds the nonclassical monocytes. Horizontal lines denote median and IQR. Wilcoxon matched pairs signed rank test was used to compare between the timepoints in each group. IgA responder V0-V7- n=21 , IgA nonresponder V0-V7-n= 24, Due to multiple comparisons (n = 2), significance level was adjusted using Bonferroni's correction and P values of < 0.025 were considered statistically significant.

When I looked at the dendritic cell populations, significant changes were only noted in the frequency of mDCs , at day 7 post vaccination in the nonresponders (Day 0 (Median (IQR)= 22.00% (16.00% and 29.73%), day 7 (Median (IQR)= 15.40% (12.43% and 22.58%), p=0.0053) (Figure 16) but not the responders (Day 0 (Median (IQR)= 24.00% (12.45% and 27.45%), day 7 (Median (IQR)= 17.40% (12.00% and 31.10%), p=0.1470). No significant change in the frequency of pDCs was observed at day 2 and 7 post vaccination for both responders and nonresponders.

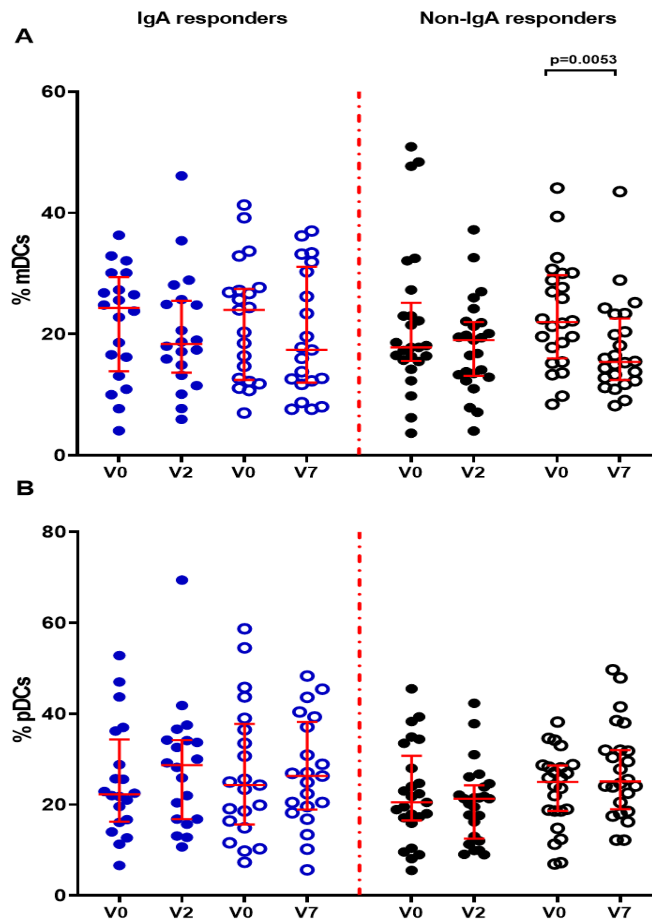


Figure 16: Changes in DC population post LAIV

Changes in the frequency of dendritic cell populations pre and post LAIV vaccination (day 2 and day 7 for Group A and B respectively). Closed symbols denote responses at baseline and Day 2 and open symbol denote responses at baseline and day 7 with IgA responders in blue and non-IgA responders in black. Horizontal lines denote median and IQR. Wilcoxon matched pairs signed rank test was used to compare between the timepoints in each group. Group A (n=45) and Group B (n=45). . IgA responder V0-V2- n=20, V0-V7-n=21, IgA nonresponder V0-V2-n= 25, V0-V7-n=24. Due to multiple comparisons (n = 2), significance level was adjusted using Bonferroni's correction and P values of < 0.025 were considered statistically significant.

3.4 Discussion

Innate immune cells play an important role in activating the adaptive arm of the immune system in response to infections and vaccination via phagocytosis, antigen presentation and cytokine production. Different monocyte subsets have been described in humans, and in line with existing data, classical monocytes were the most frequent in the blood accounting for over 70% of total monocytes, followed by the nonclassical monocytes at about 14-18% and the remaining made up of the intermediate monocytes (Ziegler-Heitbrock *et al.*, 2010). Increase or decrease in different monocyte subsets have been described in infections with various pathogens, inflammatory conditions, and vaccination (Wong *et al.*, 2012; Mohanty *et al.*, 2014). Influenza virus can infect all monocyte subsets (Hoeve *et al.*, 2012) and perturbation in monocyte subsets was reported for both infection (Oshansky *et al.*, 2014) and IIV vaccination (Mohanty *et al.*, 2014). An effect of LAIV vaccination in children on monocyte subsets has however not been reported.

3.4.1 Monocyte Perturbation

In my study, I show a decrease in frequency of classical monocytes and an increase in frequency of intermediate monocytes post LAIV. This finding is in line with findings from Mohanty et al where a decrease in frequency of classical monocytes and increase in frequency of intermediate monocytes was seen post IIV in both young and older adults (Mohanty *et al.*, 2014). A similar dynamic of change in monocyte subsets was also noted when monocytes were assessed in patients with severe or mild influenza and healthy controls (Cole *et al.*, 2017). In the blood monocytes will usually not get infected by influenza virus therefore the decrease in frequency of classical monocytes suggest that upon LAIV administration, they are possibly recruited into the respiratory mucosa where the vaccine is administered, to interact with replicating virus that are released from the infected airway epithelial cells (Cao *et al.*, 2012).

Intermediate monocytes represent the most abundant monocyte population in the nasopharynx upon influenza infection (Baharom *et al.*, 2016) and could be important in the control of virally infected cells that have escaped the site of infection. In influenza infected patients compared to controls, an increased number of intermediate monocytes is observed in both the blood and the nasopharynx compared to controls (Oshansky *et al.*, 2014; Vangeti, 2019). A similar finding of increase in intermediate monocytes was also reported in the acute phase of influenza infection with a greater conversion of classical monocytes to intermediate monocytes in seroconverters compared to nonseroconverters (Wong *et al.*, 2021). These data are in line with my findings of greater magnitude of decrease in the frequency of classical monocytes in the seroconverters compared to the

nonseroconverters and an increase in the frequency of intermediate monocytes at day 2 post LAIV in seroconverters but not the nonseroconverters. Significant increase in the frequency of intermediate monocytes in the IgA responders but not the responders suggest that early proliferation of intermediate monocytes is key for a robust antibody production as elucidated by the findings by Wong et al that seroconversion was dependent on increase in both intermediate monocytes and CD4+T cells during the acute phase of influenza infection (Wong *et al.*, 2021). Increase in frequency of intermediate monocyte and association with antibody responses was also noted in Zika virus infection (Michlmayr *et al.*, 2017). Taken together, this finding suggests that the early expansion of CD14+CD16+ cells in the blood is important for seroconversion post LAIV vaccination.

Increase in frequency of non-classical monocyte relative to classical monocytes in the nasopharynx was associated with lower cytokine levels in influenza infected individuals (Oshansky *et al.*, 2014). However, I report no change in the frequency of nonclassical monocytes in blood post vaccination with LAIV. Other studies have reported that nonclassical monocytes play a role in the immune response to viral infections such as HIV and Hepatitis B and C (Wong *et al.*, 2012). Changes in the frequency of different monocyte subsets post infection/vaccination may be due to different factors including but not limited to monocyte maturation and migration to the site of infection.

3.4.2 Monocyte maturation

Circulatory monocytes undergo a maturation process once they are released into the blood stream. After hematopoietic stem cell transplantation, monocyte repopulation was shown to begin with the classical monocytes followed by the intermediate and then the non-classical monocytes (Rogacev *et al.*, 2015). Subsequently, in a study by Patel et al, deuterium-labeled glucose was administered to healthy human volunteers to investigate monocyte maturation under normal state. Classical monocytes were the first monocyte subset in circulation after emergence from the bone marrow followed by the intermediate and the non-classical subsets. They also showed that classical monocyte spent a limited time in the blood after which majority of them will leave the circulation and the remaining cells will mature into intermediate monocytes and eventually to nonclassical monocytes by acquiring CD16 and reducing CCR2 expression (Patel *et al.*, 2017). Our finding of decreased frequency of classical monocytes and increased frequency of intermediate monocyte in blood post LAIV may therefore be as a result of maturation of monocyte from one subset to the other.

3.4.3 Chemokine receptor expression in monocyte subsets

Migration of monocytes to the sites of infection is another factor that may explain the decline in classical monocytes post LAIV vaccination in my study. Classical monocytes mainly express CCR2 and upon influenza infection, they are rapidly recruited to the site of infection (Vangeti *et al.*, 2022). CCR2 is also expressed by intermediate monocytes but at a lower level (Guermonprez *et al.*, 2019). An increase in the level of chemokines including CCL2 in the plasma of influenza patients has been reported and this correlated with the increase in intermediate monocytes in blood (Vangeti *et al.*, 2022). Influenza infection of mice lacking CCR2 showed a reduction in the number of monocytes recruited to the lungs, an increase in viral load compared to controls and a delayed virus specific CD8+ T cell response (Dawson *et al.*, 2000). This highlights the importance of CCR2 in influenza infection. Although not assessed in our study, the decrease in the frequency of classical monocytes in blood that we reported may be driven by the expression of CCR2 which allows classical monocytes to migrate from the blood circulation to tissues in this case the nasopharynx to aid in the immune response to the replicating influenza virus.

Intermediate monocytes also express high levels of CCR5 (Hijdra *et al.*, 2013). Mice lacking CCR5 showed a higher mortality rate during the first few days of influenza infection compared to controls despite having no difference in their viral loads. However, by day 2 post infection there was a huge influx of monocytes in the lung of CCR5 deficient mice compared to the controls suggesting that the lack of CCR5 did not affect recruitment of cells to the lungs (Dawson *et al.*, 2000). However, the recruitment of memory CD8+ T cells to the lung airways during virus challenge has been shown to be mediated by CCR5 (Kohlmeier *et al.*, 2008).

In humans, increased mortality was observed in Spanish patients infected with pandemic H1N1 virus that had a CCR5 mutation, (CCR5-Δ32 mutation) compared to those without the mutation suggesting that CCR5 plays a role in protection against influenza (Falcon *et al.*, 2015). Subsequent studies could however not corroborate these findings as reviewed by (Ellwanger *et al.*, 2020). CCR5 is a receptor involved in HIV pathogenesis and this may explain why intermediate monocytes are also implicated in HIV/AIDS immunopathology (Ellery *et al.*, 2007). It is important to note that although chemokine receptor expression varies among monocyte subsets, some are shared among subsets with variations in their expression levels. This allows the different subsets to carry out individual as well as collective functions as seen with migration to site of infection which can be done by both classical and intermediate monocytes. Antigen presentation however is a major function of intermediate

monocytes due to its higher expression of the MHC- class II antigen presenting molecules such as HLA-DR, CD40 and CD54 compared to other cell types (Wong *et al.*, 2011).

3.4.4 HLA-DR expression in monocyte subsets

HLA-DR is essential for antigen presentation upon infection and its expression was assessed in all monocyte subsets pre and post LAIV. The highest level of expression was seen in the intermediate and the nonclassical monocytes subsets compared to the classical subset highlighting their role in antigen presentation. A decreased HLA-DR expression at day 2 post LAIV vaccination was noted in my study. This decrease was mainly within the classical subset with no significant difference seen within the intermediate and nonclassical subsets. A reduced frequency of HLA-DR expressing classical monocytes has been previously reported in patients with severe H7N9 infection. Furthermore, a negative correlation was observed between HLADR expression in the classical monocytes and disease severity (Diao *et al.*, 2014). Decrease in HLADR expression in monocytes has also been noted in other infections such as dengue (Azeredo *et al.*, 2010), human herpes virus (Janelle and Flamand, 2006) Hepatitis C virus (Averill, Lee and Karandikar, 2007) and cytomegalovirus (CMV) infection (Averill, Lee and Karandikar, 2007) which is high in The Gambia with almost 80% of children infected by 1 year (Miles *et al.*, 2007).

3.4.5 Cytokine production in monocyte subsets

Upon activation, monocytes produce cytokines to activate other immune cells and initiate the adaptive arm of the immune system. These cytokines which include TNF- α, IL-6, IFN- γ, IL-10, IL-15, and IL-18 are produced at the early stage of the infection suggesting that they are key in viral control (Vangeti *et al.*, 2022). In my study the ability of the innate immune cells to produce IL-10 and TNF- α was measured pre and post vaccination by intracellular cytokine staining in the absence of any antigenic stimulation. I detected TNF- α and IL-10 cytokine production albeit at very low levels pre and post vaccination in all monocyte subsets. I saw a similar frequency of TNF- α producing cells in the intermediate and nonclassical subsets with the classical subset having the lowest frequency. A lower level of TNF- α production within the classical monocytes compared to other monocyte subsets upon LPS stimulation in influenza infected patients has been previously reported (Cros *et al.*, 2010).

Cytokine profile differs among monocyte subsets with conflicting data on which subsets produce the most TNF- α or IL-10 (Belge *et al.*, 2002; Wong *et al.*, 2011; Dimitrov *et al.*, 2013). Differences in expression of TNF- receptors exist within monocytes subsets with the intermediate monocytes expressing the highest level of TNFR1 and nonclassical monocytes the highest level of TNFR2 (Hijdra *et al.*, 2012).

TNF- α mediates killing of virally infected cells by antibody dependent cellular cytotoxicity (ADCC) via CD16, a major surface receptor on intermediate cells (Yeap *et al.*, 2016).

In influenza infected patients compared to controls, an increase in expression of TNF- α was seen in the absence of stimulation with the highest expression noted in the classical monocytes. A correlation between the increase in TNF- α and IL-10 seen in both blood and the nasopharynx and the frequency of classical monocytes was also observed (Vangeti *et al.*, 2022). The magnitude and time course of influenza symptoms has also been shown to correlate with TNF- α and IL-10 levels in nasopharynx (Scott Fritz *et al.*, 1998) and elevated levels of both cytokines have been observed in plasma of Influenza infected patients compared to controls (Vangeti *et al.*, 2022). The huge influx of immune cells to the site of infection and release of proinflammatory cytokines if prolonged may predisposes influenza patients to severe disease and lung damage. It is thus not surprising that as a control mechanism, effector CD4 and CD8+T cells produce large amounts of IL-10 that controls lung inflammation during influenza infection (Sun *et al.*, 2009).

3.4.6 Effect of LAIV vaccination on DC populations and cytokine production

LAIV vaccination in my cohort led to a decrease in the frequency of mDCs post vaccination (day 2, p=0.07 and day 7, p=0.02). In children (Gill *et al.*, 2008) and adults (Vangeti, 2019) infected with influenza compared with healthy controls a decrease in frequency of mDCs in the blood and an increase of this population in the nasal wash has also been reported. Antigen presentation is a major role of mDCs and the decrease in the frequency of this subset seen in my study might therefore be because of the cells homing to the site of infection or the lymphoid organs to activate other immune cells as reported in the study by (GeurtsvanKessel *et al.*, 2008).

In my study, there was no increase in pDC frequency from baseline to day 2 (p= 0.57), but a statistically significant increase was seen at day 7 (p= 0.03). In children that received measles vaccines, also a live attenuated but systemic rather than mucosal vaccine, a trend for an increase in frequency of pDCs was seen after vaccination (García-León *et al.*, 2015). Studies have shown a statistically significant decrease in frequency of mDCs but not the pDCs in the first few days following vaccination with IIV (Kobie, Treanor and Ritchlin, 2014). In a mouse model of influenza infection, despite presence of pDCs in the lymph nodes and the availability of viral antigens, pDcs did not play a role in T cell activation, viral control, and IFN-α production but a reduced antibody response to

influenza was observed when they were depleted (GeurtsvanKessel *et al.*, 2008) suggesting a division of labour within DC subsets.

I examined the frequency of TNF- α and IL-10 secreting cells pre and post vaccination without any stimulation. No difference was seen in the frequency of IL-10 and TNF- α cytokine producing cells at either day 2 or 7 post vaccination in the mDC subset whilst a significant increase in the frequency of cells producing both cytokines was seen at day 7 in the pDC subset. The mean frequency of TNF- α producing cells at any timepoint was higher in the pDC subset whilst IL-10 was higher in the mDC subset. pDCs have been shown to be the main source of TNF- α produced upon stimulation of PBMCs with influenza (Decalf *et al.*, 2007) but are poor producers of IL-10 (Sittig *et al.*, 2016).

3.4.7 Role of monocytes and DCs in humoral immune response to LAIV vaccination

I next assessed the role of monocytes and DCs in the humoral immune response to LAIV vaccination. During the acute phase of influenza infection an increase in frequency of intermediate monocytes in blood was associated with seroconversion post infection (Wong *et al.*, 2021), whilst in dengue virus infection, expansion of intermediate monocytes was associated with differentiation of B cells into plasmablasts leading to IgG and IgM secretion (Kwissa *et al.*, 2014).

CD16, the FcγRIIIa receptor found on the surface of monocytes is involved in signal transduction and plays a critical role in antibody responses. CD16+ monocytes are capable of mediating antibody dependent cellular cytotoxicity (ADCC) on parasitized cells (Royo *et al.*, 2019) or virally infected cells via TNF- α production (Yeap *et al.*, 2016). In the presence of autologous tetanus specific antibodies in plasma, CD16+CD14+ monocyte mediated activation responses were observed when tetanus vaccine antigens were induced in vitro with cord blood monocytes (Darboe et al, 2022, unpublished). Activation of monocytes can lead to its differentiation into dendritic cells (Sun *et al.*, 2009). In a study involving HIV patients, myeloid DCs from elite controllers increased the proportion of $CXCR5^{+}PD-1^{+}$ CD4+T cells in coculture experiments with B cells (Martin-Gayo *et al.*, 2017) suggesting that monocytes may contribute to antibody responses through the activation of follicular helper T cells, a subset of cells that a key in generating high avidity antibodies (Chevalier *et al.*, 2020).

DCs activate both innate and adaptive immune cells and the role of pDC in antibody responses has been reported (GeurtsvanKessel *et al.*, 2008; García-León *et al.*, 2015). I did not observe an association between mDC and pDCs with humoral and cellular immune responses in my study. A previous study of measles vaccinated children showed no correlation between T cell responses and

pDC frequency but a correlation between pDC and humoral immunity was noted (García-León *et al.*, 2015). It is important to note that although both measles and LAIV are live vaccine, their route of administration differs.

Taken together, my results show that vaccination of children with LAIV activates innate immune cells and affects their frequency in circulation. An association between the frequency of innate cells post vaccination was associated with subsequent humoral immune responses generated towards LAIV vaccination.

3.5 Conclusion

In this chapter, the aim was to characterize the innate immune cell changes post-LAIV and assess their relationship with later adaptive immune responses. Monocytes and DCs play an important role in the immune response to infections and vaccination through antigen presentation, providing the first line of defence before the adaptive immune system gets activated (phagocytosis) and production of cytokines and chemokines which will ultimately lead to activating the adaptive immune system.

The major findings from this chapter are as follows:

1. In blood, frequency of classical monocytes decreased, and Intermediate monocytes increased post vaccination.
2. HLA-DR expression is reduced post LAIV vaccination, and this was more significant within the classical monocytes.
3. There was an increase in frequency of TNF- α producing cells in all monocyte populations at day 7 post LAIV whilst the frequency of IL-10 producing cells was increased as early as day 2. This increase in frequency remained significant at day 7 post vaccination.
4. At day 7 post vaccination there was a decrease in frequency of mDCs and an increase in frequency of pDCs compared to baseline frequencies.
5. No difference in frequency of TNF- α and IL-10 producing mDCs post LAIV but an increase in frequency of pDC producing TNF- α and IL-10 at day 7 post LAIV was noted.
6. Increase in frequency of intermediate monocytes at day 2 and 7 post LAIV vaccination was associated with humoral and mucosal antibody responses to the vaccine.

4 Chapter 4: CD4+ and CD8+ T-cell responses to live attenuated influenza vaccine

4.1 Aim

The aim of this work was to characterize the frequency of CD4+ and CD8+ T-cell response to vaccination with live attenuated influenza vaccine. I specifically assessed

1. The magnitude of CD8+ and CD4+ T-cell in response to LAIV vaccination.
2. The phenotype of influenza-specific CD8+ and CD4+ T-cells in the blood at baseline and post vaccination with LAIV.

4.2 Declaration

Part of this data **(Figure 19A and C)** has already been published in the Lancet Respir Med 2019;7: 665–76, **(Figure 4C-D)**: Immunogenicity to pH1N1 with the 2016–17 and 2017–18 LAIV formulations.

The T cell phenotype data presented **(Figure: 21-24)** in this part of the thesis is from data collected for a sub project within NASIMMUNE done by Isabelle Kalra, a student from the University of Manchester whom I co-supervised in the laboratory when she came to Medical Research Council Unit, The Gambia at LSHTM for her placement in 2018. I contributed in optimizing and performing the assay when the need arose and led the data analysis.

4.3 Introduction

Young children have been identified as a high risk group for influenza vaccination with WHO estimates indicating that 10-30% of children are infected annually (Mohn *et al.*, 2020). Two types of vaccines exist, IIV and LAIV. Both vaccines have both been shown to be efficacious against both matched and mismatched strains in a study that assessed 34 RCTs involving a total of 94,821 participants, across 47 influenza seasons with data from both children and adults (Tricco *et al.*, 2013).

The assessment of cellular immune responses following LAIV has shown that both CD4+ T and CD8+ cells are induced (Hoft *et al.*, 2011), with a more pronounced response observed in the younger compared to the older age group(He *et al.*, 2006). A higher frequency of pre-existing crossreactive CD8+IFN-g+veIL-2−ve T cells has been shown to be associated with a reduced risk of fever and illness severity score and no viral shedding when infected with pandemic virus (Sridhar, Saranya *et al.*, 2013). However, the induction of CD8+T post vaccination remains questionable as whilst some studies have reported an increase in CD8+T cell responses post LAIV (He *et al.*, 2006), others saw no induction (X.-S. S. He *et al.*, 2008).

Previous studies have shown that LAIV works better in children, is easier to administer and may induces more T cell responses compared to IIV (Hoft *et al.*, 2011). Inactivated influenza vaccines induces T cell responses mostly to HA and NA proteins the major components of the vaccine, whilst LAIV induces a braoder immune response targeting both the HA and NA proteins and the more conserved internal proteins, matrix and nucleoprotein (Sridhar, Brokstad and Cox, 2015).

The role of CD4+ and CD8+T cells following LAIV administration in children in Africa has not been explored. Thus, this chapter seeks to characterize and assess CD4+ and CD8+ T cells induced by LAIV vaccination in Gambian children.

To address this question, the frequency and phenotype of CD4+ and CD8+T cells in children aged 24-59 were characterised at baseline, and day 21 after a single dose of LAIV. For the T cell phenotyping I also looked at responses at day 7 post LAIV.

4.4 Results

Figure 17 below shows the gating strategy used to identify CD4+T and CD8+ T cells producing IFN-γ and IL-2. Figure 18 shows responses in the negative control , positive control and the H3 haemagglutinin (HA) antigen stimulated condition.

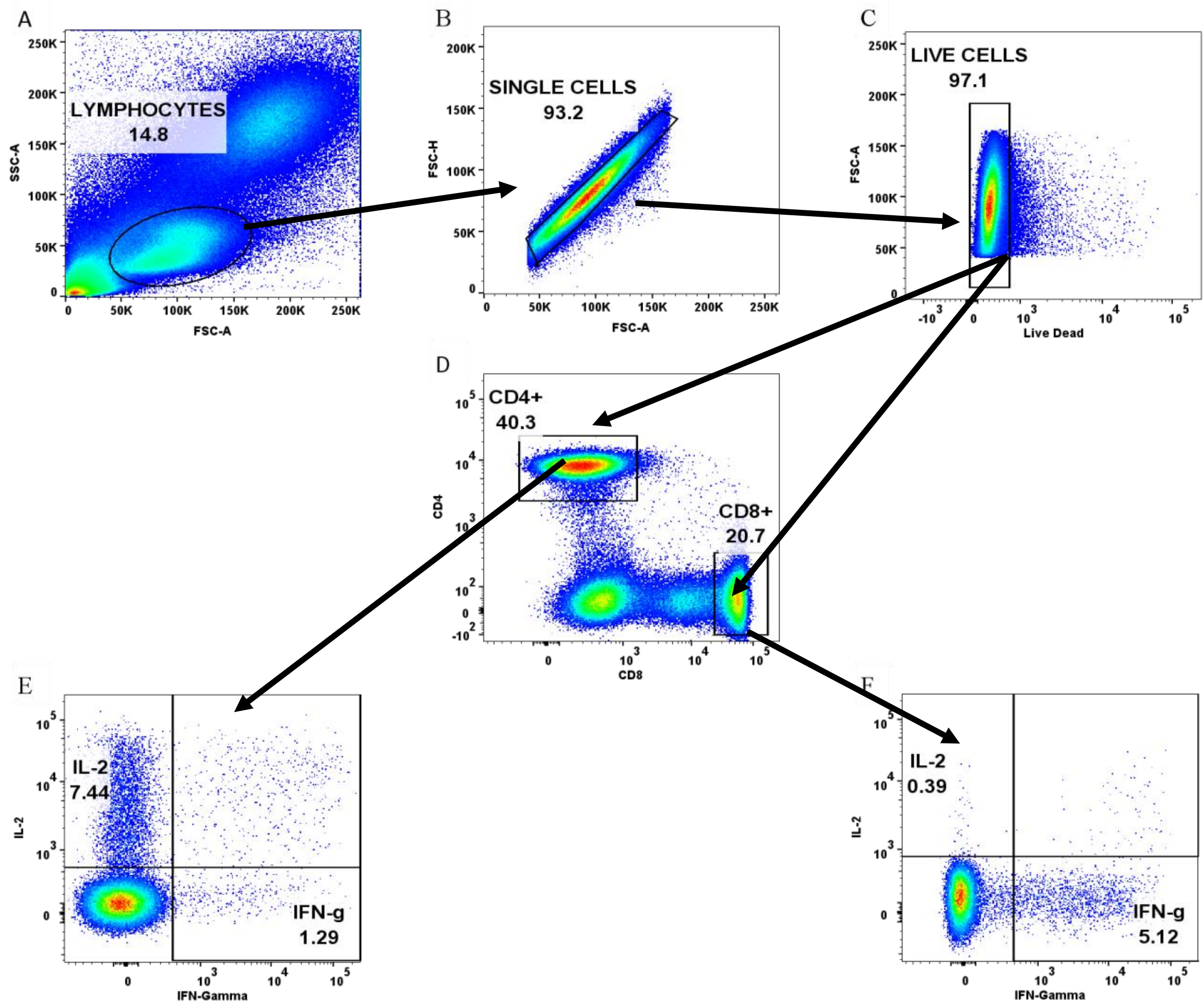


Figure 17: Gating strategy for detection of IFN- γ and IL-2 in CD4+ and CD8+ T-cells.

Displayed is from a sample following overnight stimulation with Staphylococcal Enterotoxin B (SEB). Lymphocytes were identified based on A) forward scatter area (FSC-A) versus size scatter area (SSC-A). B) Single cells were identified based on forward scatter area (FSC-A) and forward scatter height (FSC-H) C) Live were then gated on to exclude dead cells. D) CD4+ and CD8+ cells were then gated on. Within each T cell subset I gated on IL-2 and INF-Ɣ producing cells. Plot shows cytokine production in E) CD4+ and F) CD8+ T cells at day 21 post LAIV following SEB stimulation.

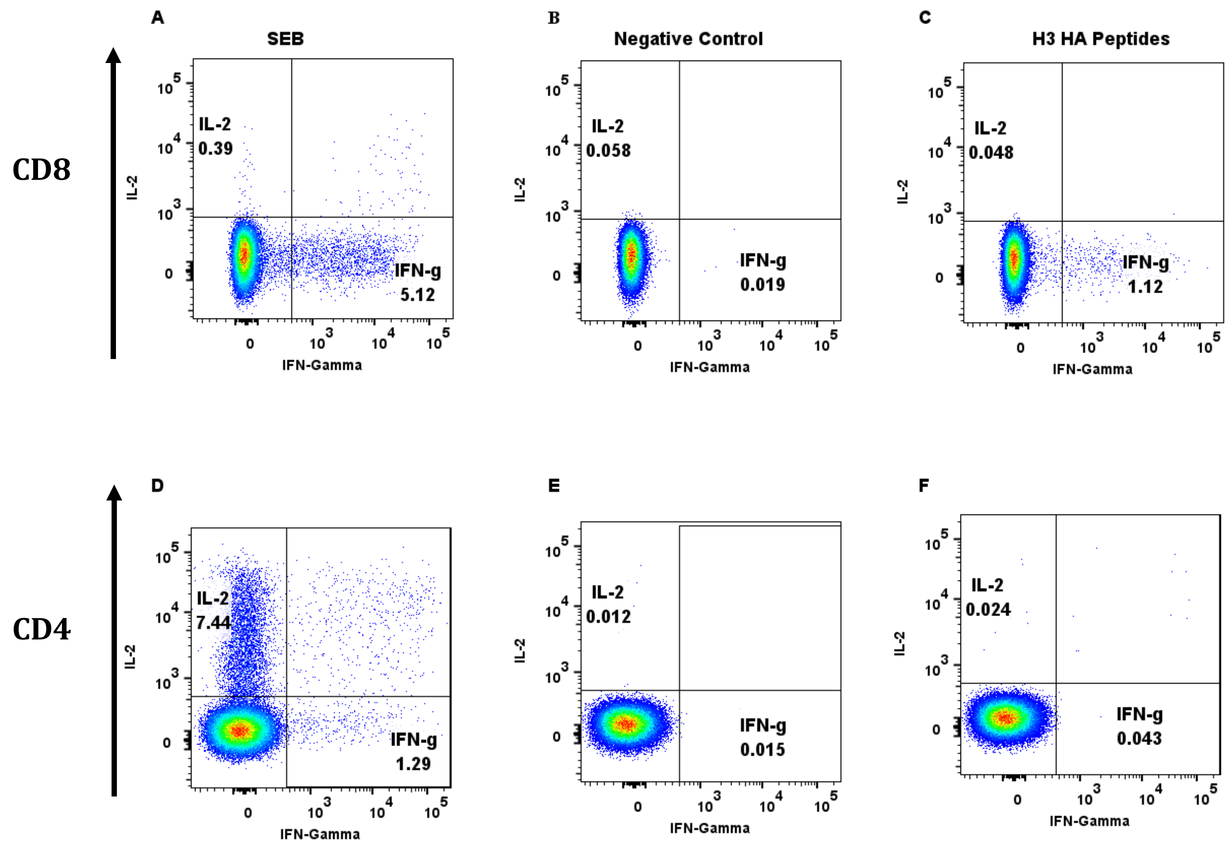


Figure 18: CD8+ and CD4+ T-cell responses (IFN- γ and IL-2) following stimulation with Staphylococcal Enterotoxin B (SEB), negative control (co-stimulatory antibodies anti-CD28 and anti-CD48 alone) and an overlapping peptide pool matched to H3 haemagglutinin protein (from A/H3N2 Hong Kong/4801/2014) at day 21 post LAIV .

4.4.1 LAIV vaccination induced increased frequencies of CD4+ IFN-γ+ and IL2+ T cells but not CD8+ IFN-γ+ and IL2+ T cells

In 2017, one hundred and eighteen (118) children were enrolled between February and April and one dose of 2016-17 NH formulation LAIV was administered. In 2018, 135 children were enrolled and received one dose of 2017-18 NH formulation LAIV. The aim of the study was to look at CD4+ and CD8+ T cells in responses to LAIV vaccination. IFN-γ and IL-2 production by CD4+ and CD8+ T cells were assessed by stimulating whole blood with influenza A specific pH1 HA, H3 HA and matrix and nucleoprotein (MNP) peptide pools for both 2016-2017 and 2017-18 LAIV responses. In addition to the Influenza A antigens, in 2018, Influenza B specific HA (BHA) and matrix and nucleprotein (BMNP) was also used.

In 2016-2017, pre-existing IL-2+ and IFN- γ + CD4+ and CD8+ T cell responses were detected (Figure 19A-E). The magnitude of pre-existing CD8+IFN-γ+T cells specific to pH1 HA, H3 and MNP was higher than CD4+ IFN-γ+T cells for all antigens (Figure 19A and 19D). After vaccination with 2016-17 LAIV, CD4+IFN- γ + T cells were significantly increased for H3 HA ($p<0.001$) and MNP ($p=0.002$) but not pH1 HA1 ($p=1.00$) (Figure 19A). pH1 HA specific CD4+IL2+ responses were not boosted post vaccination with the 2016-17 LAIV ($p=1.00$) but similar to CD4+IFN- γ + T cells, a significant increase in H3 HA ($p=0.02$) and MNP ($p=0.001$) CD4+IL2+ T cells was seen at day 21 post vaccination (Figure 19B). No increase in CD8+IFN-γ+ or CD8+IL2+ T cells post vaccination to any of the antigens was seen (Figure 19D and 19E). With the 2017-18 LAIV, the frequency of pre-existing CD8+IFN-γ+T cells specific to pH1 HA, H3 HA and MNP was also higher than the antigen specific CD4+ IFN-γ+T cell responses in all antigens at baseline (Figure 19A and 19D). After vaccination with LAIV, CD4+IFN- γ + T cells were significantly increased for all three antigens, pH1 HA ($p<0.001$), H3 HA ($p=0.002$) and MNP ($p<0.001$) (Figure 19A). An increase in CD4+IL2+T cells was seen post vaccination with pH1 HA ($p<0.001$), H3 HA ($p<0.001$) and MNP ($p<0.001$) (Figure 19B). There was no increase observed in CD8+IFN-γ+ or CD8+IL2+ T cells post vaccination to any of the antigens (Figure 19C- D).

A significant increase in number of children that had >2 fold increase in pH1 HA specific CD4+IFN-γ+ was seen with the 2017-2018 LAIV formulation ($p=0.018$) and MNP specific CD4+IL2+ responses ($p=0.002$) (Figure 19C). In 2016-2017, the CD4+IFN-γ response was highest in the H3 HA and CD4+IL2+ responses were highest with MNP. In 2017-2018 both IFN-γ and IL2 production by CD4+T cells was greater upon MNP stimulation (Figure 19C).

In 2017-18, Influenza B HA and MNP antigens were included in the assay and baseline responses were detected for both CD4+ IFN- γ + and CD4+IL-2+ T cells. Post vaccination a statistically significant increase in BHA specific CD4+ IFN- γ + ($p=0.01$) and CD4+IL-2+ T cells ($p=0.001$) were detected a significant increase in BMNP specific CD4+ IFN- γ + ($p=0.008$) and CD4+IL-2+ T ($p=0.008$) cells was also observed post LAIV vaccination (Figures 19F).

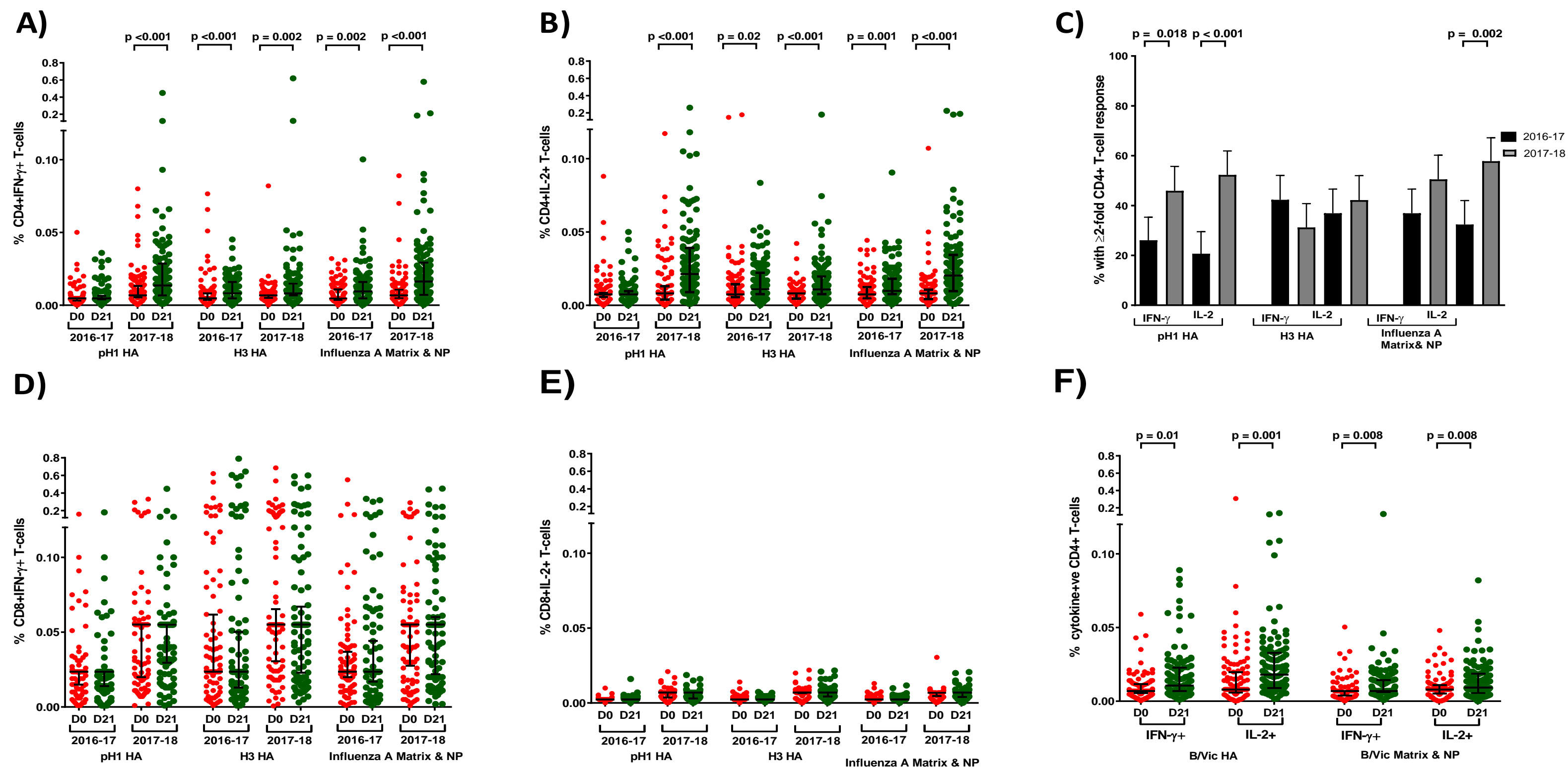


Figure 19: LAIV vaccination induced increased frequencies of CD4+IFN- γ + and IL2+ T cells but not CD8+ IFN- γ + and IL2+T cells

T-cell responses to vaccine strain-matched pH1 haemagglutinin (HA; Cal09 or NY15 in respective years), H3 HA , influenza A matrix and nucleoprotein (both matched to LAIV backbone) peptide pools, comparing 2016-17 and 2017-18 LAIVs. A) Frequency of influenza-specific CD4+ T-cell responses B) Frequency of CD4+IL2+ T-cell responses C) Percentage of children with a 2-fold rise in influenza-specific CD4+ T-cell responses at day 21 after 2016-17 and 2017-18 LAIV D) Frequency of children with CD8+ IFN-γ + T-cell responses E) Frequency of CD8+ 1L2+ cell responses. F) IFN-γ and IL-2 CD4+ T-cell responses to influenza B Victoria lineage (B/Vic) haemagglutinin (HA), as well as matrix and nucleoprotein (NP) antigen from Russian-backbone LAIV . Displayed p values are Bonferroni-adjusted for multiplicity within each group of analyses. Error bars on plots displaying percentage of responders represent the upper 95% confidence interval. NP = nucleoprotein. IFN-γ = interferon gamma.

4.4.2 The proportion of mono- and dual-functional CD4+ T-cell responses to influenza antigens tested from baseline to day 21 post-LAIV

The proportion of CD4+T cells that produce either IFN-γ or IL-2 (mono) or a combination of IFN-γ and IL-2 (dual) was assessed pre and post vaccination using Boolean gating on FlowJo and SPICE (V6.0). There was no significant change in the proportion of mono/dual-functional CD4+ T-cell responses post vaccination (Figure 20).

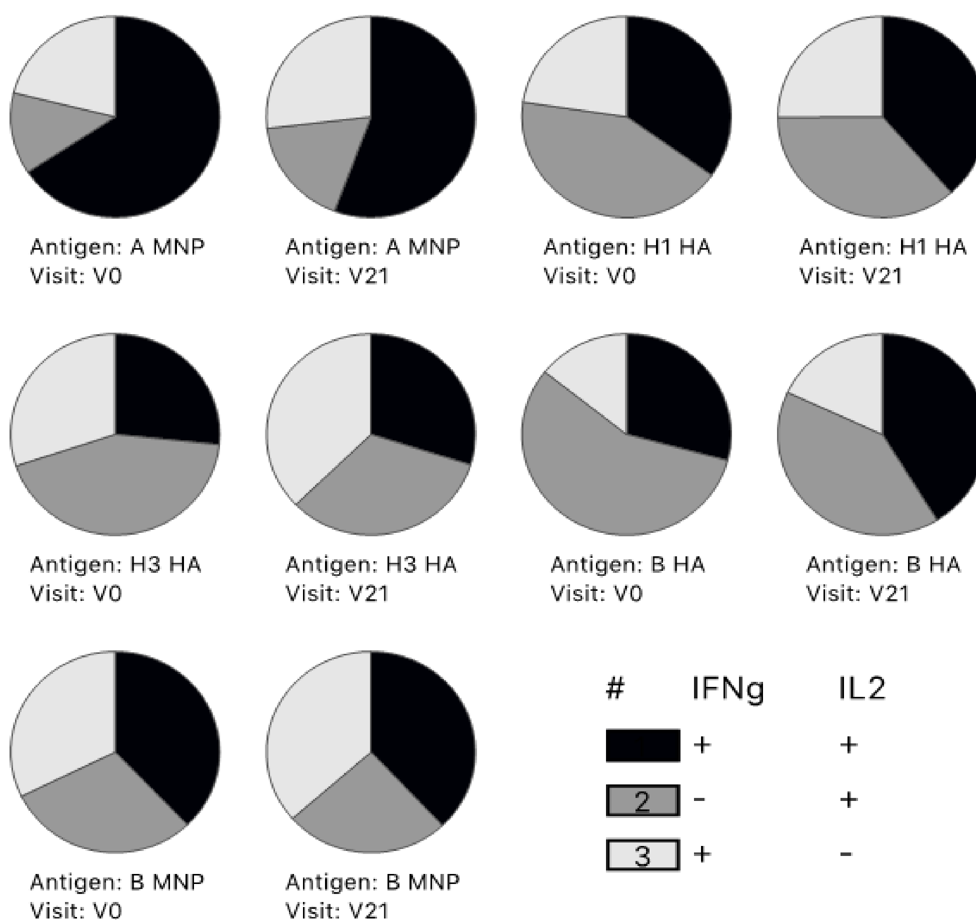


Figure 20: The proportion of mono- and dual-functional CD4+ T-cell responses to influenza antigens tested from baseline to day 21 post-LAIV

Black represents the proportion of CD4+T cells producing both IFN-γ and IL-2, dark grey represnts CD4+T cells producing IL-2 only and light grey represents CD4+T cells producing IFN-γ only.

4.4.3 CD4+ and CD8+ T cell phenotypes pre and post LAIV vaccination

CD8+T cells were induced post LAIV but no significant change in cell frequencies was noted. The phenotype of CD4+ and CD8+ T cells pre and post vaccination was therefore assessed to see if vaccination induced a change in T cell phenotype. A gating strategy for the detection of influenza specific cells and T cell activation and proliferation is shown below in Figure 21.

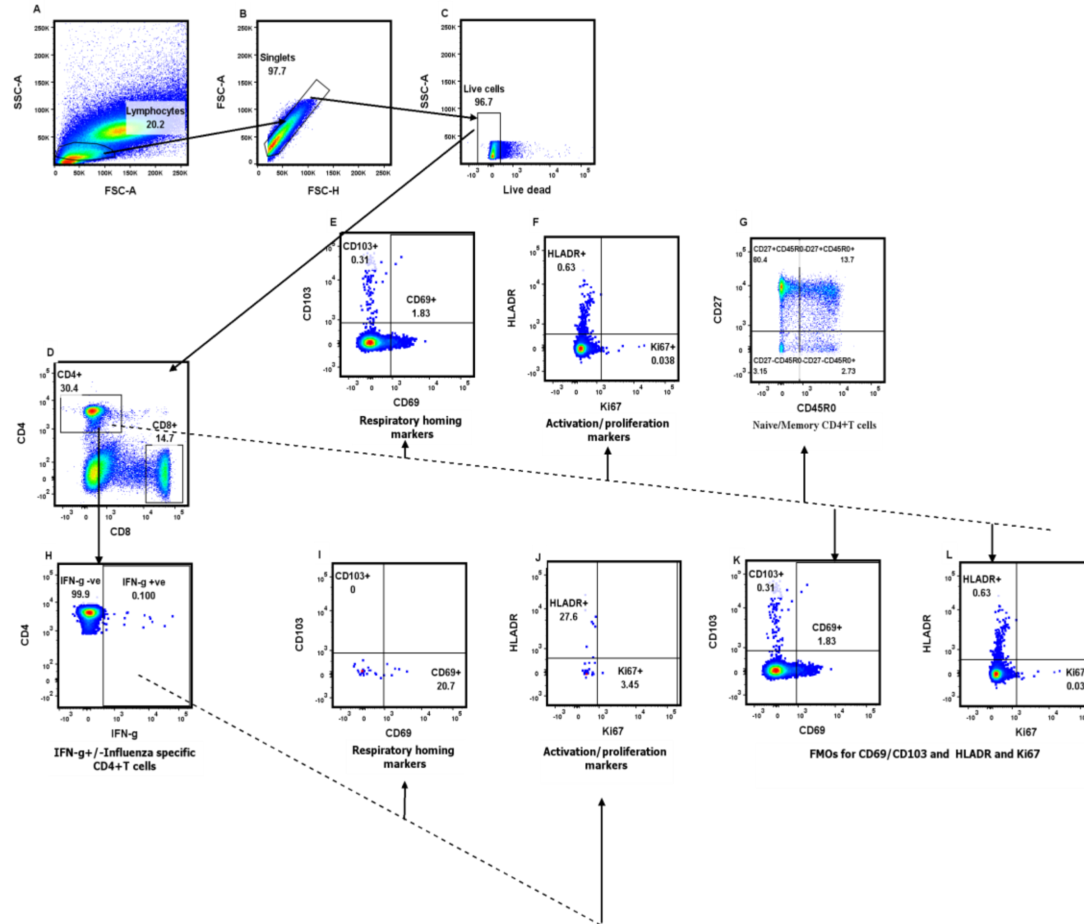


Figure 21: Gating strategy for detection of influenza specific T cells

A) Lymphocytes were identified based on forward scatter-area (FSC-A) versus side scatter-area (SSC-A). B) Single cells were then gated on C)Live cells were gated on D) CD4+ and CD8+ T cells were then gated on E) CD4+T cells expressing respiratory homing markers CD69 and CD103 F) CD4+ T cells, expressing activation and proliferation markers HLADR and Ki67 G)CD27 and CD45R0 was used to identify naïve and different subsets of memory T cells within CD4+T cells H) IFN-gamma expression in influenza specific CD4+T cells was assessed I) CD4+IFN-g+ T cells expressing respiratory homing markers CD69 and CD103 J) CD4+ IFN-g+ T cells, expressing activation and proliferation markers HLADR and Ki67 K-L) FMOs showing CD103,CD69,HLADR and Ki67 gating from CD4+T cells from HA3 stimulated sample at day 21 post vaccination.

CD27 and CD45R0 was used to identify naive T cells (CD27+CD45R0-), central memory T cells (CD27+CD45R0+), effector memory T cells (CD27-CD45R0+) and terminal effector memory cells (CD27-CD45R0-). In both CD4+ and CD8+T cells, there was a higher frequency of naive T cells and lower frequencies of central and effector memory T cells in the blood and the frequencies were higher for CD4+ T cells than CD8+T. However there was a statistically significant increase in terminal effector CD8+T cells compared to CD4+T cells (Figure 22A).

Activation of the CD4+T and CD8+T cells was assessed based on HLA-DR and CD69 expression whilst Ki67 was used to assess proliferation. At baseline CD8+T cells were more activated and expressed higher levels of both HLA-DR and CD69 compared to CD4+T cells. There was no difference in Ki67 expression between CD4+T and CD8+T cells (Figure 22B). CD103 is a respiratory homing marker and this was assessed either alone or together with CD69. There was a significant increase in CD8+T cells expressing CD103, CD69 and coexpression of CD103 and CD69 compared to CD4+T cells (Figure 22C).

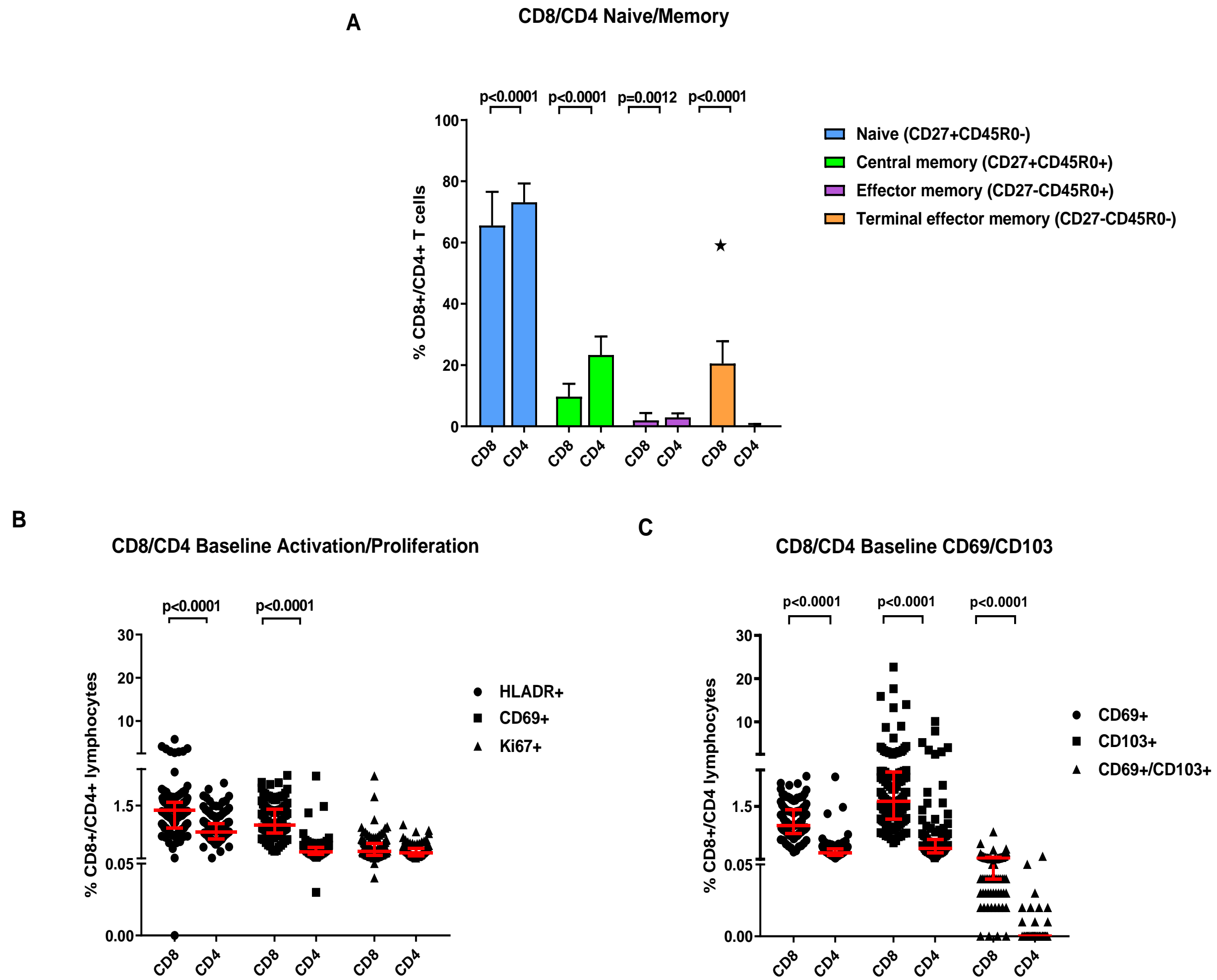


Figure 22: Total CD8 and CD4 T cell phenotype in Gambian children aged 24-59 months at baseline

Large terminal effector memory population, more activation and higher expression of respiratory homing markers in CD8+T cells than CD4+T cells. A) The proportions of naïve (CD27+CD45R0-), central memory (CD27+CD45R0+), effector memory (CD27-CD45R0+) and terminal effector memory (CD27-CD45R0-) cells in the total CD8 and CD4 T-cell populations. Median ± Q3 is shown. N=123. B)Expression of HLADR, CD69 and Ki67 in the total CD8 and CD4 T-cell populations. Line denotes median and n=123. C) Expression and co-expression of respiratory homing markers CD69 and CD103 in the total CD8 and CD4 T cell populations. Line denotes median and n=120. Data analysis was done using Wilcoxon matched pairs signed rank test. Due to multiple comparisons (n = 2), significance level was adjusted using Bonferroni's correction and P values of < 0.025 were considered statistically significant.

Influenza A H3 and MNP was used to stimulate whole blood and IFN-γ production assessed within the different T cell compartments at V0, V7 and V21. Following H3 and MNP stimulation, a similar frequency of naive, central and terminal memory CD4+T (Figure 23A-B) and CD8+T (Figure 24A-B) cells producing IFN-γ was seen post vaccination. A higher frequency of terminal effector CD8+ IFN-γ+ T cells was seen pre and post vaccination with LAIV compared to that observed within the CD4+IFN-γ + effector T cells at a similar timepoint (Figure 24A) . Terminal effector CD8+ IFN-γ+ T cells appeared to increase at day 7 and reduce at day 21 but similar to the other subsets this was not significant (Figure 24B). There was no significant change in CD4+IFN-γ and CD8+IFN-γ T cells expressing HLA-DR and Ki-67 (Figure 23B and 24B) or CD69 and CD103 (Figure 23C and 24C) at any timepoint post vaccination but it appears that the magnitude of responses were higher for CD8 than CD4. Overall, there was no significant change in phenotype of influenza-specific CD8+ and CD4+ T-cells post vaccination with LAIV.

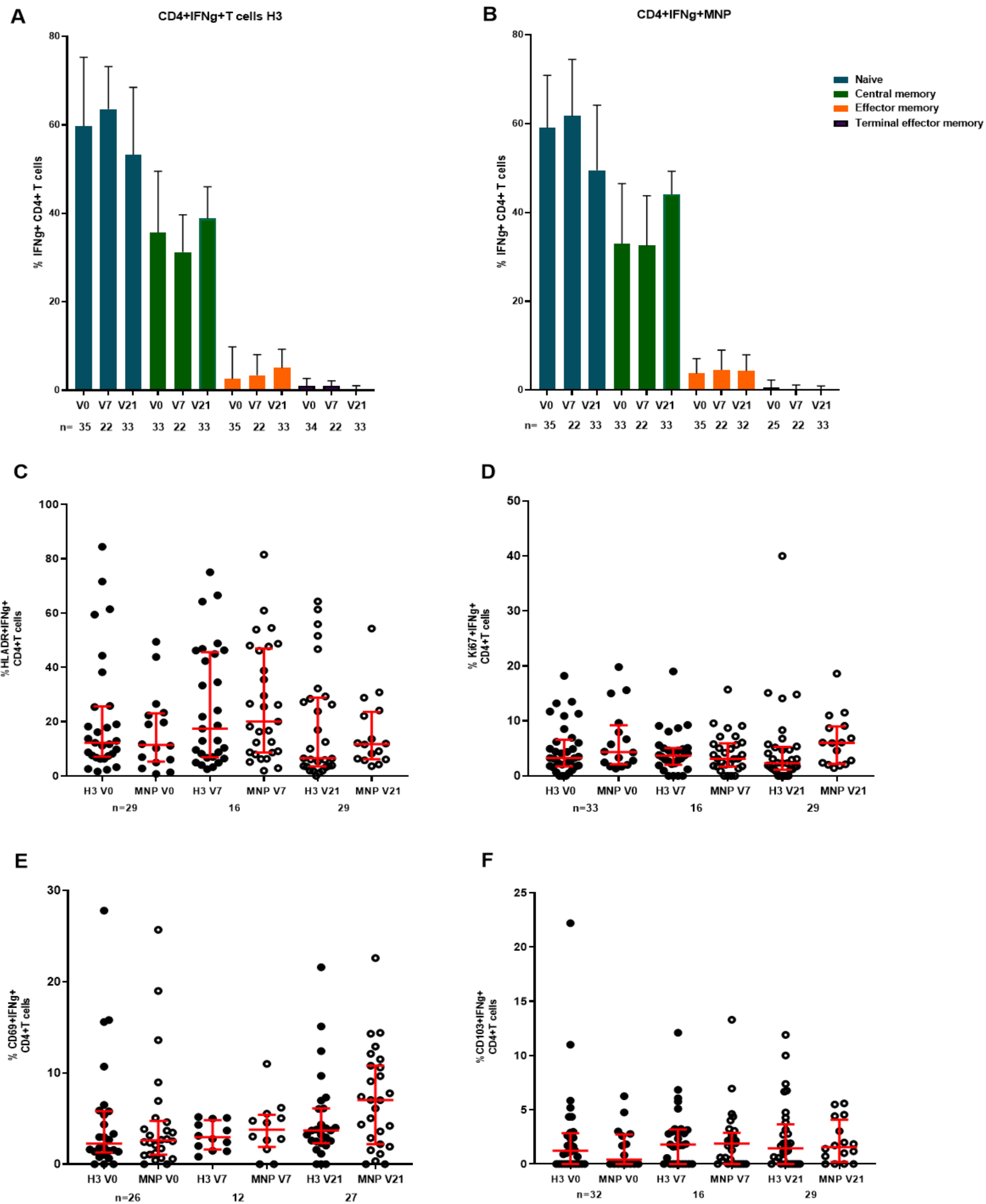


Figure 23: No change in phenotype of flu specific CD4+T cells in response to LAIV

The flu-specific CD4 populations of naïve (CD27+CD45RO-), central memory (CD27+CD45RO+), effector memory (CD27-CD45RO+) and terminal effector memory (CD27-CD45RO-) cells in response to A) haemagglutinin 3 (H3) and B) matrix and nuclear protein (MNP) at baseline (V0), 7 days (V7) and 21 days after vaccination (V21). Median + Q_3 is shown. (B) The flu-specific CD8 cell populations expressing the activation marker C) HLA-DR and proliferation marker D) Ki67 in response to H3 and MNP at V0, V7 and V21. Median ± IQR is shown. (E) The flu-specific CD4+ T cell populations expressing the respiratory homing markers CD69* and IFN- ϒ and F) CD103 and IFN- ϒ in response to H3 and MNP at V0, V7 and V21. Median ± IQR is shown. Data analysis was done using Wilcoxon matched pairs signed rank test.

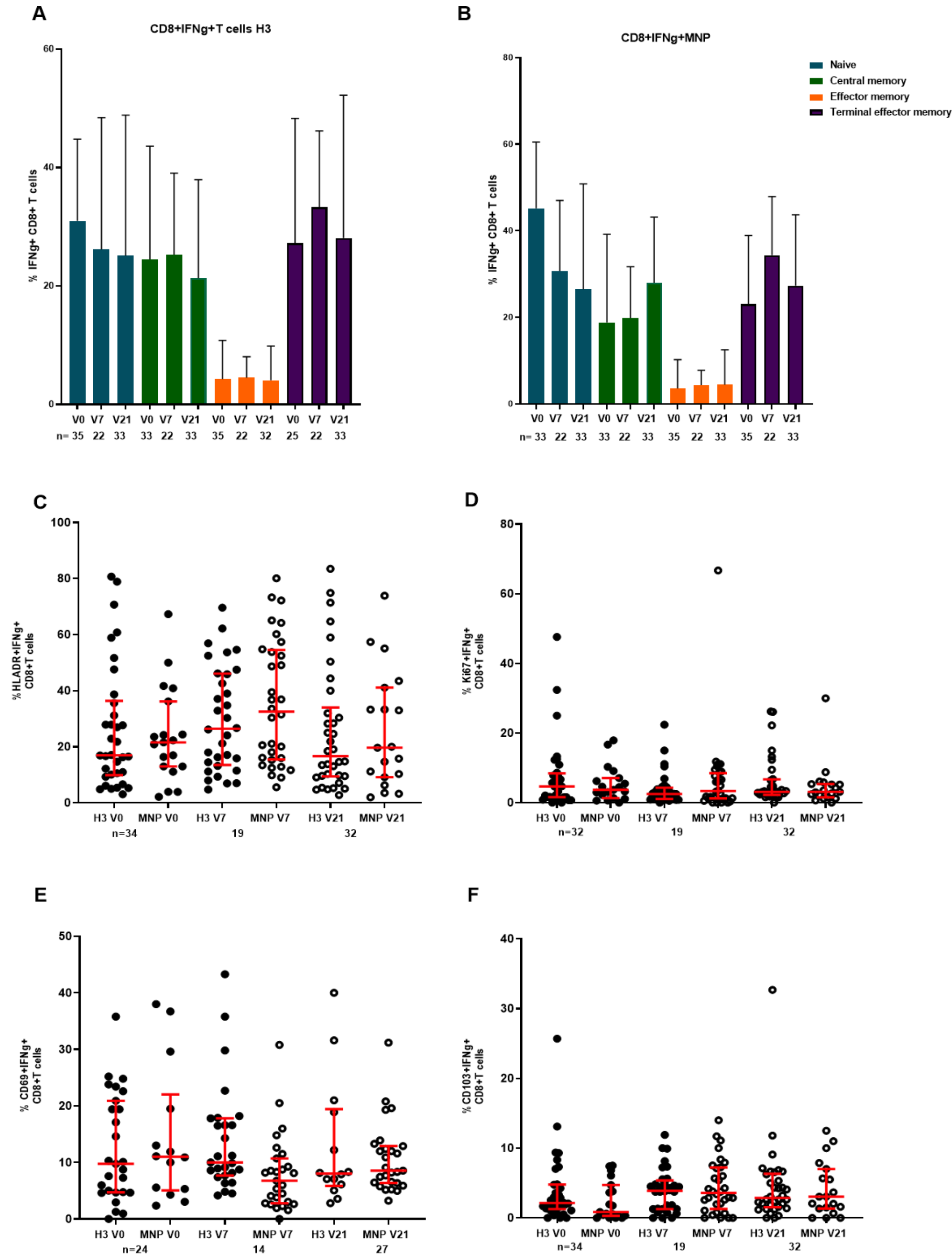


Figure 24: No change in phenotype of flu-specific CD8+T cells in response to LAIV

The flu-specific CD8 populations of naïve (CD27+CD45RO-), central memory (CD27+CD45RO+), effector memory (CD27-CD45RO+) and terminal effector memory (CD27-CD45RO-) cells in response to A) haemagglutinin 3 (H3) and B) matrix and nuclear protein (MNP) at baseline (V0), 7 days (V7) and 21 days after vaccination (V21). Median + Q_3 is shown. (B) The flu-specific CD8 cell populations expressing the activation marker C) HLA-DR and proliferation marker D)Ki67 in response to H3 and MNP at V0, V7 and V21. Median ± IQR is shown. (E) The flu-specific CD8+T cell populations expressing the respiratory homing markers CD69* and IFN- ϒ and F) CD103 and IFN- ϒ in response to H3 and MNP at V0, V7 and V21. Median ± IQR is shown. Data analysis was done using Wilcoxon matched pairs signed rank test.

4.5 Discussion

4.5.1 Greater increase in frequency of CD4+T cells after LAIV vaccination

This is the first study of LAIV immunogenicity in Africa. Our data shows that LAIV vaccination led to an increase in the frequency of CD4+T cell post vaccination in young children. Although CD8+T cells were induced, the difference in frequency post vaccination was not significant compared to baseline frequency. Others studies have also reported a lack of CD8+ T cell induction post vaccination with LAIV (C. Eichelberger, 2012; Mohn *et al.*, 2015) whilst others have shown CD8+ T cell induction (He et al., 2006). This may be because CD8+T cells act at the site of infection and in the case of LAIV vaccination, it will be the mucosal tissue so analyzing them in blood may not give a true reflection of induction after vaccination. But the Mohn et al study where PBMCs were used showed CD8+ T cell induction thus this possibilty may be an unlikely reason for the lack of induction seen. It is also worth noting that at baseline CD8+T cell responses were higher than the CD4+T cell responses (presumably induced by previous wild type influenza infection). A previous study by He et al showed that lower baseline responses were associated with higher T cell responses post vaccination with LAIV (He et al., 2008) suggesting that the high baseline CD8+T cell responses may impact CD8+T cell response post LAIV vaccination. However, boosting of pre-existing T cells post vaccination has been previously reported in studies where over 70% of the children had pre-existing CD8+ T cell responses (He et al., 2006; Mohn et al., 2017). There is also a possibility that the one dose of LAIV was not enough to induce significant CD8+T cell responses as in the studies that reported CD8+T cell responses, most of the children within this age group who had not been previously vaccinated will received 2 doses of LAIV as opposed to the 1 dose of LAIV that was administered in this study. CD4+T cells play a role in influenza infection in multiple ways and the 3 fold increase in the number of influenza epitopes identified for CD4+T cells compared to CD8+T cells suggests that the lack of increase in frequencies of CD8+ T cell post LAIV may be because they are less frequently induced compared to CD4+T cells (Chen *et al.*, 2014).

4.5.2 Effect of LAIV vaccination on cytokine production

Mono as well as dual cytokine producing CD4+T cells were detected post LAIV, similar to previous reports (Mohn *et al.*, 2015). Cytokine production from CD4+ T cells induce innate immune cell activation with these cells in turn producing IFN-γ to activate other T cells thus potentiating the immune response (McKinstry et al., 2011). Multifunctional cytokines are thought to be more superior than mono cytokines in initiating immune responses (Kannanganat et al., 2007).

In children who received the 2016-2017 LAIV formulation, the CD4+ IFN-γ response was slightly higher for H3 HA compared to MNP with the lowest responses seen with H1 HA . In 2017-2018 the pH1N1 component of LAIV was updated and the A/California/07/2009-like (Cal09) portion was changed to A/Michigan/45/2015-like strain (A/17/New York/15/5364, NY15). This led to a significant increase in the number of children with a >2 fold increase in CD4+T cell responses. When this change occurred, the CD4+ T cell response against MNP for both IFN-γ and IL2 production also increased. The MNP antigen induced high frequency of CD4+IFN-γ+ and CD4+IL2+ T cells consistent with data showing that M and NP are the main targets of CD4+T cell response to Influenza (Chen *et al.*, 2014) but high levels of both CD4+IFN-γ+ and CD4+IL2+ were also induced by HA which has also been shown to be a target of CD4+T cell responses (Babon *et al.*, 2009). This increase in responses may also be due to the two viruses (pH1N1 and H3N2) now inducing a robust immune response, both of which have the same matrix and nucleproteins due to the shared master donor virus. Significant pre-existing IFN-γ+CD4+T cells was shown to correlate with a decrease in severity of infection and viral shedding in a human challenge model (Wilkinson *et al.*, 2012). Even with the change in the H1N1 portion of the vaccine, CD8+ T cells were not induced post LAIV vaccination. We saw a difference in MNP responses specific to Influenza A and Influenza B antigens. There was no increase in BMNP specific CD4+T cells producing IFN-γ or IL2 as opposed to the high levels of cytokines secreted when Influenza A MNP was used for the stimulations.

The ability of LAIV to exert an immune response is dependent on the ability of the virus to replicate. In the wider Nasimmune study within which this PhD is nested, we reported that there was a difference in replicative capacity between the two pH1N1 influenza strains in the 2016-17 and 2017-18 LAIVs (Lindsey *et al.*, 2019). This may well have led to the differences in cytokine profiles seen in our analysis. In addition, viral strain circulation may also have an impact on immune responses to LAIV vaccination (Mohn *et al.*, 2015) as higher responses are generated to strains that circulate less.

4.5.3 Phenotype of CD4+ and CD8+ T cells post LAIV vaccination

When phenotypes of antigen specific CD4+ and CD8+ T cells were assessed we saw no change post vaccination with LAIV. This is consistent with findings from a previous study which showed that in children, there was no increase in influenza specific CD8+CD27+ T cells with both LAIV and IIV vaccination (He et al., 2008). More effector memory CD8+T cells were seen pre and post vaccination with LAIV but this was not statistically significant consistent with reports from previous studies which have shown that this memory T cell compartments produces the highest amount of IFN-γ despite no change in their frequencies post vaccination (Sridhar *et al.*, 2012). There was a higher

frequency of CD8 terminal effector cells compared to CD4+T cells at baseline. This may be due to a high prevalence of cytomegalovirus (CMV) in this population, as reported by Miles et al, who showed that CMV drives the CD8+ T-cell compartment towards a more terminally differentiated phenotype. By the age of 1 year, almost 85% of children in Sukuta (where our study was also conducted) are infected with CMV (Miles *et al.*, 2007). It is worth noting that this change in phenotype of the CD8+T reduces the ability of the T cell to produce cytokines and may thus affect vaccine responses. To conclude, compared to IIV, the ability of LAIV to induce better celluar responses and induce production of polyfunctional cytokines may make LAIV an ideal vaccine candidate, especially in young children who will have lower levels of pre-existing T cells and antibodies that are generated through years of natural exposure or vaccination. The function that these LAIV-induced CD4+ T-cells are playing is currently unclear and I will characterise them further (i.e. by looking at CD4+ T-follicular helper cells) and assess the relationship with other arms of immunity like serum and mucosal antibodies.

4.6 Conclusion

In this chapter, the aim was to characterize the CD4+ and CD8+ T-cell response to vaccination with live attenuated influenza vaccine. I assessed the magnitude of CD8+ and CD4+ T-cell in response to LAIV vaccination. I also assesed the phenotype of influenza-specific CD8+ and CD4+ T-cells in the blood at baseline and post vaccination with LAIV.

The major findings from this chapter are as follows:

1. At baseline we observed higher CD8+T cell responses to influenza antigens in our study population.
2. A single dose of LAIV led to significantly increased frequency of CD4+T cells but not CD8+T cells in Gambian children post vaccination.
3. No change in the phenotypes of CD4+ and CD8+ T cells was observed post LAIV.

5 Chapter 5: Follicular helper T cell responses to LAIV vaccination

5.1 Aim

The aim of this work was to characterize the detailed phenotype of circulating Tfh cells pre- and post-vaccination with LAIV. I specifically assessed:

1. Whether specific circulating Tfh subsets are induced by LAIV.
2. Whether these changes correlate with mucosal IgA titres and serum hemagglutination inhibition (HAI) titres.

5.2 Introduction

Follicular helper T (Tfh) cells are a subset of CD4+T cells that are found localized in B cell follicles. They are essential for germinal center formation and contribute to the development of high affinity antibodies, plasma cells and memory B cells (Schmitt et al., 2013; Crotty et al., 2014). Markers such as CXCR5, CCR7, BCL-6, IL-21, ICOS and PD1 are used in Tfh cell identification (Breitfeld *et al.*, 2000; Bentebibel *et al.*, 2013; Locci *et al.*, 2013; Schmitt, Bentebibel et al., 2014). CXCR5 plays a role in B cell migration (Moser et al., 2015) and within Tfh cells, the CXCR5+CD4+T Tfh cells have been shown to be superior in inducing antibody production compared to their CXCR5- counterparts (Morita *et al.*, 2011; Schmitt, Bentebibel and Ueno, 2014).

A population of Tfh cells has also been described in blood with similarities in some surface marker expression and function (Morita *et al.*, 2011). However, a higher expression of these markers is seen in germinal centers (GC) compared to circulating blood Tfh cells (He *et al.*, 2013; Crotty, 2014).

In blood, three different subsets of Tfh cells, Tfh-1, Tfh-2, and Tfh-17 have been identified based on expression of the chemokine receptors CXCR3 and CCR6. These Tfh subsets produce different cytokines and have different capacities to provide B cell help for antibody responses post vaccination (Morita *et al.*, 2011). Within Tfh cells, Tfh-1 subset is the population that expresses both ICOS and PD1 and with higher expression of CXCR5 (Schmitt et al., 2014). The ability of Tfh cells to provide B cell help is ICOS dependent and a reduced frequency of circulating Tfh cells and defects in germinal center formation has been reported in patients with ICOS deficiency (Bossaller *et al.*, 2006). Increased expression of ICOS in Tfh cells post vaccination with the inactivated influenza vaccine has previously been observed and this was confined to the Tfh 1 subset (Bentebibel *et al.*, 2013; Spensieri *et al.*, 2016). Increase in ICOS expression has also been shown to correlate with total IgG and IgM responses following inactivated influenza vaccination in a subsequent study (Herati *et al.*, 2014).

A correlation between subsets of circulating Tfh cells and antibody responses post IIV vaccination (Bentebibel *et al.*, 2013; Spensieri *et al.*, 2016) has been previously reported. In contrast, a study using the inactivated influenza vaccine, reported a lack of correlation between Tfh cells and HAI titres as well as antigen specific total IgG and IgM post vaccination (Herati *et al.*, 2014).

Serum antibody responses measured by HAI assay represent an agreed correlate of protection for IIV. However, at present, no correlate of protection has been identified for LAIV. Given that mucosal

antibody responses dominate in LAIV vaccinees, this parameter may be useful in defining correlates of protection for LAIV. A major hurdle however is the difficulty to analyse mucosal samples and the assays available are not standardized (Sridhar *et a*l., 2015). The identification of a circulating phenotype of Tfh cells which contribute to the humoral response to influenza vaccination (Bentebibel *et al.*, 2013, 2016; Herati *et al.*, 2014; Spensieri *et al.*, 2016) therefore provides an opportunity to explore its suitability to serve as a biomarker to assess LAIV immunogenicity.

Very few studies have explored Tfh responses in LAIV vaccinated children: a recent study of LAIV vaccinated children reported an induction of tonsillar Tfh cells which correlated with systemic IgG but not HAI titres post vaccination (Lartey *et al.*, 2020). In PBMCs stimulated with LAIV, an induction of Tfh cells was seen at day 7 leading to the detection of anti HA-IgG and IgM in the culture supernatants. Depletion of CD45R0+, ICOS+, IL21+ and BCL-6+ cells led to a reduction in antibody responses highlighting the important role of these markers in the Tfh dependent antibody responses (Aljurayyan *et al.*, 2018).

No studies have assessed circulating Tfh responses in children vaccinated with LAIV. This chapter therefore investigates whether circulating Tfh cells provide T cell help towards generation of mucosal and systemic antibody responses post LAIV vaccination. This is currently unknown.

To address this question, I used the serum and mucosal antibody data generated within the NASIMMUNE study. Serum antibody responses were measured at baseline and on day 21 post-LAIV using the HAI assay and as previously reported, a ≥ 4-fold increase in HAI titre from baseline is defined as seroconversion. Mucosal influenza-specific IgA was measured in oral fluid samples at baseline and day 21 post-LAIV using a protein microarray. Mucosal influenza-specific IgA responders were defined as those with at least a two- fold change in antibody concentration from baseline. The detailed phenotype of circulating follicular helper T cells (Tfh) in a total of 130 children aged 24-59 months (56 in 2017 and 74 in 2018) vaccinated with a single dose of LAIV (Nasovac-S) was assessed using *ex-vivo* cytokine staining as described in Section 2- Materials and methods. In 68 of the children vaccinated in 2018, the activation induced marker (AIM) assay was used to assess antigen specific Tfh cells post LAIV vaccination. To limit the number of bleeds, children were randomized into different groups for the various immunology assays to be conducted. Figure 1 shows the number of children and type of assays done to assess Tfh cells in both years.

5.3 Results

5.3.1 *Ex-vivo* Tfh changes post LAIV vaccination

Whole blood samples collected from LAIV vaccinated children were stained *ex-vivo*. To identify Tfh cells, gating was initially performed to focus on CD4+CD45R0+CXCR5+ cells as shown in Figure 25. CXCR3, ICOS and PD1 have also been previously identified as Tfh markers (He *et al.*, 2013; Schmitt *et al.,* 2013; Crotty *et al,*, 2014). As previous studies have used CXCR3 and ICOS alone or with the addition of PD1, analysis was done using both approaches. Previous studies have reported that Tfh cells especially the Tfh-1 subset play a role in the antibody responses generated post IIV vaccination(Bentebibel *et al.*, 2016), I therefore looked at the frequency of Tfh cells within responders and nonresponders using both the serum and mucosal antibody data. Baseline immune response determines the subsequent immune response to vaccination (X. S. He *et al.*, 2008), I will therefore look at the frequency of Tfh cells in children that had responses to influenza antigens at baseline compared to children that did not based on HI titres.

Within CXCR3+ cells, ICOS expression and the co-expression of PD1 and ICOS was assessed using *ex-vivo* cytokine staining. At day 7 post vaccination, there was a significant increase compared to baseline in both the frequencies of CXCR3+ICOS+ Tfh cells ($p = 0.0011$) and CXCR3+ICOS+PD1+Tfh cells ($p = 0.005$). No significant difference was seen between baseline and day 21 post vaccination, consistent with circulating Tfh-like cells in the blood by day 21 (Figure 26A and 26B).

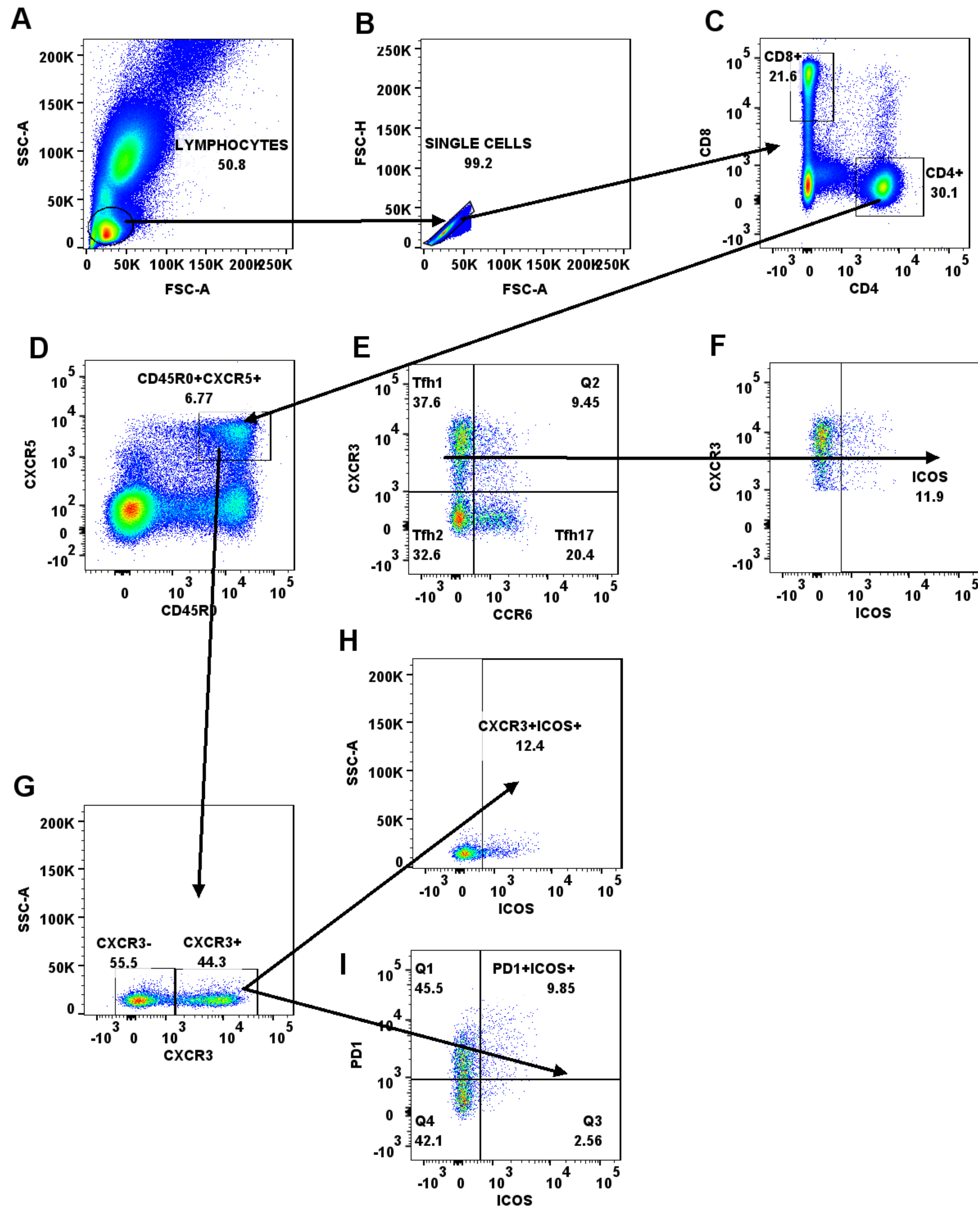


Figure 25 A-I: Gating strategy to identify circulating follicular helper T cells (Tfh) in blood and different Tfh subsets following ex-vivo staining of whole blood in LAIV vaccinated children

Ex-vivo staining was done using whole blood samples collected from children vaccinated with LAIV. A) Lymphocytes were identified based on forward scatter-area (FSC-A) versus side scatter-area (SSC-A). B) Single cells were then gated on C)CD4+ and CD8+ T cells were then gated on D) Using CD45R0 and CXCR5, CD4+T cells expressing CD45R0 and CXCR5 (CD45R0+CXCR5+) were gated on. This population represents circulating follicular helper T cells (Tfh) E) Within Tfh cells, the markers CXCR3 and CCR6 were used to identify 3 Tfh subsets. These are CXCR3+CCR6- (Tfh 1), CXCR3-CCR6- (Tfh 2) and CXCR3-CCR6+ (Tfh 17) subsets. F) ICOS+ cells were then gated on from each Tfh subset. Plot shows gating from Tfh1 subset. G) Within Tfh cells, CXCR3 expressing cells were identified and H) shows expression of CXCR3+ICOS+ whilst I) shows PD1+ICOS+ expression.

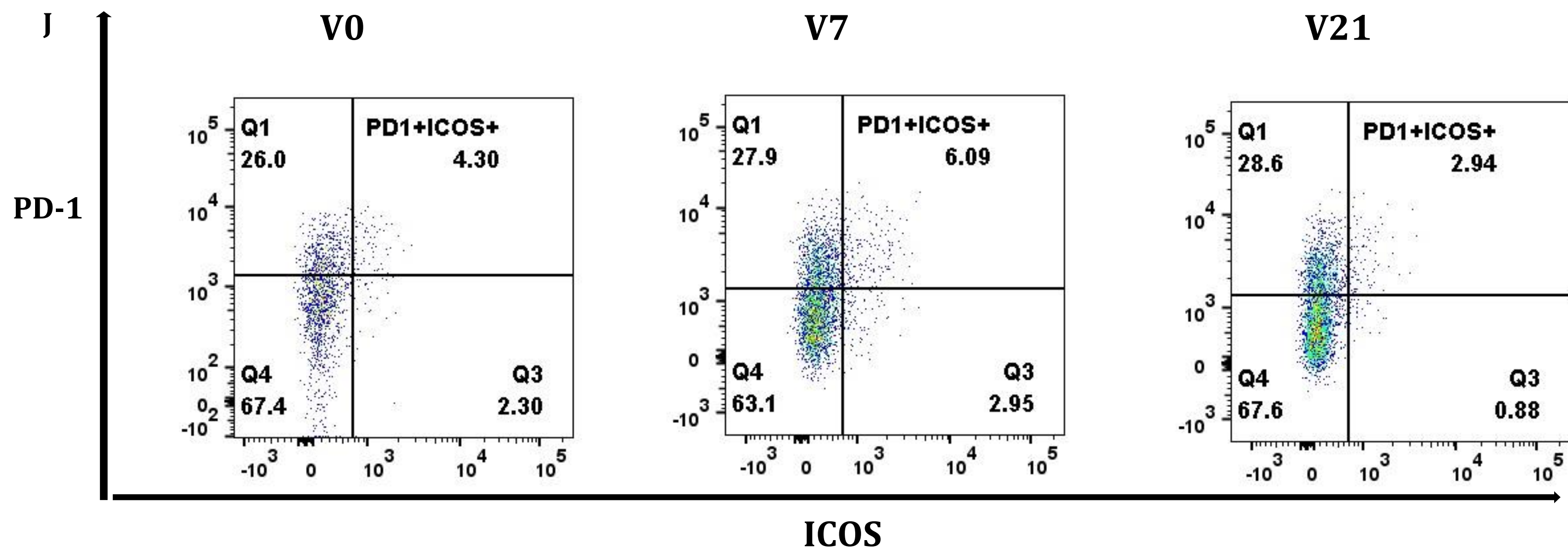


Figure 25J: Representative plots showing PD1+ICOS+ expression on CXCR3+ Tfh cells following *ex-vivo* staining of whole blood pre and post LAIV vaccination. J)Frequency of CXCR3+PD1+ICOS+ cells within CD45R0+CXCR5+ Tfh cells at day 0, day 7 and 21 post LAIV vaccination.

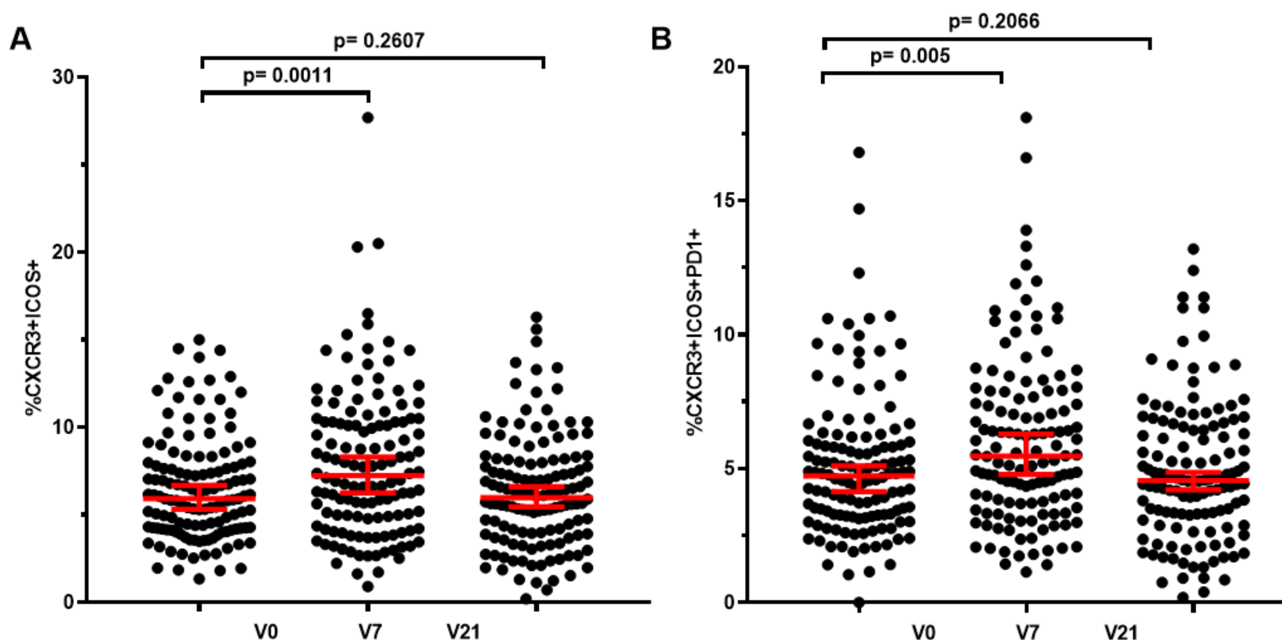


Figure 26: Live attenuated influenza (LAIV) vaccination induces Tfh cells in blood

Whole-blood samples obtained from healthy children were stained to assess A) CXCR3+ICOS+ cells within CD45R0+CXCR5+ CD4+T cells B) PD1+ICOS+ cells within CD45R0+CXCR5+ CXCR3+ CD4+T cells and analysed by flow cytometry on day 0 (i.e., baseline), day 7 and day 21 after vaccination. Data is shown for 130 vaccinated children from both years. The graphs consist of column scatter plots representing percentage of CXCR3+ICOS+ and CXCR3+ PD1+ICOS+ cells within Tfh cells over the 3 different time points. Horizontal lines represent the median with 95% CI. Data analysis was done using Wilcoxon matched pairs signed rank test. Due to multiple comparisons (n = 2), significance level was adjusted using Bonferroni's correction and P values of < 0.025 were considered statistically significant.

5.3.2 Increase in frequencies of CXCR3+ ICOS+ and CXCR3+ICOS+PD1+ Tfh cells in seroconverters but not in non seroconverters

Cells co-expressing CXCR3 and ICOS or CXCR3, ICOS and PD1 had increased by day 7 (Figure 26A and 26B) post vaccination. The association between the increase in Tfh cells and antibody responses was assessed using the serum and mucosal antibody data. I compared the frequency of these Tfh cells in a sub-population of children who seroconverted to any of the three influenza strains included in the vaccine with those who did not seroconvert to any strain.

An increased frequency of CXCR3+ICOS+ (p = 0.0009) (Figure 27A) and PD1+ICOS+ (p = 0.0076) (Figure 27B) expressing Tfh cells was seen at day 7 in seroconverters. In the non-seroconverters, the increase was not statistically significant for both CXCR3+ICOS+ (Figure 27A) and PD1+ICOS+ (Figure 27B).

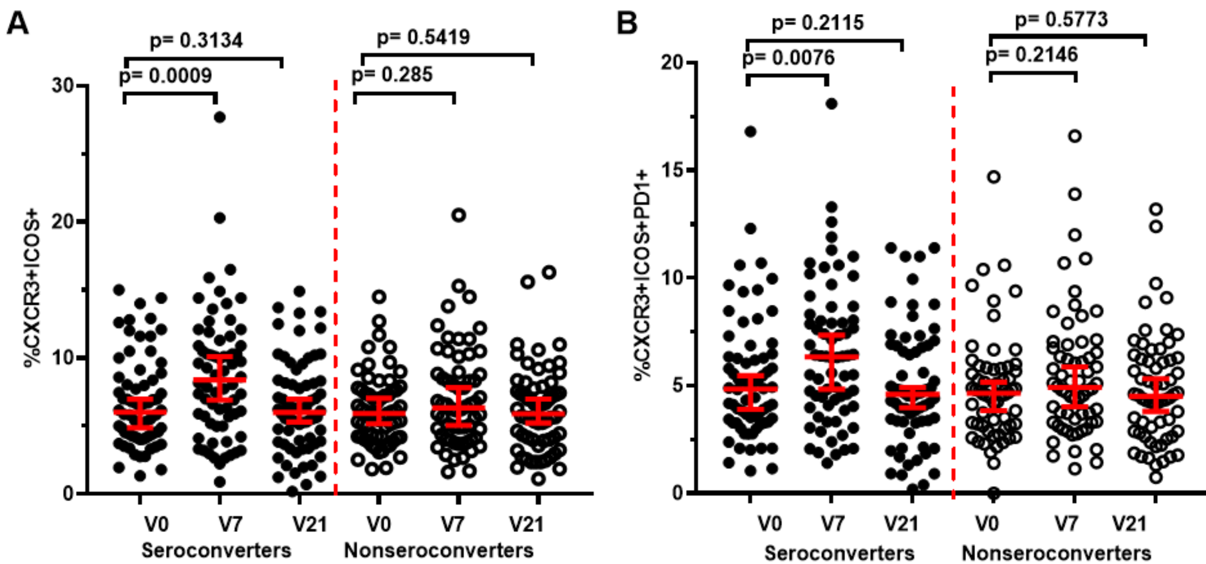


Figure 27: Increase in frequencies of CXCR3+ICOS+ and CXCR3+ICOS+PD1+ Tfh cells in seroconverters but not in non seroconverters

CD45R0+CXCR5+ cells expressing A) CXCR3+ICOS+ in seroconverters and non seroconverters B) CXCR3+ICOS+ PD1+ cells in seroconverters and non seroconverters. Frequency of Tfh cells was assessed at day 0, 7 and 21. Data is shown for 130 vaccinated children. For seroconverters, n= 68 and n=62 for nonseroconveters. Seroconverters had a 4-fold increase in antibody titres at Day 21 post vaccination. The closed circles represent the seroconverters whilst nonseroconverters are represented by the open circles. Horizontal lines represent the median with 95% CI. Data analysis was done using Wilcoxon matched pairs signed rank test. Due to multiple comparisons (n = 2 for the two timepoints compared to baseline), significance level was adjusted using Bonferroni's correction and P values of < 0.025 were considered statistically significant.

5.3.3 No difference in frequencies of CXCR3+ ICOS+ and CXCR3+ICOS+PD1+ Tfh cells in IgA responders and non-IgA responders

When the frequency of Tfh cells was assessed in IgA responders and nonresponders, no clear difference between responders and non-responders was observed. For IgA responders, an increase in the frequency of CXCR3+ICOS+ ($p = 0.0487$) (Figure 28A) and PD1+ICOS+ ($p = 0.0356$) (Figure 28B), expressing Tfh cells at day 7 post vaccination was seen, but this did not reach statistical significance based on the threshold set, which included the Bonferroni correction. In the non- IgA responders, the increase seen in both CXCR3+ICOS+ (Figure 28A) and PD1+ICOS+ (Figure 28B) expressing Tfh cells post LAIV vaccination was also not significant.

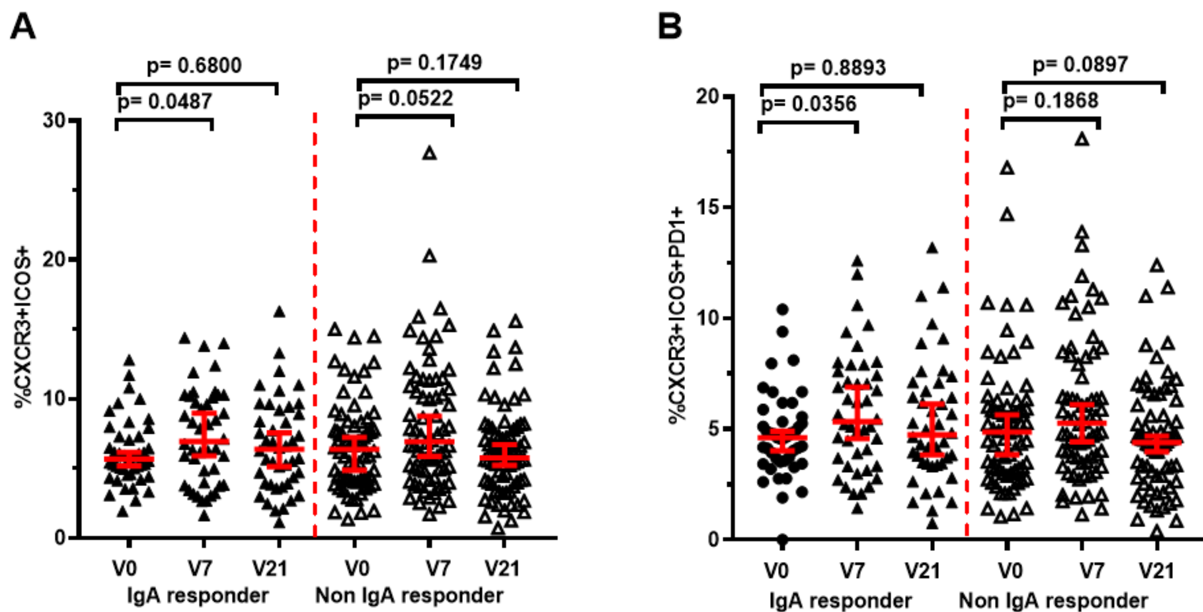


Figure 28: Frequencies of CXCR3+ICOS+ and CXCR3+ICOS+PD1+ Tfh cells post LAIV vaccination in IgA responders and nonresponders.

CD45R0+CXCR5+ cells expressing A) CXCR3+ICOS+ in IgA responders and non-responders B) CXCR3+ICOS+ PD1+ in IgA responders and non-responders were assessed. IgA response was based on mucosal influenza-specific IgA measured in oral fluid samples at baseline and D21 post-LAIV using a protein microarray. Frequency of these cells was assessed at day 0, 7 and 21. Data is shown for 114 vaccinated children. For IgA responders, n= 44 and n=70 for non IgA responders. The graphs consist of column scatter plots representing percentage of CXCR3+ICOS+ and CXCR3+ICOS+PD1+ cells within Tfh cells over the 3 different time points. IgA responders are those that had a 2-fold increase in IgA response from day 0 to day 21. The closed triangles represent the IgA responders whilst nonresponders are represented by the open triangles. Horizontal lines represent the median with 95% CI. Data analysis was done using Wilcoxon matched pairs signed rank test. Due to multiple comparisons for the two timepoints compared to baseline (n = 2), significance level was adjusted using Bonferroni's correction and P values of < 0.025 were considered statistically significant.

5.3.4 Correlation between Tfh cells and antibody responses post vaccination

The next aim was to assess whether LAIV induced Tfh cells correlated with serum and mucosal antibody responses post vaccination. HAI data was available for 130 children, 68 children seroconverted to any of the 3 antigens contained in the vaccine whilst 62 did not seroconvert to any antigen. For mucosal antibody responses, data was available for 114 children, out of which 44 had a 2-fold increase in IgA responses whilst 70 did not. The correlation analysis was done by looking at the fold change increase in Tfh responses compared to fold change increase in serum and mucosal antibody responses and pooling the data from both years. There was no correlation between the fold change in both CXCR3+ICOS+ and CXCR3+ICOS+PD1+ at day 7 post vaccination and the serum and mucosal antibody responses (Figure 29A-H).

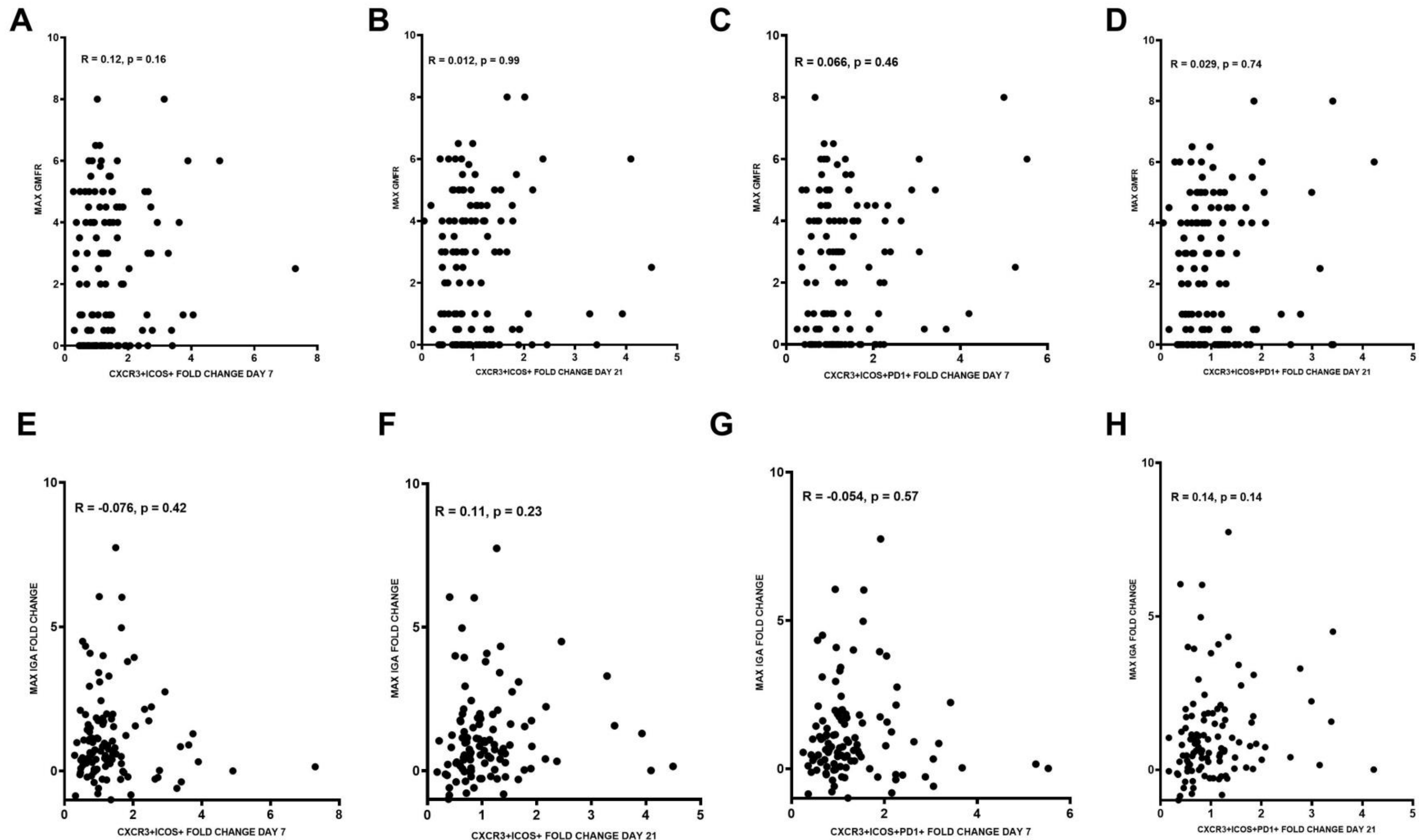


Figure 29: Correlation between fold change in CXCR3+ICOS+ and CXCR3+ICOS+PD1+ Tfh cells and maximum GMFR post LAIV vaccination

Graph shows correlation between maximum GMFR (fold change between baseline and serum antibody responses at day 21) in A) CXCR3+ICOS+ day 7 fold change responses B) CXCR3+ICOS+ day 21 fold change responses C) CXCR3+ICOS+ PD1+ day 7 fold change responses D) CXCR3+ICOS+PD1+ day 21 fold change response and fold change in mucosal antibody responses at day 21 with E) CXCR3+ICOS+ day 7 fold change responses f) CXCR3+ICOS+ day 21 fold change responses g) CXCR3+ICOS+ PD1+ day 7 fold change responses h) CXCR3+ICOS+PD1+ day 21 fold change.

5.4 Effect of LAIV vaccination on Tfh subsets

With the increase in CXCR3+ cells observed and the increased frequency of CXCR3+ICOS+ cells in seroconverters, frequency of ICOS+ cells was assessed in the Tfh subsets (Tfh-1, Tfh-2 and Tfh-17-like) at baseline and post vaccination. Identification of Tfh-1, Tfh-2, and Tfh- 17-like subsets was done using CXCR3 and CCR6 as markers, with Tfh-17 subset having the lowest frequency compared to Tfh-1 and Tfh-2. As previously reported by Bentebibel et al (Bentebibel *et al.*, 2013), no significant change in the frequency of bulk Tfh cells or Tfh subsets was observed at any time point after vaccination . At baseline,frequency of ICOS+ cells was highest in the Tfh-1 subset compared to Tfh-2 ($p = 0.0039$) and Tfh-17 subsets ($p = 0.0380$) (Figure 30A). At day 7 post vaccination, an increased frequency of ICOS+ cells was seen in only the Tfh-1 subset ($p = 0.0189$) (Figure 30B) but not Tfh-2 ($p = 0.5382$) (Figure 30C) or Tfh 17 ($p = 0.6506$) (Figure 30D) subsets. At day 21 post vaccination, a significant decrease in frequency of ICOS expressing cells was observed in all Tfh subsets.

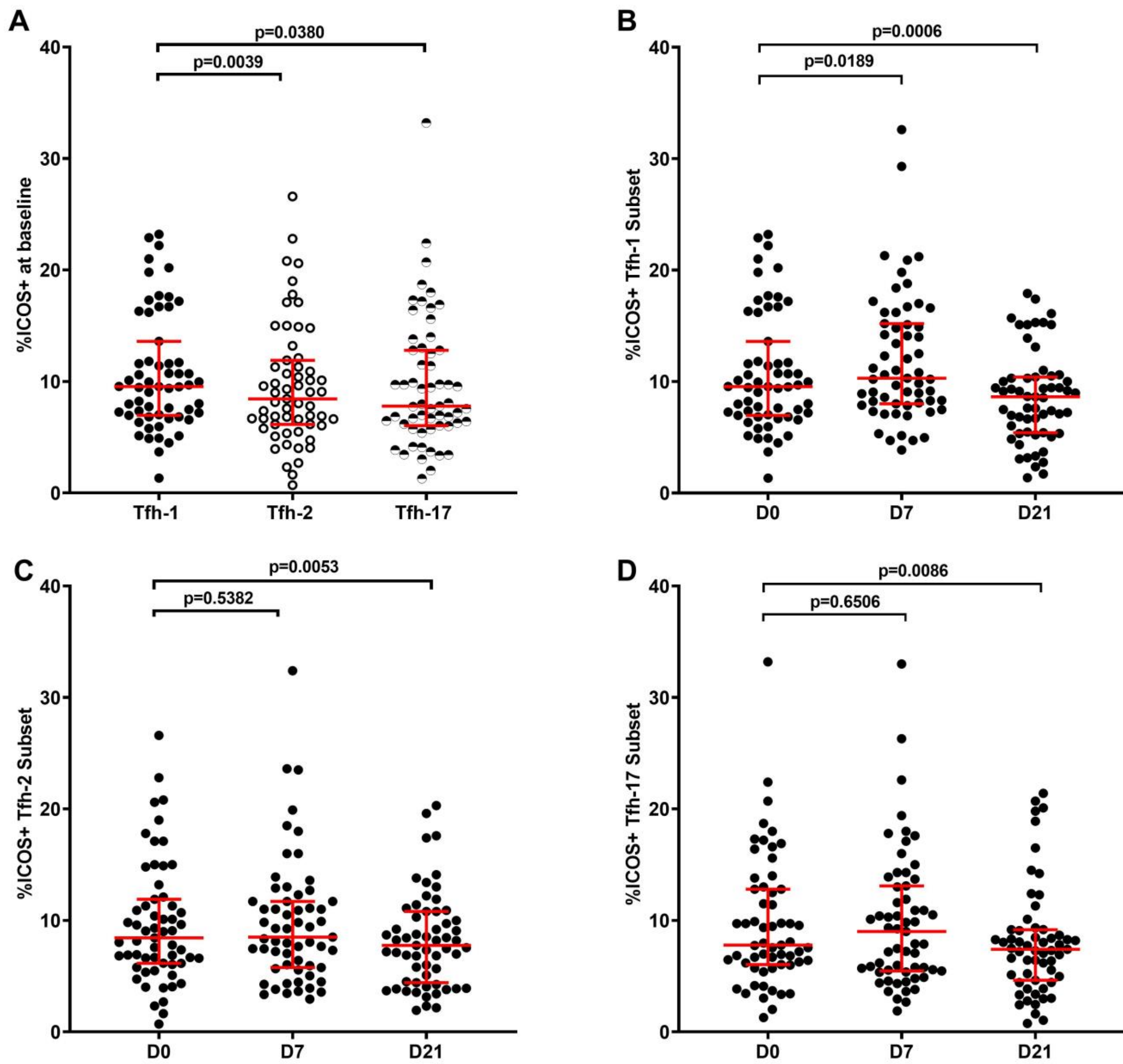


Figure 30: Frequency of ICOS+ cells in circulating Tfh subsets

The graphs show the percentage of cells expressing ICOS. Tfh cell subsets were identified based on expression of CD45RO and CXCR5 and from this population, CXCR3 and CCR6 expression was used to identify the 3 Tfh subsets as shown in Figure 23 A) %ICOS expressing cells in all Tfh subsets at baseline B) %ICOS expressing cells in Tfh 1 cells C) %ICOS expressing cells in Tfh 2 cells D) %ICOS expressing cells in Tfh 17 cells. Data is shown for 58 individuals (2017 data). Horizontal lines represent the median with 95% CI. Data analysis was done using Wilcoxon matched pairs signed rank test. Due to multiple comparisons for the two timepoints compared to baseline (n = 2), significance level was adjusted and P values of < 0.025 were considered statistically significant.

5.4.1 Frequency of ICOS+ cells in Tfh subsets in seroconverters and non seroconverters

In children that seroconverted, a greater increase in ICOS+ cells was seen in the Tfh-1 subset (p = 0.0882) (Figure 31A), at day 7 post vaccination compared to that seen in the nonseroconverters (p = 0.1624) (Figure 31A), although this did not reach statistical significance for both groups. In the Tfh-2 (Figure 31B) and Tfh-17 (Figure 31C) subsets no significant difference in baseline and day 7 ICOS+ cells was observed for both seroconverters and non seroconverters.

At day 21 post LAIV vaccination, in the seroconverters, a significant reduction in frequency of ICOS+ cells in the Tfh-1 subset (p = 0.0078) (Figure 31A) was observed but this was not seen with the Tfh-2 (p= 0.0708) and Tfh-17 (0.0325) subsets. In the non-seroconverters, a similar trend of reduced frequency of ICOS+ cells at day 21 in the Tfh-1 subset (p = 0.0208) (Figure 31A) but not Tfh-2 (p = 0.0272) and Tfh 17 (p = 0.0962) subsets (Figure 31A-C) was also noted.

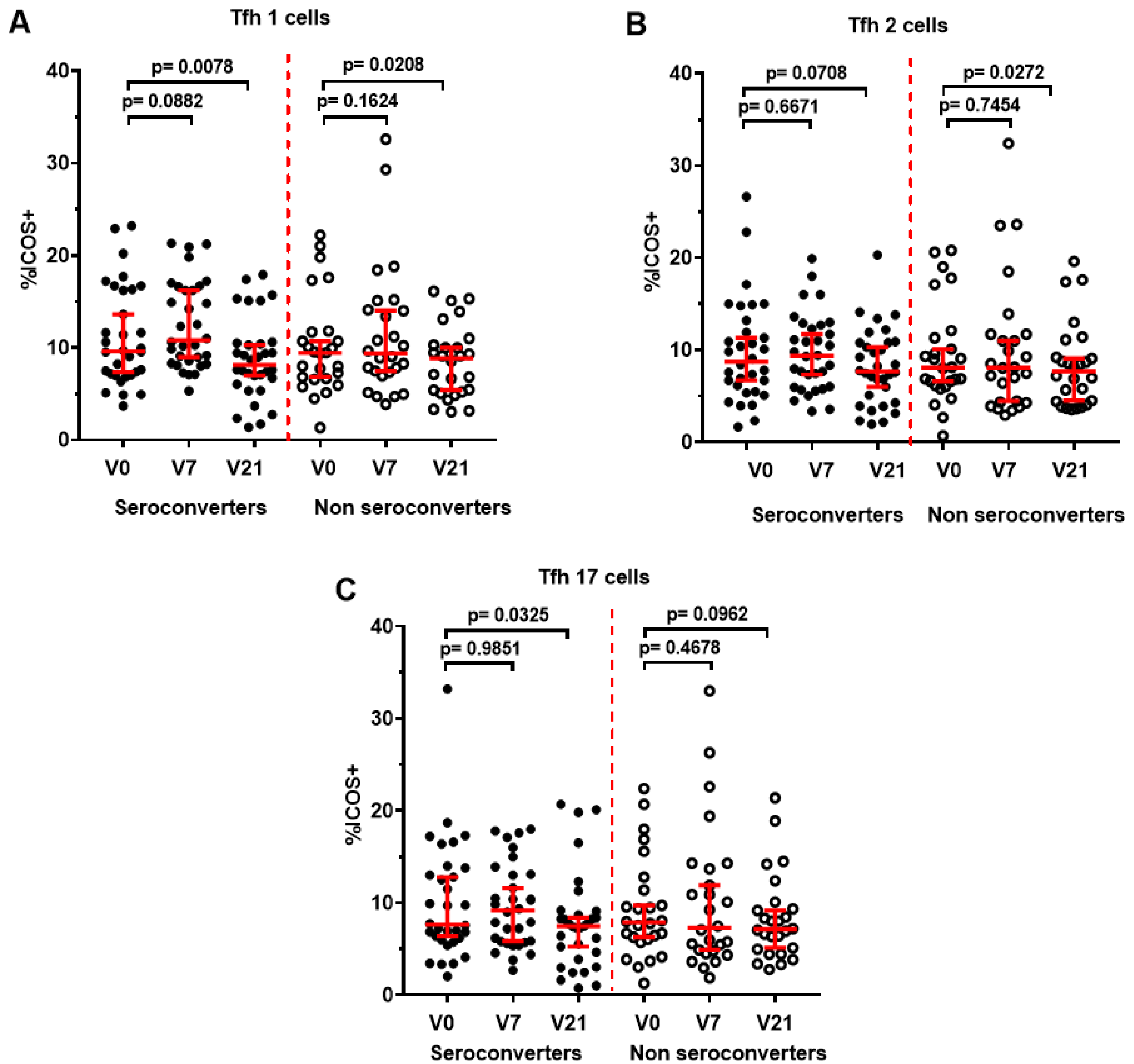


Figure 31:Frequency of ICOS+cells in Tfh subsets in seroconverters and nonseroconverters

Frequency of ICOS+ cells were gated on within A) Tfh 1 cells B) Tfh 2 cells C) Tfh 17 cells of seroconverters and non seroconverters at day 0, 7 and 21 post vaccination with LAIV. Seroconversion was based on HAI titres from HAI assay. Data is shown for 58 individuals (2017 data). Seroconverters had a 4-fold increase in antibody titres at day 21 post vaccination. In each graph, closed circles represent seroconverters and open circles non-seroconverters. Horizontal lines represent the median with 95% CI. Data analysis was done using Wilcoxon matched pairs signed rank test. Due to multiple comparisons for the two timepoints compared to baseline (n = 2), significance level was adjusted and P values of < 0.025 were considered statistically significant.

5.4.2 Increased frequency of ICOS+ cells in Tfh subsets in IgA responders but not in nonresponders

When children were stratified based on IgA response or no response, an increase in frequency of ICOS+ cells was seen within the Tfh-1 subset (p = 0.0037) but not Tfh-2 (p = 0.5057) and Tfh-17 subsets (p = 0.1375), (Figure 32A-C) at day 7 post vaccination, in IgA responders. In the IgA nonresponders, a decrease in frequency of ICOS expressing cells was seen in the Tfh-1 and Tfh-2 subsets but not theTfh-17 at day 7 post LAIV vaccination in but this was not statistically significant.

At day 21 post LAIV, an increased frequency of ICOS+ cells n compared to baseline frequencies was noted in the Tfh -1 and Tfh-2 subsets in the IgA responders (Figure 32A-C). However, in the non-IgA responders, there was a significant decrease in frequency of ICOS expressing cells at day 21 post LAIV compared to baseline levels in all 3 subsets (Tfh-1, p = 0.0002), (Tfh-2, p = 0.0007), (Tfh-17, p = 0.0006) (Figure 32A-C).

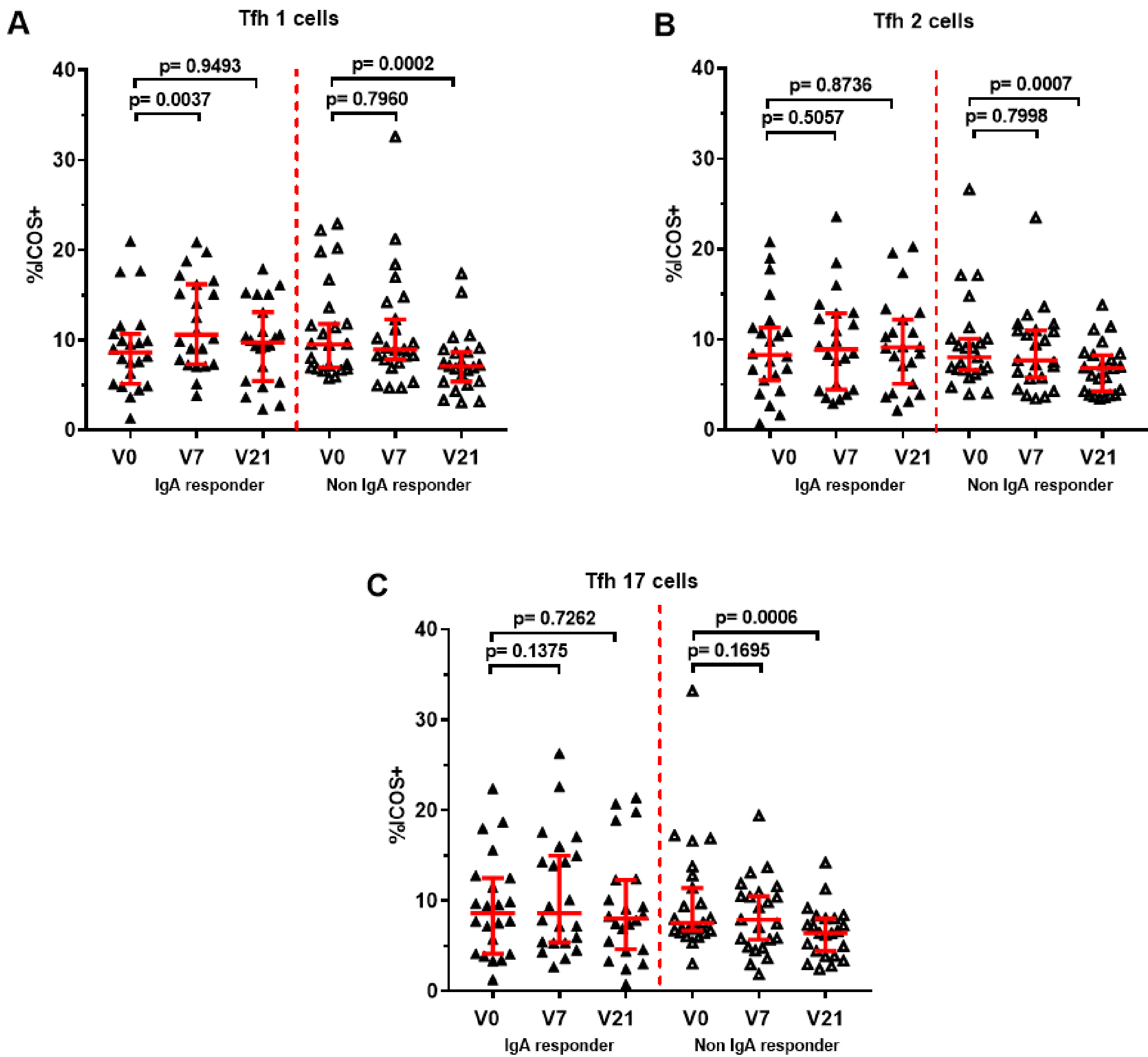


Figure 32:Frequency of ICOS+ cells in Tfh subsets in IgA responders and nonresponders.

Frequency of ICOS+ cells were gated on within a) Tfh-1 cells b) Tfh-2 cells c) Tfh-17 cells of IgA responders and non-responders at day 0, 7 and 21 post vaccination with LAIV. Mucosal influenza-specific IgA was measured in oral fluid samples at baseline and D21 post-LAIV using a protein microarray. Data is shown for 58 individuals (2017 data). IgA responders were donors that had a 2-fold increase in IgA response from day 0 to day 21 and non- responders are those with less than 2-fold increase in IgA response. In each graph, closed triangles represent IgA responders and open triangles non-responders (V21). Horizontal lines represent the median with 95% CI. Data analysis was done using Wilcoxon matched pairs signed rank test Due to multiple comparisons for the two timepoints compared to baseline (n = 2), significance level was adjusted and P values of < 0.025 were considered statistically significant.

5.4.3 Detection of LAIV induced Tfh cells by activation marker induction

In addition to the *ex-vivo* analysis of Tfh responses performed on samples collected during the entire study (2017 and 2018), I used the activation induced marker (AIM) assay to assess Tfh responses post vaccination in the 2018 cohort. This assays has been previously shown to enhance the detection of antigen specific cells such as Tfh cells in blood (Jennifer M Dan *et al.*, 2016). The assay is cytokine independent and based on T cell receptor (TCR-) upregulation using surface markers such as CD25, OX40 and PDL1. As with the *ex vivo* panel, Tfh cells were identified as CD4+T cells expressing CD45R0 and CXCR5. The expression of surface markers CD25, OX40 and PDL1 either alone or in combination on Tfh cells was then assessed after an 18-hour stimulation with various influenza antigens. Gating strategy to identify antigen specific cells is shown in Figure 33 below.

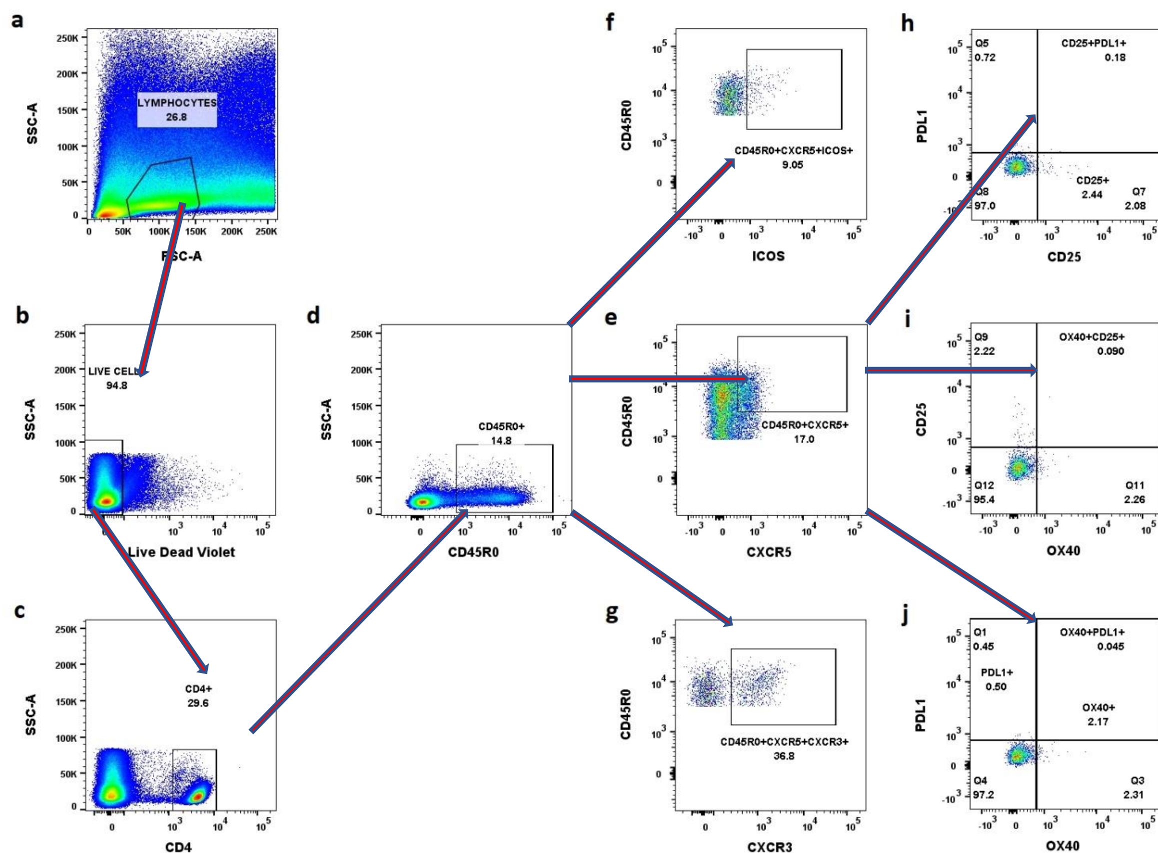


Figure 33 A-J: Activation induced marker (AIM) gating strategy to identify antigen specific follicular helper T cells (Tfh) in blood.

Whole blood samples collected from children vaccinated with LAIV was stimulated with H1, H3 and influenza B HA influenza antigens. a) Lymphocytes were identified based on forward scatter-area (FSC-A) versus side scatter-area (SSC-A). b) From the lymphocyte population, live cells were then gated on c) CD4+T cells were identified from live cells d) CD45R0+ cells were identified within CD4+T cells e) Using CD45R0 and CXCR5, CD4+T cells that expressed both CD45R0 and CXCR5 (CD45R0+CXCR5+) were gated on. This population represents circulating follicular helper T cells (Tfh). f) Tfh cells expressing ICOS and g) CXCR3 were identified. Within the CD45R0+CXCR5+, cells expressing h) CD25+PDL1+ i) OX40+CD25+ j) OX40+PDL1 were gated on.

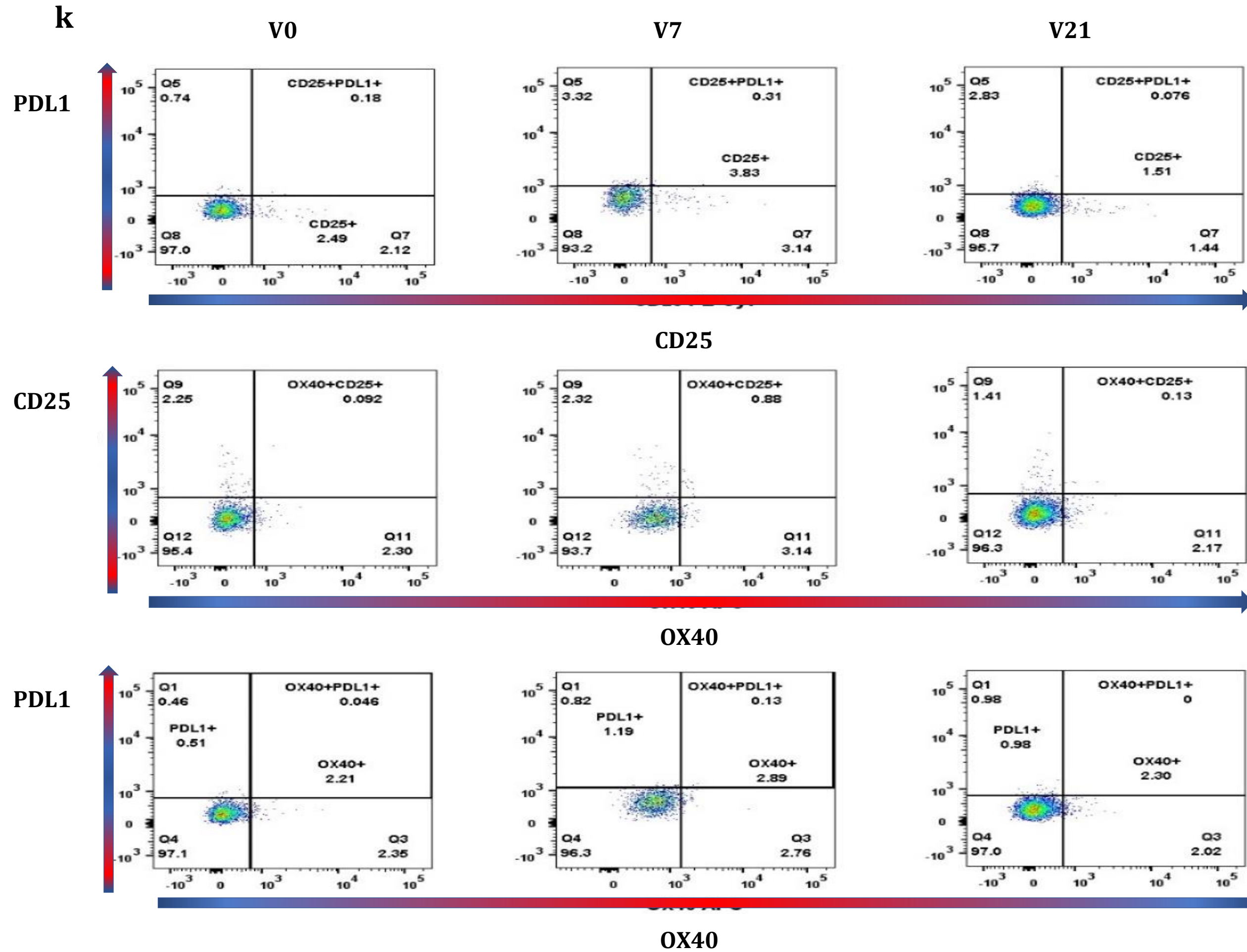


Figure 33K: Representative plots showing AIM markers expression on antigen specific Tfh cells after stimulation with Influenza H1 HA pre and post LAIV vaccination.

Frequency of CD25+PDL1+, OX40+CD25+ and OX40+PDL1+ Tfh cells were gated on within CD45R0+CXCR5+ Tfh cells and responses shown upon stimulation with H1 HA at day 0, 7 and 21 post LAIV.

5.4.4 Increase in antigen specific Tfh cells after LAIV vaccination

The frequency of CD25+ Tfh cells increased at day 7 post vaccination upon stimulation with H1 HA ($p = 0.0018$) and H3 HA ($p = 0.0016$) antigens but did not reach statistical significance with HAB stimulation after correcting for multiple testing (Figure 34A). A significant increase in frequency of CD25+ antigen specific Tfh cells was noted for all 3 antigens at day 21 post vaccination (H1- $p = 0.00006$, (H3-, $p = 0.00005$, (HAB-, $p = 0.0236$) (Figure 34A).

Despite an increase in frequency of CD25+PDL1+ Tfh cells at day 7 and 21 post vaccination for all antigens, the increase was only significant with H1 HA (day 7- $p = 0.0088$, day 21 - $p = 0.0036$) and H3 HA stimulation (day 7-$p = 0.0006$, day 21, $p= 0.0036$) but not HAB (Figure 34B).

At day 21, an increased frequency of OX40+CD25+ H1 HA ($p= 0.0267$) and H3 HA ($p= 0.0036$) antigen specific Tfh cells was observed post vaccination. However, the increase in frequency of OX40+CD25+ HAB specific Tfh cells was not significant (Figure 34C).

No increase in frequency of OX40+PDL1+ Tfh cells was seen at day 7 or day 21 following LAIV in response to stimulation with any of the three antigens (data not shown).

For CD25+OX40+PDL1+ Tfh cells, an increase was seen at day 21 post vaccination upon stimulation with H1 HA ($p = 0.0067$) and H3 HA antigens ($p = 0.\ 0144$) but not HAB. Whilst at day 7, the increase in frequency observed did not reach statistical significance after correcting for multiple testing for all 3 antigens (Figure 34D).

Apart from the trend for increased frequency of CD25+ Tfh cells seen at day 7 and 21 post vaccination, no increase in frequency of influenza B specific Tfh was seen post vaccination using any combination of CD25, OX40 or PDL1.

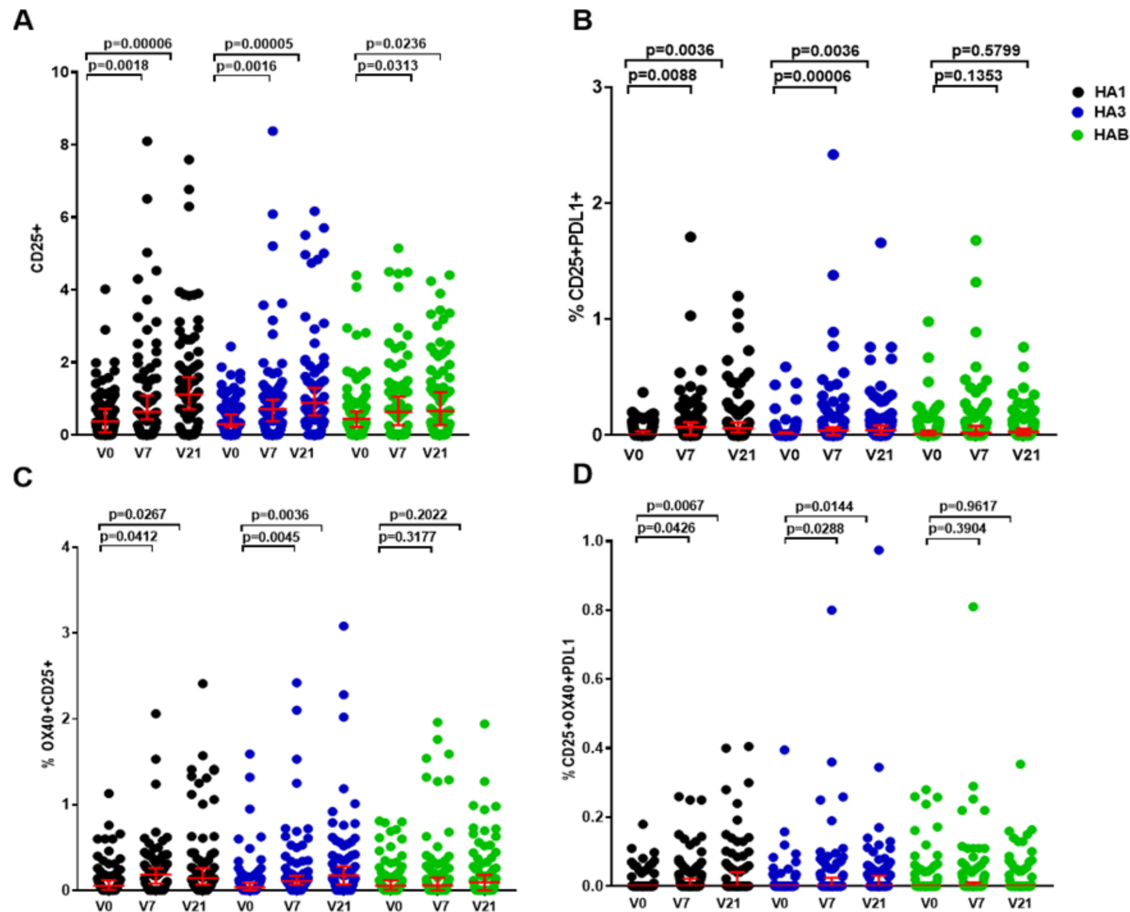


Figure 34: Induction of antigen specific Tfh cells after stimulation with influenza antigens

Frequency of A) CD25+ B) CD25+PDL1+ C) OX40+CD25+ and D) CD25+OX40+PDL1+ cells were gated on within CD45R0+CXCR5+ Tfh cells and responses shown upon stimulation with H1 HA, H3 HA and B HA. Data is shown for 68 individual donors. In each graph, the black circles represent stimulation with HA1, blue with HA3 and green with HAB. Horizontal lines represent the median with 95% CI. Data analysis was done using Wilcoxon matched pairs signed rank test. Due to multiple comparisons for the two timepoints compared to baseline (n = 2), significance level was adjusted and P values of < 0.025 were considered statistically significant.

5.4.5 Increased activation of antigen specific Tfh cells in seroconverters but not in non seroconverters

As previously seen with the *ex-vivo* data, increased activation of Tfh cells was seen in seroconverters but not the non-seroconverters post LAIV vaccination. Increased frequency of CD25+Tfh cells at day 7 ($p = 0.0002$) and 21 ($p < 0.0001$) upon stimulation with H1 HA and H3 HA (day 7- $p = 0.0042$, day 21- $p = 0.0168$) but not influenza B HA (Figure 35A) was noted.

In the nonseroconverters, an increased frequency of CD25+ Tfh cells was seen with H1 HA stimulation at day 21 post vaccination but this was not statistically significant ($p = 0.0543$). A significant increase in frequency of theses cells was also observed at day 21 with H3 HA stimulation ($p = 0.0016$) whilst stimulation with HAB led to an increase at day 7 ($p = 0.0212$) but not at day 21 ($p = 0.1768$) (Figure 35A).

At day 7 post vaccination, an increased frequency of OX40+CD25+ Tfh cells was noted in seroconverters with H1 HA stimulation ($p = 0.0372$) but not the non seroconverters. However, this increase was not statistically significant. A significant increase in frequency was also seen with H3 HA stimulation at day 21 post vaccination in the non seroconverters ($p = 0.0108$), but not the seroconverters ($p = 0.1945$) (Figure 35B).

In seroconverters, the frequency of CD25+PDL1+ Tfh cells increased at day 7 ($p = 0.0034$) and 21 ($p = 0.0047$) post vaccination for H1 HA and H3 HA stimulation (day 7, $p = 0.0004$, day 21, $p = 0.0051$) but not HAB. The increase in frequency of CD25+PDL1+ Tfh cells at day 7 and 21 post vaccination was not significant in the nonseroconverters for all three antigens (Figure 35C).

No significant increase in the frequency of OX40+PDL1+ Tfh cells was noted at day 7 and 21 post vaccination in both seroconverters and nonseroconverters for all three antigens (data not shown).

For CD25+OX40+PDL1+Tfh cells, (Figure 35D), no significant increase in frequency of this population was noted in both seroconverters and non seroconverters with H1 HA stimulation.

An increased frequency of CD25+OX40+PDL1+ Tfh cells was seen with H3 HA stimulation at day 7 ($p = 0.0108$) and 21 ($p = 0.0140$) post LAIV in seroconverters but not in the nonseroconverters (Figure 35D).

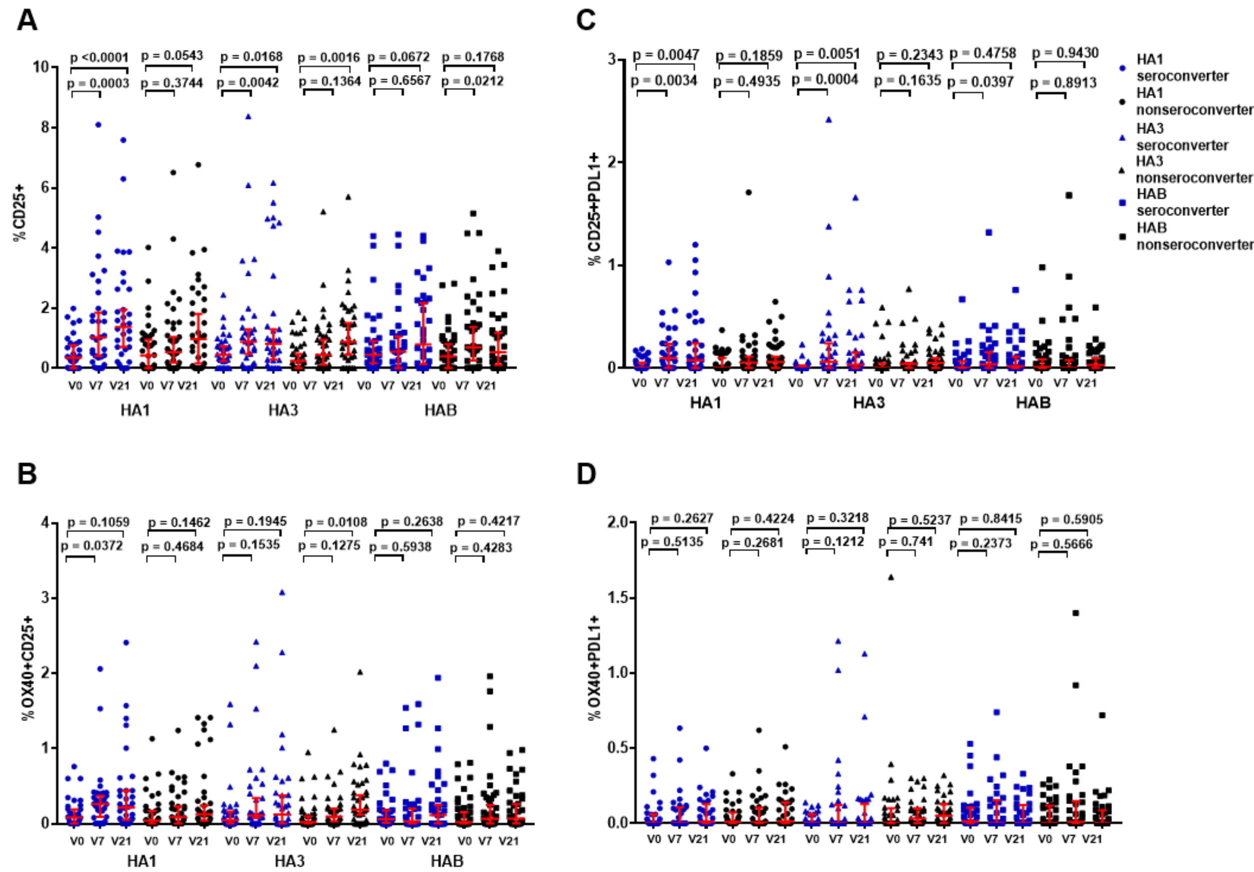


Figure 35: Induction of antigen specific Tfh cells after stimulation with influenza antigens in seroconverters and non seroconverters

Frequency of A) CD25+ B) OX40+CD25+ C) CD25+PDL1+ and D) CD25+OX40+PDL1+ cells were gated on within CD45R0+CXCR5+ Tfh cells and responses shown upon stimulation with HA1 (circles), HA3 (triangles) and HAB (squares). Seroconversion was based on HI titres from HAI assay. Seroconverters had a 4-fold increase in antibody titres at day 21 post vaccination. Data is shown for 68 individual donors. In each graph, the blue circles represent seroconverters n= 32 and black circles represent non seroconverters n=36. Horizontal lines represent the median with 95% CI. Data analysis was done using Wilcoxon matched pairs signed rank test. Due to multiple comparisons for the two timepoints compared to baseline (n = 2), significance level was adjusted and P values of < 0.025 were considered statistically significant.

5.4.6 Increased frequency of CD25+, CD25+PDL1+ and OX40+CD25+ antigen specific Tfh cells in IgA nonresponders at Day 21 post LAIV

As seen with the *ex-vivo* data when bulk Tfh was assessed, the clear difference observed with seroconverters and non seroconverters was not evident when the frequency of antigen specific Tfh cells was assessed in IgA responders and nonresponders (Figure 36A-D). At day 7 ($p = 0.0105$) and 21 ($p = 0.0250$) post vaccination, an increase in frequency of H1 HA specific CD25+ Tfh cells was seen in IgA responders, whilst in non-responders the increase was only significant at day 21 ($p = 0.0029$) (Figure 36A). For H3 HA stimulation, the increase in frequency of CD25+ Tfh cells was not significant post LAIV vaccination in IgA responders, but an increase was noted in the non-IgA responders at day 7 ($p = 0.0097$) and 21 ($p = 0.0005$) post LAIV vaccination (Figure 36A). For Influenza B HA stimulation, no significant increase in frequency of CD25+ Tfh cells was noted in both the IgA and non-IgA responders at both day 7 and 21 post LAIV (Figure 36A).

For the frequency of OX40+CD25+ antigen specific Tfh cells, no significant increase in frequency was noted with H1 HA stimulation at day 7 and 21 post LAIV vaccination in IgA responders and non-responders (Figure 36B). In the IgA responders, no significant increase in frequency of OX40+CD25+ Tfh cells was noted at day 21 with H3 HA stimulation but in the non-IgA responders, a significant increase was noted at day 21 post LAIV ($p = 0.0008$) (Figure 36B).

For frequency of CD25+PDL1+ H1 HA antigen specific Tfh cells, no statistically significant increase was seen in both IgA and non-IgA responders. An increase in the frequency of CD25+PDL1+ antigen specific Tfh cells at day 7 ($p = 0.0166$) was noted in IgA responders with H3 HA stimulation but in the non-IgA responders the increase was noted at both day 7 ($p = 0.0157$) and 21 ($p = 0.0120$) post LAIV vaccination. No significant increase in frequency of CD25+PDL1+ Tfh cells was noted with Influenza B HA stimulation for both groups at day 7 and 21 post LAIV vaccination (Figure 36C).

No significant increase in frequency of OX40+PDL1+ Tfh cells (data not shown) was noted at day 7 and 21 post vaccination in both IgA responders and nonresponders with all three antigens.

For CD25+OX40+PDL1+ Tfh cells, an increase in frequency was noted with H1 HA stimulation in the IgA nonresponders at day 21 post vaccination ($p = 0.0114$) but not the responders. No increase in frequency of CD25+OX40+PDL1+ Tfh cells was noted with H3 HA and HAB stimulation for both IgA responders and non-responders (Figure 36D).

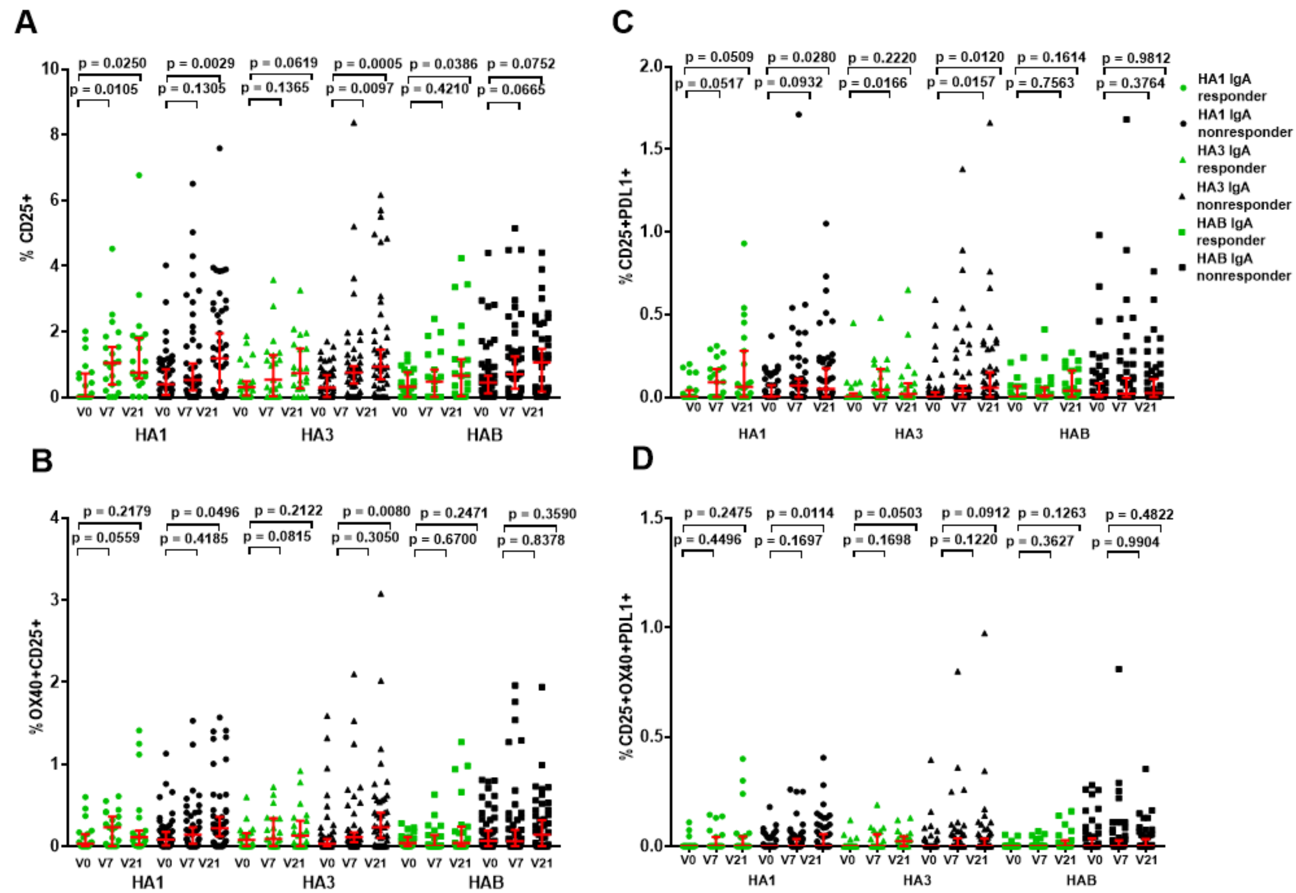


Figure 36: Induction of antigen specific Tfh cells after stimulation with influenza antigens in IgA responders and nonresponders

Frequency of A) CD25+, B) OX40+CD25+, C) CD25+PDL1+ and D) CD25+OX40+PDL1+ Tfh cells were gated on within CD45R0+CXCR5+ Tfh cells and responses shown upon stimulation with HA1 (circles), HA3 (triangles) and HAB (squares). Data is shown for 64 individual donors. In each graph, the green circles represent IgA responders n=21 and black circles represent non-IgA responders n=43. Horizontal lines represent the median with 95% CI. Data analysis was done using Wilcoxon matched pairs signed rank test. Due to multiple comparisons for the two timepoints compared to baseline (n = 2), significance level was adjusted and P values of < 0.025 were considered statistically significant.

5.4.7 Frequency of LAIV induced Tfh cells in children with or without pre-existing antibody responses within CD45R0+CXCR5+Tfh cells

Previous studies have shown that pre-existing antibody responses affect the subsequent immune response to vaccination (Coelingh *et al.*, 2014). I therefore stratified children based on baseline serum antibody responses with children having HI titres ≥ 40 for each of the influenza antigens assessed termed seropositive at baseline and those with HI titres < 40 as seronegative at baseline.

In baseline seropositive children, no significant increase in frequency of CD25+ H1 HA specific Tfh cells was noted post vaccination. However, in the children seronegative at baseline, a marked increase in frequency of CD25+ antigen specific Tfh cells was seen at day 7 ($p = 0.0064$) and 21 ($p < 0.0001$) post LAIV with H1 HA stimulation (Figure 37A).

For H3 HA stimulation, an increase in frequency of CD25+ Tfh cells was observed post LAIV in both the children seropositive at baseline (day 7- p= 0.0202 and 21- p = 0.0014) and those seronegative at baseline at day 21 ($p = 0.0155$) (Figure 37A).

With HAB stimulation, the increase in frequency of CD25+ Tfh cells was not significant in children seropositive at baseline, whilst in children that were seronegative at baseline, an increased frequency of these cells was noted post vaccination at day 21 ($p = 0.0016$) (Figure 37A).

No difference in the frequency of OX40+CD25+ antigen specific Tfh cells was observed in children that were seropositive at baseline with H1 HA stimulation. However, an increased frequency of OX40+CD25+ in antigen specific Tfh cells was observed at day 21 post LAIV in children seronegative at baseline ($p = 0.0103$) (Figure 37B).

There was an increase in frequency of 0X40+CD25+ Tfh cells observed with H3 HA stimulation, in the children seropositive at baseline ($p = 0.0051$) at day 21 post vaccination but not in those that were seronegative at baseline (Figure 37B).

No increase in frequency of OX40+CD25+ Tfh cells was seen with influenza B HA stimulation in children that were seropositive at baseline. However, an increased frequency of OX40+CD25+ in antigen specific Tfh cells was seen in children seronegative at baseline with Influenza B HA stimulation at day 7 post vaccination (p = 0.0201) (Figure 37B).

For CD25+PDL1+Tfh cells, no statistically significant increase in frequency of these cells was noted in children that were seropositive at baseline with H1 HA stimulation but in children that were seronegative at baseline, an increased frequency was seen at day 7 (p = 0.0150) and 21 post LAIV (p = 0.0005) (Figure 37C).

With H3 HA stimulation no significant increase in frequency of CD25+PDL1+ antigen specific Tfh cell was noted at day 7 post LAIV in children that were seropositive at baseline whilst in children that were seronegative at baseline, increased frequency of these cells was seen at day 7 (p = 0.0018) and 21 post LAIV. In both baseline seropositive and seronegative children, no difference in frequency of CD25+PDL1 Tfh cells was seen with Influenza B HA stimulation post LAIV (Figure 37C).

No difference in the frequency of OX40+PDL1+ antigen specific Tfh cells was observed in children that were seropositive at baseline and those that were not for all three antigens.

In children seropositive at baseline, no increase in frequency of CD25+OX40+PDL1+ antigen specific Tfh cells was noted post LAIV with H1 HA stimulation but an increased frequency of CD25+OX40+PDL1+ Tfh cells was noted in children seronegative at baseline at day 21 post LAIV (p = 0.0015) (Figure 37D).

At day 7 and 21 post vaccination, there was no increase in frequency of CD25+OX40+PDL1+ Tfh cells with H3 HA stimulation in children seropositive at baseline but an increased frequency of these cells was seen in the children that were seronegative at baseline at day 7 (p = 0.0144) and 21 (p = 0.0032) post LAIV. In both groups no difference in frequency of CD25+OX40+PDL1 Tfh cells was seen with Influenza B HA post LAIV (Figure 37D).

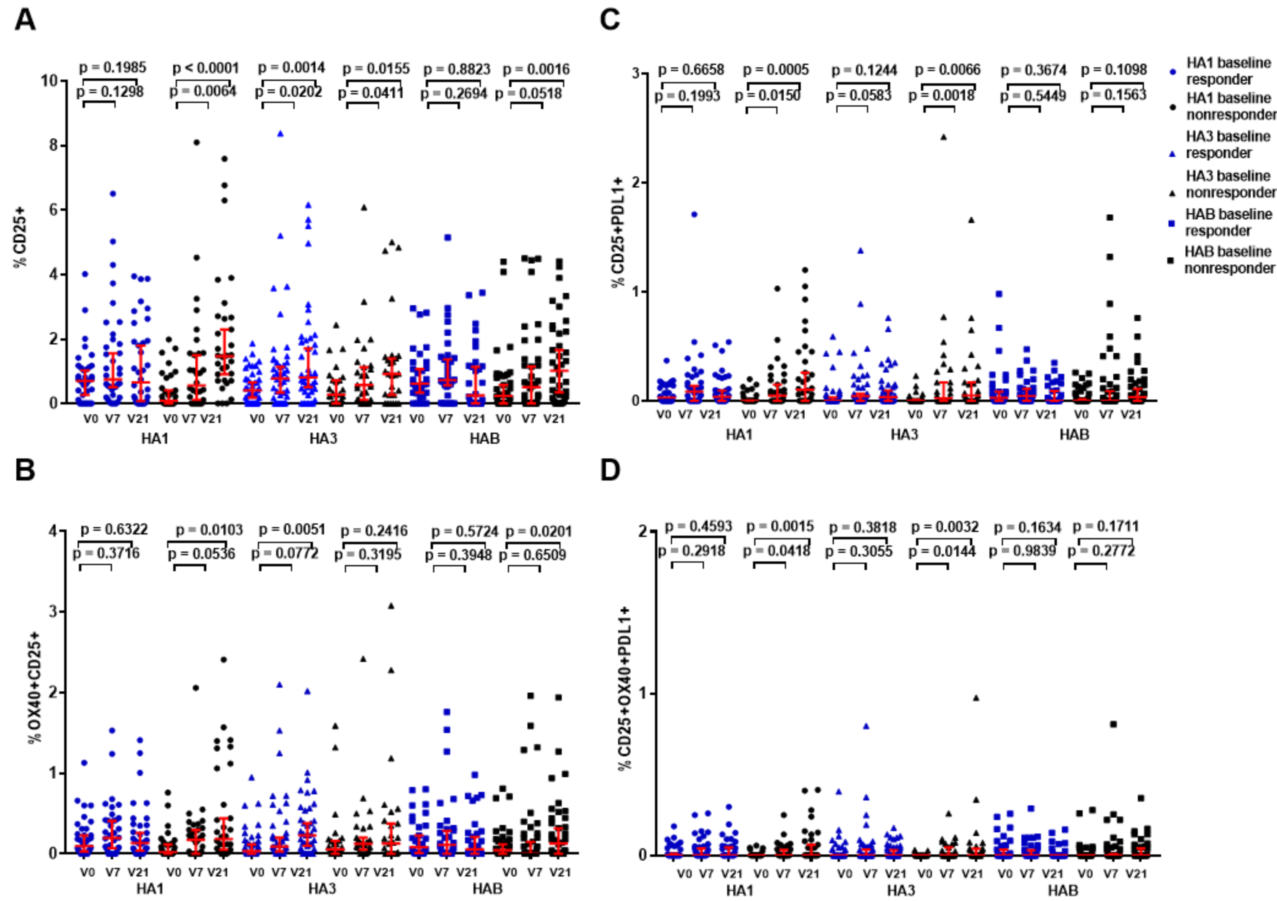


Figure 37: Detection of AIM markers on antigen specific Tfh cells after stimulation with influenza antigens in baseline responders and nonresponders

Frequency of A) CD25+ B) OX40+ CD25+ C) CD25+PDL1+ and D) CD25+OX40+PDL1+ Tfh cells were gated on within CD45R0+CXCR5+ Tfh cells and responses shown upon stimulation with HA1 (circles) baseline responder-n=36 , nonresponder =32, HA3 (triangles) baseline responder-n=41, nonresponder =27 and HAB (squares) baseline responder-n=30, nonresponder =38. Data is shown for 68 individual donors. In each graph, the blue circles represent participants with baseline antibody responses and black circles represent beasline nonresponders. Horizontal lines represent the median with 95% CI. Data analysis was done using Wilcoxon matched pairs signed rank test. Due to multiple comparisons for the two timepoints compared to baseline (n = 2), significance level was adjusted and P values of < 0.025 were considered statistically significant.

5.4.8 Increase in frequency of OX40+CD25+ antigen specific Tfh-1 cells post LAIV

Although prior studies have not used CXCR3 to further define Tfh in AIM studies (Havenar-Daughton et al. 2016; Dan et al. 2016; Bowyer et al. 2018), this was included in the gating strategy (i.e. assessing AIM markers on CD4+ CD45RO+CXCR5+ CXCR3+ T-cells) to see if increasing the specificity of Tfh classification in this way would reveal expansions in antigen-specific Tfh cells.

The increased frequency of CD25+ in Tfh cells upon stimulation with H1 HA was only significant at day 7 ($p = 0.0002$) whilst with H3 HA stimulation, the increase was significant at both day 7 ($p = 0.0247$) and 21 ($p = 0.0015$) (Figure 38A).

However, with the addition of CXCR3, increase in the frequency of OX40+CD25+ Tfh cells was now observed at day 21 with H1 HA stimulation ($p = 0.0233$) and at day 7 ($p = 0.0058$) and 21 ($p = 0.0003$) post vaccination with H3 HA stimulation (Figure 38B).

CD25+PDL1+ Tfh cells increased in frequency only at day 21 post vaccination upon H1 HA ($p = 0.0136$) but not H3 HA stimulation as previously observed (Figure 38C).

As previously reported, no increase in frequency of OX40+PDL1+ Tfh cells was seen at day 7 or day 21 following LAIV in response to stimulation with any of the three antigens (Figure 38D).

The frequency of CD25+OX40+PDL1+ cells within the CD45RO+CXCR5+CXCR3+ Tfh cells was not assessed because of low frequency of these cells after gating. Addition of CXCR3, had no effect on detection of influenza B antigen specific Tfh cell responses post vaccination using any combination of CD25, OX40 or PDL1 to define antigen specific T-cells.

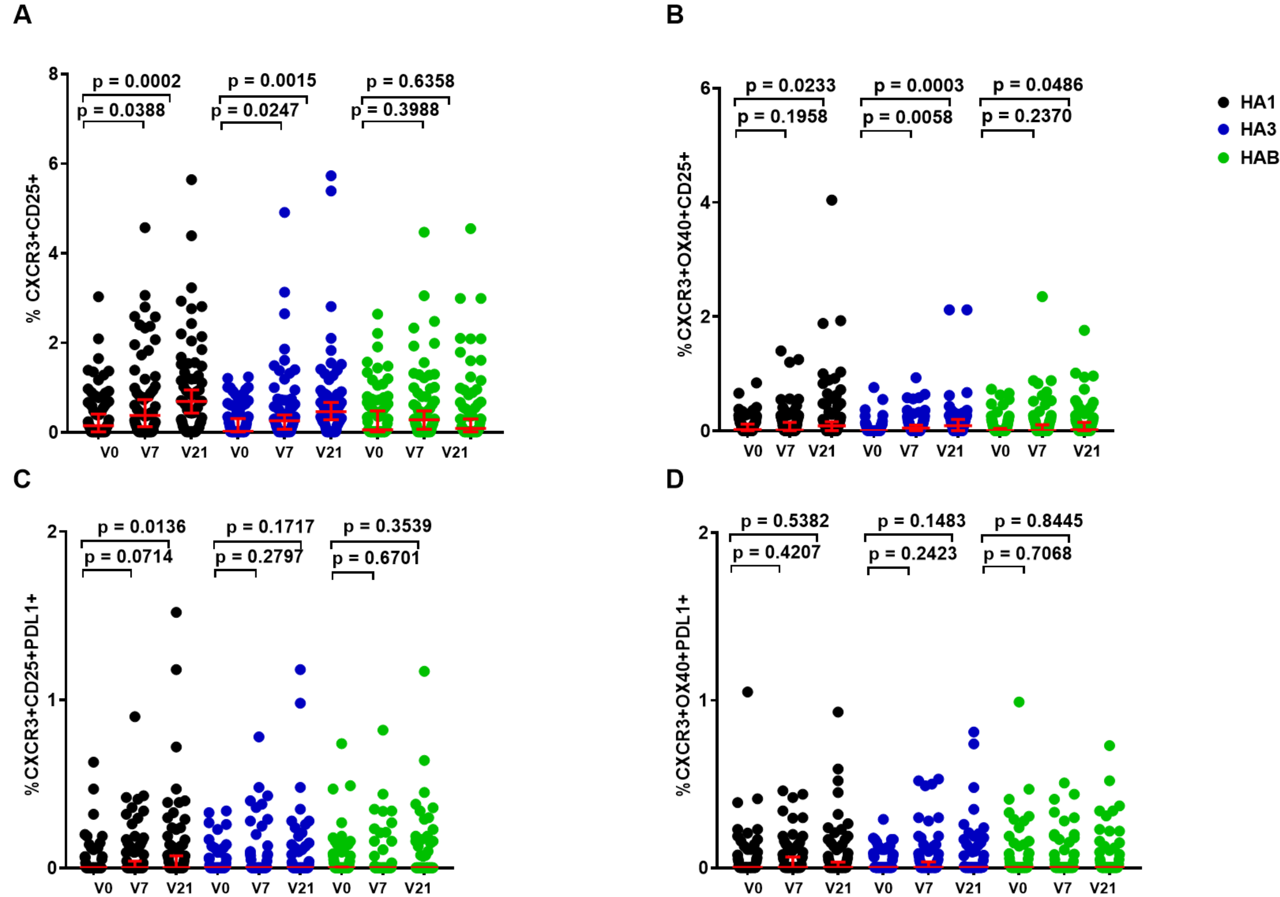


Figure 38: Induction of antigen specific CXCR3+Tfh cells after stimulation with influenza antigens

Frequency of A) CD25+ B) OX40+CD25+ C) CD25+PDL1+ D) OX40+PDL1+ cells were gated on within CD45R0+CXCR5+CXCR3+ Tfh cells and responses shown upon stimulation with H1 HA, H3 HA and B HA. Data is shown for 68 individual donors. In each graph, the black circles represent stimulation with HA1, blue with HA3 and green with HAB. Horizontal lines represent the median with 95% CI. Data analysis was done using Wilcoxon matched pairs signed rank test. Due to multiple comparisons for the two timepoints compared to baseline (n = 2), significance level was adjusted and P values of < 0.025 were considered statistically significant.

5.4.9 Increase in frequency of CD25+ antigen specific Tfh-1 cells in seroconverters but not in non seroconverters

Post LAIV vaccination, an increased frequency of CD25+ antigen specific Tfh cells upon H1 HA stimulation at day 7 (p = 0.0046) and 21 (p = 0.0003) was observed. However, in the non-seroconverters, no significant increase was noted (Figure 39A).

For H3 HA stimulation, there was no significant increase in frequency of CD25+ Tfh cells at day 7 post vaccination but a significant increase at day 21 (p = 0.0207) was noted in seroconverters, but not the nonseroconverters (p = 0.0292). No difference in frequency of CD25+ Tfh cells was noted post vaccination in both groups with Influenza B HA stimulation (Figure 39A).

No difference in frequency of OX40+CD25+, H1 HA specific Tfh cells was seen post LAIV vaccination in both seroconverters and non seroconverters. Increased frequency of OX40+CD25+ antigen specific Tfh cells at day 21 post vaccination in seroconverters (p = 0.0181) and day 7 (p = 0.0238) and 21 (p = 0.0089) in non-seroconverters with H3 HA stimulation was noted. No difference in frequency of OX40+CD25+ influenza B HA specific Tfh cells was seen post LAIV vaccination in both seroconverters and non seroconverters (Figure 39B).

No difference in the frequency of CD25+PDL1+ (Figure 39C), and OX40+PDL1+ (Figure 39D) antigen specific Tfh cells, was noted for both seroconverters and nonseroconverters for all three antigens post LAIV.

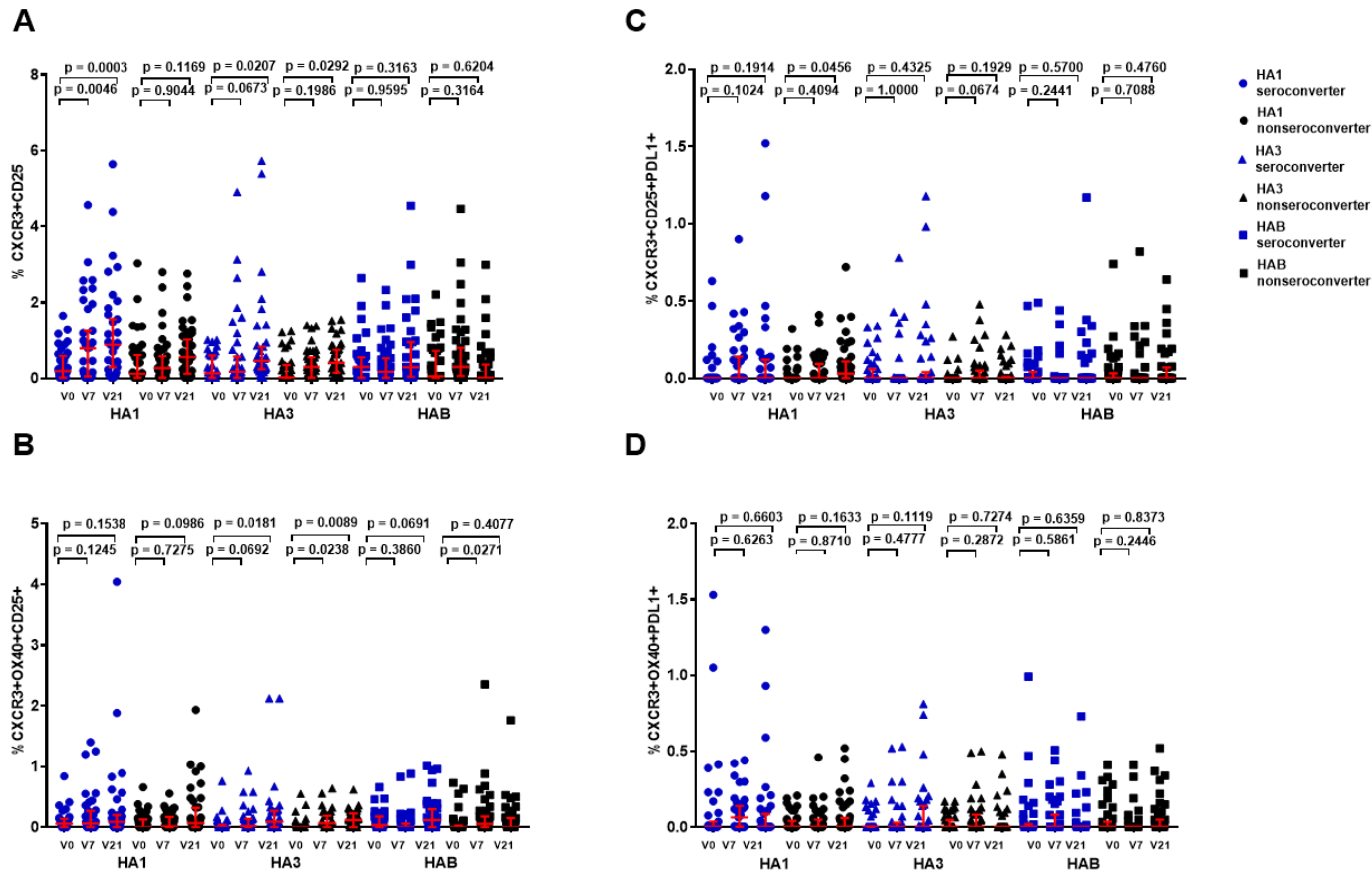


Figure 39: Induction of antigen specific CXCR3+Tfh cells after stimulation with influenza antigens in seroconverters and non seroconverters

Frequency of A) CD25+ B) OX40+CD25+ C) CD25+PDL1+ and D) OX40+PDL1+ cells were gated on within CD45R0+CXCR5+CXCR3+ Tfh cells and responses shown upon stimulation with HA1 (circles), HA3 (triangles) and HAB (squares). Seroconversion was based on HI titres from HAI assay. Seroconverters had a 4-fold increase in antibody titres at day 21 post vaccination. Data is shown for 64 individual donors. In each graph, the blue circles represent seroconverters, n= 31 and black circles represent non seroconverters, n= 33 . Horizontal lines represent the median with 95% CI. Data analysis was done using Wilcoxon matched pairs signed rank test. Due to multiple comparisons for the two timepoints compared to baseline (n = 2), significance level was adjusted and P values of < 0.025 were considered statistically significant.

5.4.10 Increased frequency of CD25+, CD25+PDL1+ and OX40+CD25+ antigen specific Tfh-1 cells in IgA nonresponders at Day 21 post LAIV

Analysis of antigen specific Tfh cells was again assessed in children stratified as IgA responders and non-responders as previously described. In the IgA responders, no significant increase was seen in the frequency of CD25+Tfh cells with H1 HA stimulation at day 7 and 21 post LAIV, whilst in the IgA non-responders, an increased frequency of CD25+ antigen specific Tfh cells was seen at day 21 ($p = 0.0014$) with H1 HA stimulation (Figure 40A).

For H3 HA antigen specific Tfh cells, no increase in frequency of CD25+ cells was observed post LAIV in IgA responders but in IgA nonresponders a significant increase was noted at day 7 ($p = 0.0040$) and 21 ($p = 0.0015$) post LAIV (Figure 40A). No increase in the frequency of CD25+ antigen specific Tfh cells was seen post vaccination in IgA responders and non-responders with Influenza B HA stimulation.

For OX40+CD25+ antigen specific Tfh cells, no significant increase in frequency was noted post LAIV in both IgA responders and nonresponders with H1 HA stimulation (Figure 38B). Stimulation with H3 HA resulted in an increased frequency of OX40+CD25+ Tfh cells at day 21 in IgA responders although this did not reach statistical significance after correcting for multiple testing ($p = 0.0280$). However, an increase in these OX40+CD25+ antigen specific Tfh cells was noted in the IgA non responders at both day 7 ($p = 0.0213$) and 21 ($p = 0.0034$) post vaccination (Figure 40B).

Stimulation with Influenza B HA led to an increase in frequency of OX40+CD25+ in Tfh cells at day 21 in IgA responders although this did not reach statistical significance after correcting for multiple testing ($p = 0.0238$). There was no difference in baseline and post vaccination frequencies of OX40+CD25+ Tfh cells in the IgA non responders with influenza B HA stimulation (Figure 40B).

No increase in frequency of CD25+PDL1+ antigen specific Tfh cells was seen at day 21 post vaccination in IgA responders for both H1 and H3 HA stimulation. In non-IgA responders, an increase in frequency of CD25+PDL1+ antigen specific Tfh cells was noted with both H1 ($p = 0.0170$) and H3 HA stimulation ($p = 0.0249$) at day 21 post vaccination. No increase in frequency of CD25+PDL1+ antigen specific Tfh cells was seen post vaccination in IgA responders and non-responders with Influenza B HA stimulation (Figure 40C). No difference in frequency of OX40+PDL1+ antigen specific Tfh cells was noted in both groups post vaccination for all three antigens (Figure 40D).

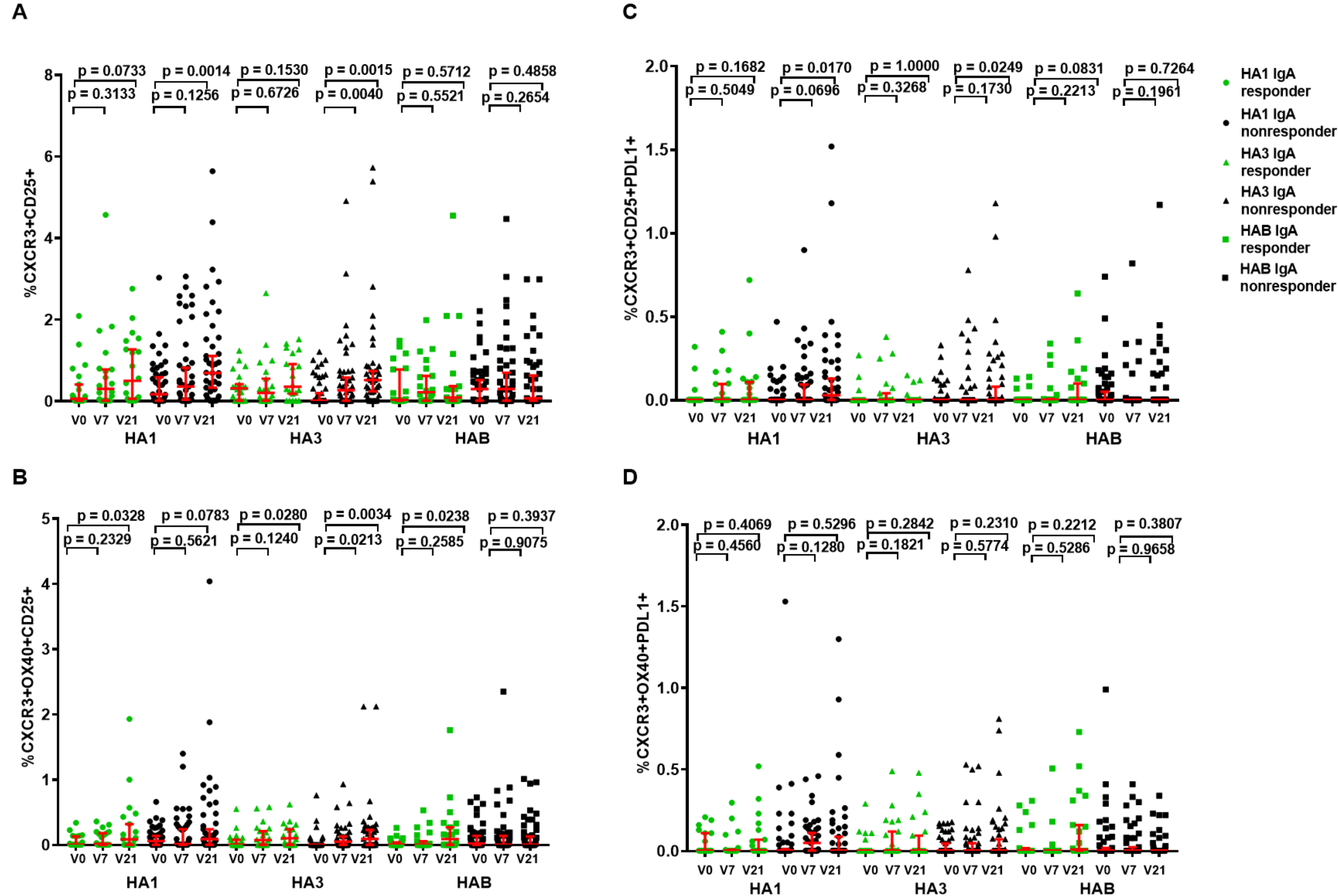


Figure 40: Induction of antigen specific CXCR3+Tfh cells after stimulation with influenza antigens in IgA responders and nonresponders

Frequency of A) CD25+, B) OX40+CD25+, C) CD25+PDL1+ and D)OX40+PDL1+ Tfh cells were gated on within CD45R0+CXCR5+ CXCR3+ Tfh cells and responses shown upon stimulation with HA1 (circles), HA3 (triangles) and HAB (squares). Data is shown for 61 individual donors. In each graph, the green circles represent IgA responders n= 20 and black circles represent non-IgA responders n= 41. Horizontal lines represent the median with 95% CI. Data analysis was done using Wilcoxon matched pairs signed rank test. Due to multiple comparisons for the two timepoints compared to baseline (n = 2), significance level was adjusted and P values of < 0.025 were considered statistically significant.

5.4.11 Detection of LAIV induced Tfh cells in children with or without pre-existing antibody responses within CD45R0+CXCR5+CXCR3+ Tfh cells

In CD45RO+CXCR5+CXCR3+ Tfh cells, the frequency of antigen specific cells post LAIV was assessed using AIM markers in children with or without baseline serum antibody responses as previously described. In children that were seropositive at baseline no increase in frequency of CD25+ Tfh cells was noted post vaccination. In children that were seronegative at baseline, a marked increase in frequency of CD25+ antigen specific Tfh cells was seen at day 21 post LAIV with H1 stimulation ($p < 0.0001$) (Figure 41A). However, with H3 HA stimulation an increase in frequency of CD25 expression at day 7 ($p = 0.0140$) and 21 ($p = 0.0035$) post LAIV was only seen with those that were seropositive at baseline but not those that were seronegative at baseline. No difference in frequenc of CD25+antigen specific Tfh cells was seen with Influenza B HA stimulation post LAIV in both groups (Figure 41A).

In children that were seropositive at baseline, no increase in frequency of OX40+CD25+ antigen specific Tfh cells was noted with H1 HA stimulation but an increased frequency was seen at day 21 post LAIV in children seronegative at baseline ($p = 0.0110$) (Figure 41B). For H3 HA stimulation, increased frequency of 0X40+CD25+ Tfh cells at day 7 ($p = 0.0044$) and 21 ($p = 0.0040$) post vaccination was observed in the children seropositive at baseline, whilst increased frequencies at day 21 was seen in children seronegative at baseline ($p = 0.0235$) (Figure 41B). For influenza B HA stimulation, no increase was noted for frequency of OX40+CD25+ Tfh cells in children seropositive at baseline, whilst in children seronegative at baseline an increase in frequency was seen at day 21 ($p = 0.0082$) post LAIV (Figure 41B).

When frequency of CD25+PDL1+Tfh cells was assessed, no increase was seen in the children that were seropositive at baseline, whilst increase in frequency at day 21 ($p = 0.0097$) was noted in children that were seronegative at baseline with H1 HA stimulation. No difference in frequency of CD25+ antigen specific Tfh cells was seen with H3 and influenza B HA stimulation post LAIV in both groups (Figure 41C).

No difference in the frequency of OX40+PDL1+ antigen specific Tfh cells was observed in children that were seropositive at baseline and those that were not for all three antigens (Figure 41D). A summary of the results obtained in the analysis of both bulk Tfh, subsets and antigen specific Tfh cells is shown in (Figure 42A and B) and (Figure 43-44A-D) below.

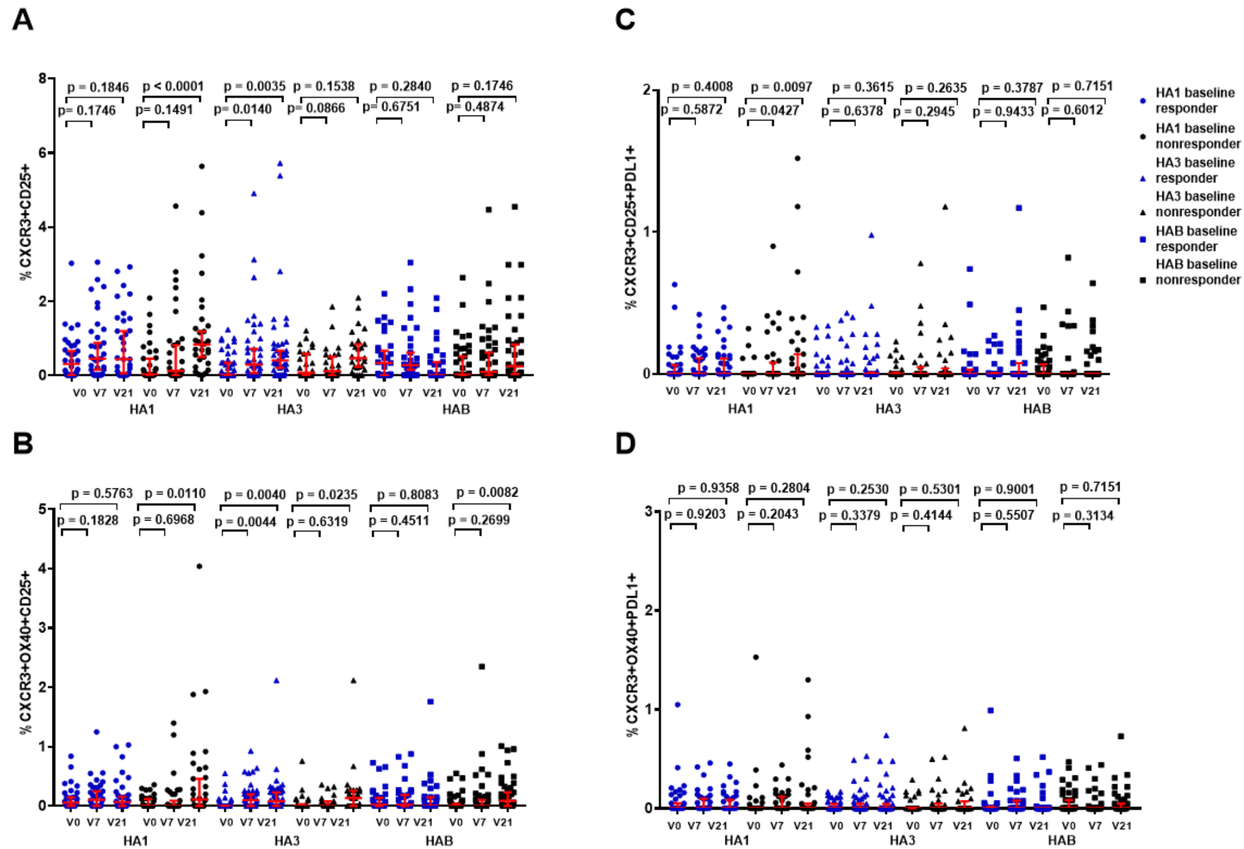


Figure 41: Detection of AIM markers on antigen specific Tfh cells after stimulation with influenza antigens in baseline responders and nonresponders

Frequency of A) CD25+, B) OX40+CD25+, C) CD25+PDL1+ and D)OX40+PDL1+ Tfh cells were gated on within CD45R0+CXCR5+CXCR3+ Tfh cells and responses shown upon stimulation with HA1 (circles), baseline responder-n=34 , nonresponder =31, HA3 (triangles) baseline responder-n=39, nonresponder =26 and HAB (squares) baseline responder-n=29, nonresponder =36. Data is shown for 65 individual donors. In each graph, the blue circles represent participants with baseline antibody responses and black circles represent beasline nonresponders. Horizontal lines represent the median with 95% CI. Data analysis was done using Wilcoxon matched pairs signed rank test. Due to multiple comparisons for the two timepoints compared to baseline (n = 2), significance level was adjusted and P values of < 0.025 were considered statistically significant.

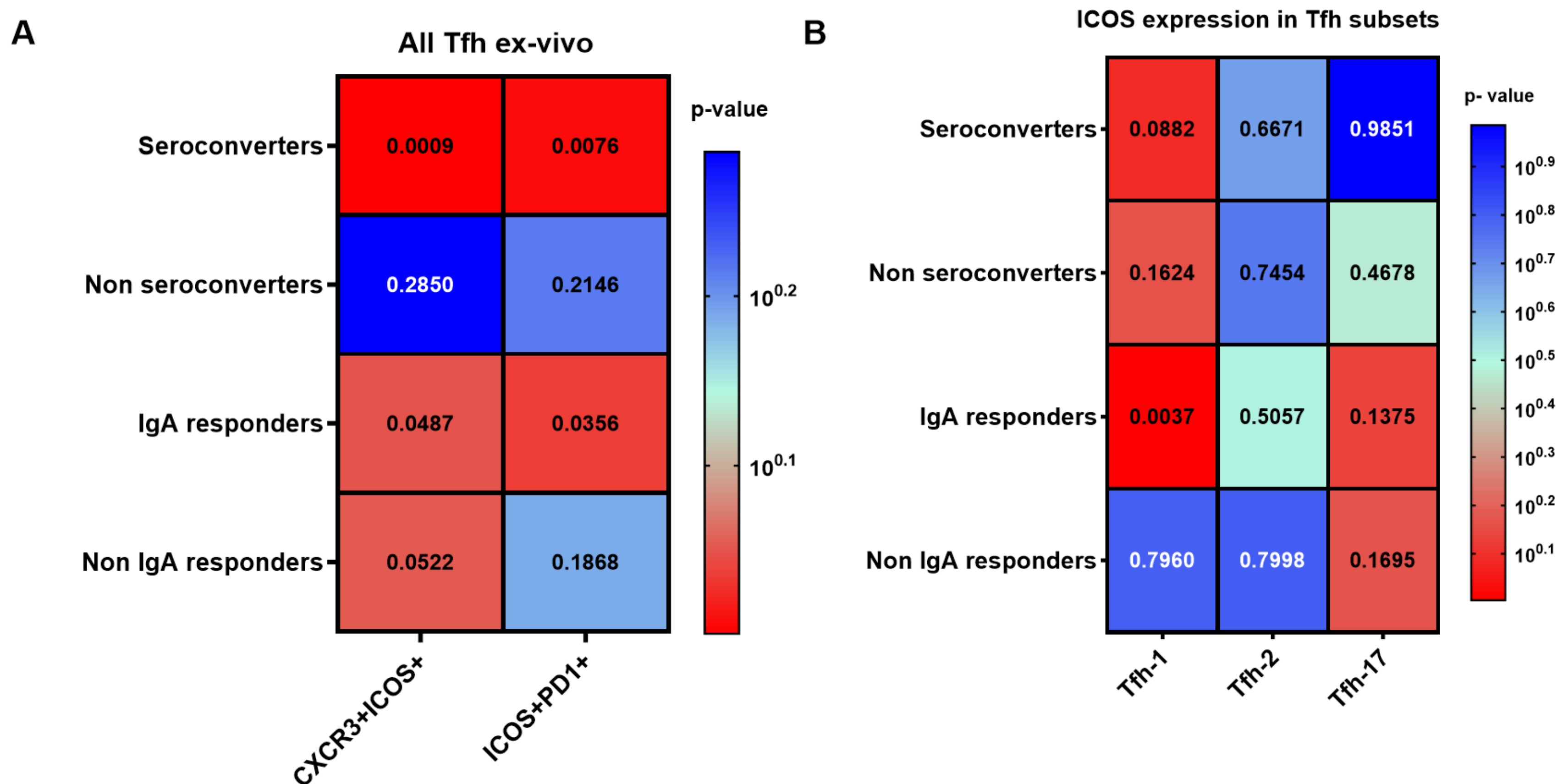


Figure 42: Heatmap showing p values of the frequency of circulating Tfh cells at baseline and day 7 post LAIV vaccination

In A) All Tfh ex-vivo (2017 and 2018) B) Tfh subsets (2017 data only) Due to multiple comparisons for the two timepoints compared to baseline (n = 2), significance level was adjusted and P values of < 0.025 were considered statistically significant.

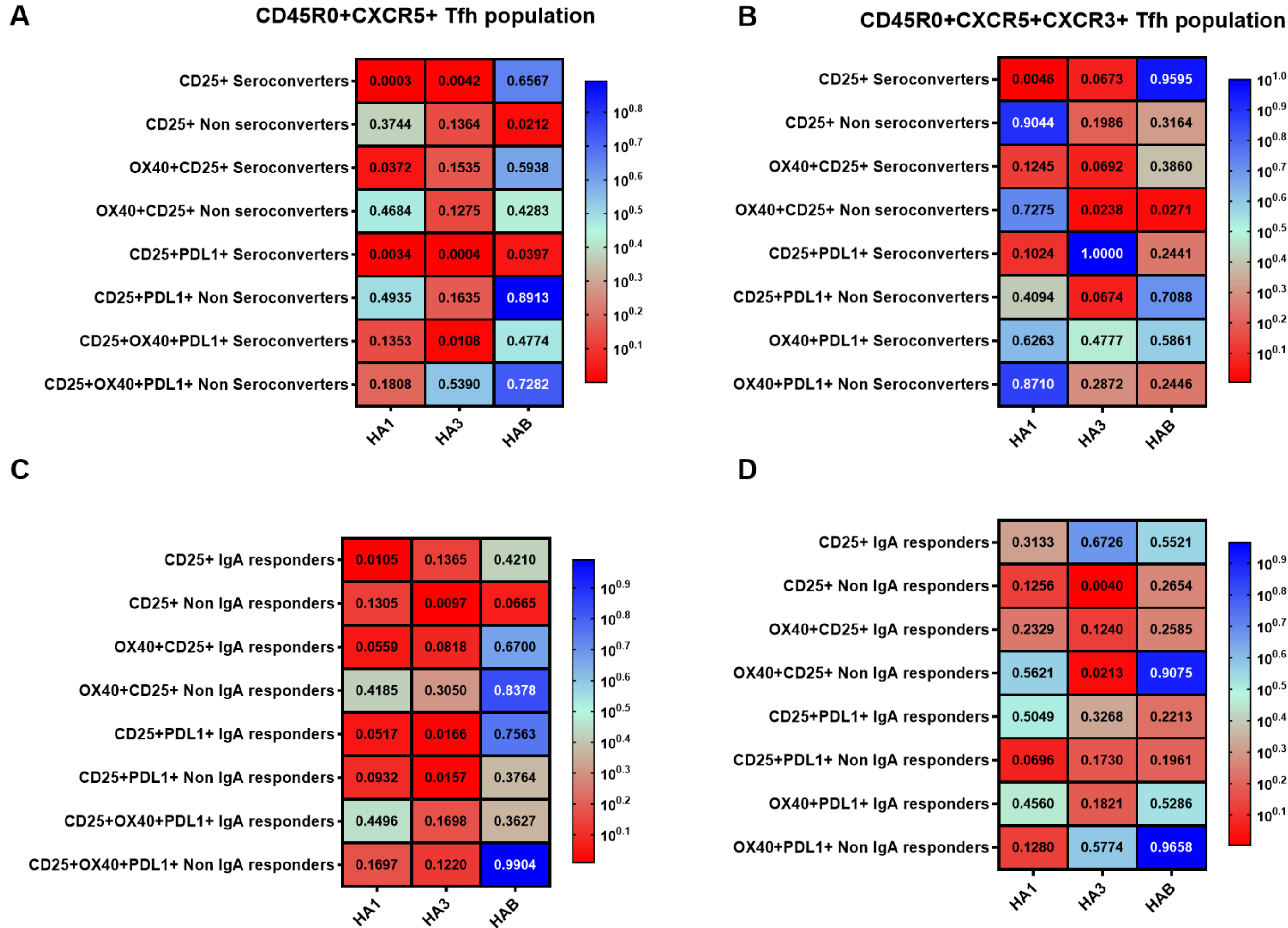


Figure 43: Heatmap showing p values of the percentage of activation marker expression on CD45R0+CXCR5+ and CD45R0+CXCR5+CXCR3+ Tfh cells at baseline and day 7 post LAIV vaccination after stimulation with different influenza antigens

In children that were seroconverters or non seroconverters in A) CD45R0+CXCR5+ Tfh and B) CD45R0+CXCR5+ CXCR3+Tfh cells and IgA responders or non-responders in C) CD45R0+CXCR5+ Tfh and D) CD45R0+CXCR5+ CXCR3+Tfh cells post LAIV vaccination. Due to multiple comparisons for the two timepoints compared to baseline (n = 2), significance level was adjusted and P values of < 0.025 were considered statistically significant.

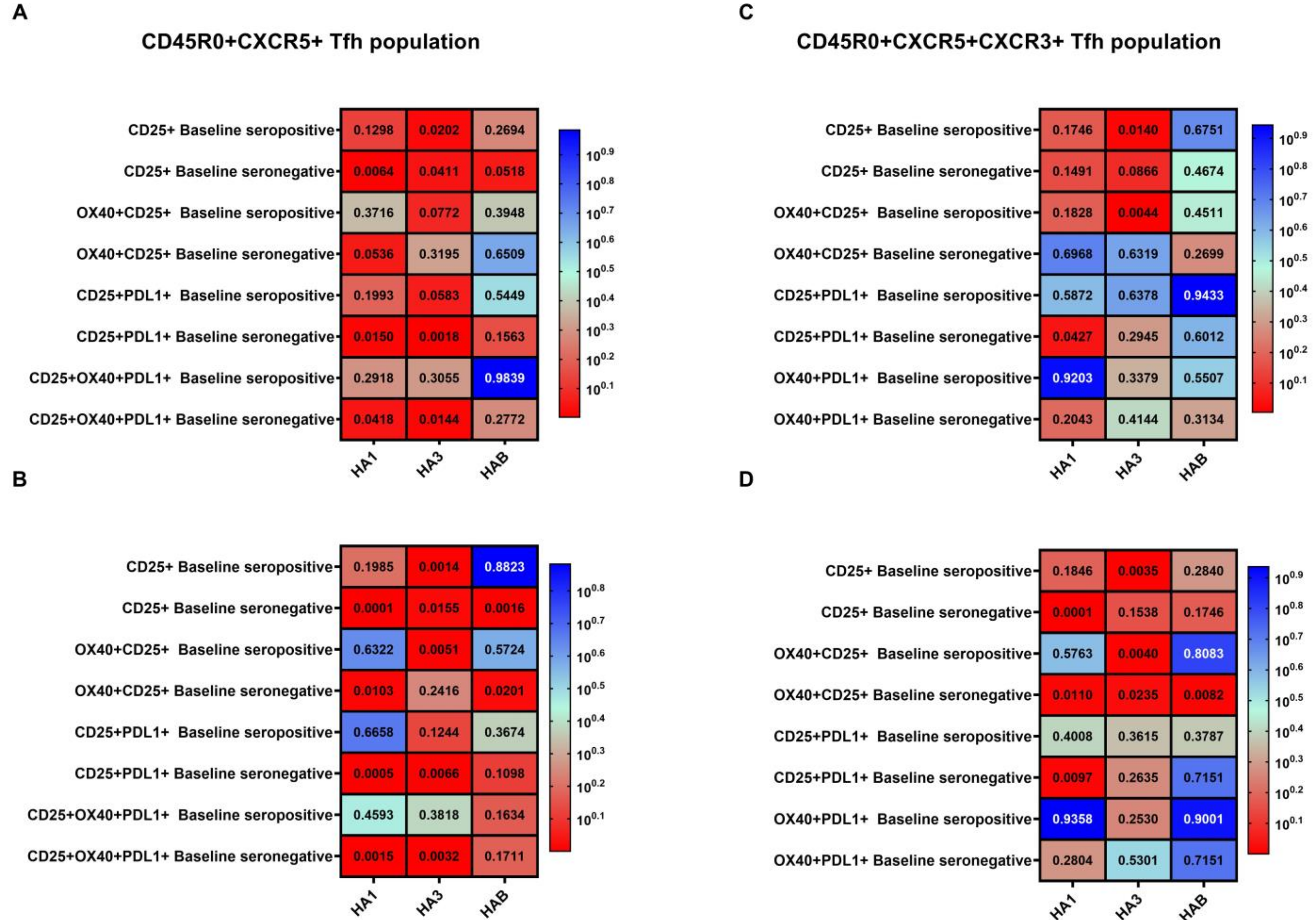


Figure 44: Heatmap showing p values of the percentage of activation marker expression on CD45R0+ CXCR5+ and CD45R0+CXCR5+CXCR3+ Tfh cells at baseline and day 7 and 21 post LAIV vaccination after stimulation with different influenza antigens

In children that were baseline responders or nonresponders in A) CD45R0+CXCR5+ Tfh at day 7 and B) CD45R0+CXCR5+ Tfh cells at day 21 C) CD45R0+CXCR5+CXCR3+ Tfh at day 7 and D) CD45R0+CXCR5+ CXCR3+Tfh cells at 21 post LAIV vaccination. Due to multiple comparisons for the two timepoints compared to baseline (n = 2), significance level was adjusted and P values of < 0.025 were considered statistically significant.

5.5 Discussion

I investigated the role of Tfh in LAIV-induced immune responses in LAIV vaccine naive children using *ex-vivo* staining and detected an increase in frequency of circulating Tfh cells in blood post LAIV vaccination. The increase in the frequency of circulating Tfh cells at day 7 post LAIV vaccination was statistically significant in the children that seroconverted to one or more antigens at day 7 post vaccination but not those that did not seroconvert. This is consistent with findings from previous studies were expansion of circulating Tfh cells was only observed in vaccine responders but not the nonresponders (Pallikkuth *et al.*, 2012). When IgA response and frequency of Tfh cells was assessed, no difference in frequency of circulating Tfh cells at day 7 post LAIV vaccination was observed in IgA responders and non-responders. No correlation between the fold change in circulating Tfh cells at day 7 post vaccination and the serum and mucosal antibody responses was observed.

The three different subsets of Tfh cells in blood based on expression of the chemokine receptors CXCR3 and CCR6 were identified in this study. Post vaccination, the frequency of ICOS+ cells increased in the Tfh-1 but not Tfh-2 or Tfh-17 cells, consistent with findings from previous studies (Bentebibel *et al.*, 2013). Tfh-1 subset is the only subset that can provide help to memory B cells for production of high avidity antibodies which occurs within 7 days post vaccination as demonstrated by Bentebibel et al (Bentebibel *et al.*, 2016). At day 21, a significant decrease in frequency of ICOS+ cells was observed in all Tfh subsets suggesting that these cells are involved in antibody responses and once this is completed there may be a memory pool maintained in blood especially with Tfh-1 cells which lack the ability to activate naive B cells (Schmitt, Bentebibel and Ueno, 2014).

No difference in frequency of ICOS+ cells within Tfh subsets was observed, whether children had seroconverted or not. This may be because LAIV does not induce significant serum antibodies responses compared to IIV (Sridhar, Brokstad and Cox, 2015). When children where stratified based on IgA response or no response, an increase in the frequency of ICOS+ cells was seen in IgA responders only within the Tfh-1 subset but not Tfh-2 and Tfh-17 at day 7 post vaccination.

In addition to the *ex-vivo* staining, I used the more sensitive activation induced marker (AIM) assay to detect antigen specific Tfh cells in blood. This assay is cytokine independent and is based on T cell receptor (TCR-) upregulation using surface markers such as CD25, OX40 and PDL1. The AIM assay has been used in the detection of antigen specific CD4+T cells in animals (Jennifer M Dan *et al.*, 2016) and humans (Zaunders *et al.*, 2009; Reiss *et al.*, 2017; Bowyer *et al.*, 2018). GC and blood Tfh cells and rare dengue and pertussis antigen specific T cells have also been detected with the AIM assay after

antigenic stimulation (Jennifer M Dan *et al.*, 2016). An increased frequency of antigen specific cells is detected by AIM compared to other traditional assays such as ELISPOT and ICS (Jennifer M. Dan *et al.*, 2016; Reiss *et al.*, 2017; Bowyer *et al.*, 2018).

Activation of antigen specific circulating Tfh cells could be detected using various combinations of the AIM markers post LAIV vaccination although responses differed depending on the antigen used for stimulation. No increase in frequency of OX40+PDL1+ antigen specific Tfh cells was seen post LAIV vaccination with any of the 3 antigens. This was quite unexpected as previous studies have shown that OX40+CD25+ and OX40+PDL1+ could both detect antigen specific CD4-T cells. Reiss et al reported that OX40+PDL1+ marker combination detected fewer antigen specific cells compared to OX40+CD25+ (Reiss *et al.*, 2017), whilst Bowyer et al reported similar level of detection between the two markers (Bowyer *et al.*, 2018). The difference in the results obtained may be due to differences in methodology. Whilst both studies used cryopreserved PBMC in their study to assess AIM, whole blood was used in this study. There was also a difference in the population of interest: we looked at CXCR5+Tfh cells whilst they focused on CD4+T cells, a much bigger population. Differences in the clones of antibody used may also be a reason for the differences in identifying OX40+PDL1+ cells. There were differences in the type of antigen used for stimulation between all three studies.

Post vaccination, no increase in the expression of AIM markers was detected with Influenza B HA antigen. The reason for this is unclear but may be due to viral replication of Influenza B. A lower replicative capacity of Influenza B virus compared to seasonal Influenza A viruses and inhibition of viral replication in strains belonging to the Victoria-like lineage compared to those of the Yamagata lineage in the presence of mucin, which is present in the airways has been previously reported (Bui *et al.*, 2019).

There was no correlation between AIM markers and antibody responses post LAIV. In young children less than 8 years old with no history of LAIV it is recommended to administer 2 doses of the LAIV four (4) weeks apart, but a single dose was used in our study based on recommendation by the vaccine manufacturers. Therefore, it is possible that the single dose did not induce sufficient mucosal antibody responses in these children. In our study, when children were stratified based on mucosal responses for the AIM assay data analysis, 21 children were IgA responders whilst 43 were nonresponders. Previous studies have shown that Tfh-1 cells are poor at providing help to B cells (Morita *et al.*, 2011; Locci *et al.*, 2013) and as such the dominance of Tfh-1 response seen in our study

as well as others studies of influenza vaccination and infection may be the reason for the poor induction of antibody responses post vaccination, highlighting the need for further research on how to improve the current influenza vaccines.

It is also worth noting that in the published studies showing correlation between blood Tfh and antibody responses (Bentebibel *et al.*, 2013, 2016), IIV vaccination was used. We know from previous studies that LAIV is a mucosal vaccine and is better at inducing mucosal antibody responses than serum antibody responses (Sridhar, Brokstad and Cox, 2015). Therefore, the lower level of serum antibody in these children may also be responsible for the difference in our results and the lack of correlation between Tfh induction and antibody responses.

Viral replication in the nose is key for the induction of the immune response to LAIV and baseline responses affect the subsequent immune response to vaccination (Shannon, White and Nayak, 2020). Increased expression of some activation markers on antigen specific Tfh cells post LAIV was seen in children seronegative at baseline whilst in the children that were seropositive, the increase was not statistically significant. In a recent study by Lartey et al, the authors showed that LAIV led to increased expression of ICOS in the follicles post vaccination. Further analysis revealed an inverse relationship between follicular helper T cell responses and pre-existing mucosal responses (Lartey *et al.*, 2020). Increase in frequency of influenza specific CD4+T cell responses post vaccinations was shown using tetramers in participants vaccinated with the split influenza vaccine however the authors noted difference in the responses elicited post vaccination. This was shown to be dependent on both vaccination history and pre-existing immunity (Kissling *et al.*, 2023). Masking of epitopes by pre-existing antibody responses have been previously described and this could potentially reduce stimulation of antigen presenting cells as well as antigen specific B and T cells. This will ultimately affect activation of follicular helper T cells, a subset of activated CD4+T cells. Our findings are therefore in line with previous results where pre-existing immune responses is shown to limit the subsequent immune response by neutralizing the virus and inhibiting further replication (Coelingh *et al.*, 2014; Mohn *et al.*, 2015).

Previous studies have looked at functionality of Tfh cells through assessment of cytokine secretion. One limitation in our study was that this was not assessed due to the limitation in the number of markers that can be assessed in our flow panel and the volume of sample that can be collected from the children. Co-culture experiments with B cells have also been done to assess the ability of Tfh cells

to provide help to B cells in antibody production. This will require a cell sorter which at the time of the study was not available at MRC The Gambia.

5.6 Conclusion

To my knowledge, this is the first study in children to report Tfh induction in blood post LAIV using both the ex-vivo and AIM assay. This study therefore provides evidence that Tfh cells are induced by LAIV and AIM assay can be successfully used to identify antigen specific Tfh cells using whole blood.

Our results shows that LAIV in children induced circulating follicular helper T cells at day 7 post vaccination. Difference in Tfh induction in seroconverters and non seroconverters was noted, but this was not seen when we looked at IgA responders and non-responders. Using the AIM assay, we showed that influenza specific Tfh cells were induced post LAIV.

Key questions remain to be answered regarding Tfh induction and LAIV. What is the functionality of these Tfh cells identified? Does the mode of delivery of a vaccine affect the subsequent Tfh induction? Will a booster dose of LAIV in vaccine naïve children enhance Tfh and the subsequent humoral immune response to the vaccine?

6 General Discussion

6.1 Influenza Burden

Despite the high burden of influenza among children under 5 years living in Africa, there is limited focus on research that would faciliate the expansion of influenza vaccine use in the continent (Hirve, 2015; Ortiz *et al.*, 2016; Lindsey *et al.*, 2018). South Africa is among the few countries with an established influenza vaccination programme and to reduce the burden of influenza in high risk groups, there is an urgent need to scale up influenza research in Africa. Efforts to increase access to influenza vaccines in LMIC have led to the production of NASOVAC-S, an LAIV manufactured at SIIPL (Rudenko *et al.*, 2016). This vaccine has gone through various clinical trials in Asia (Brooks *et al.*, 2016; KDC, Lewis *et al.*, 2018; Krishnan *et al.*, 2021) and Africa (Victor *et al.*, 2016) with good safety profiles. However, there was no immunogenicity data reported for the African cohort. This PhD project-nested within the the NASIMMUNE study, aimed to explore the innate and adaptive immune responses induced by LAIV in Gambian children and how early immune responses impact induction of subsequent adaptive immunity that provides clinical protection.

6.2 Influenza vaccines

Two types of influenza vaccines exist, LAIV and IIV with differences in their cost, mode of delivery and the immune responses induced. LAIV works better in children compared to adults, but despite its widespread use, no correlates of protection have been defined (Sridhar, Brokstad and Cox, 2015). HAI titres have been used as a measure of vaccine efficacy for IIV, especially in adults however this measure is less effective in predicting vaccine efficacy in high risk groups for influenza infection including children (Reber and Katz, 2013).

6.3 Immune correlates of protection

Studies have shown that HAI on its own does not accurately predict LAIV efficacy (Hobson *et al.*, 1972; Wright *et al.*, 2016). This is possibly due to the fact that in addition to systemic antibody responses, local/mucosal IgA and T cell responses are also induced by LAIV; and more so than with IIV, where HAI is a better correlate of protection (He *et al.*, 2006; Sridhar, Brokstad and Cox, 2015; Mohn *et al.*, 2018). Identifying the cells in the different arms of the immune system induced by LAIV vaccination and how they relate to each other is key towards defining the correlates of protection for LAIV. This project is the first study to assess immune response to LAIV in Gambian children and will provide the first immunogenicity data on LAIV in Africa.

6.4 Increase in frequency of intermediate monocytes and their role in the adaptive immune response post LAIV

In Chapter 3, I looked at the effect of LAIV on the frequency and function of innate immune cells with a focus on the monocyte and dendritic cell subsets. LAIV induced increase in frequency of the intermediate monocyte population and a decline in the classical monocyte subset. Increase in frequency of intermediate monocytes has been described in multiple studies of infection, inflammation, and vaccination. Intermediate monocytes comprise a very small proportion of the monocyte population suggesting that their expansion results in a functional role. Functionality of these monocytes is mediated by their surface receptors including HLA-DR, CD54, CD86 and CD40 for antigen presentation to other immune cells, migration to site of infection via chemokine receptors and integrins which allows interaction with T cells to provide help to B cells for antibody responses and killing of virally infected cells. CXCR5 has in fact been shown to aid the recruitment of CD8 memory T cells to the site of infection resulting in the control of viral replication (Kohlmeier *et al.*, 2008), whilst CXCR2 allows the migration of monocytes to the site of infection (Dawson *et al.*, 2000). The observed increase in frequency of intermediate monocytes at day 2, the earliest timepoint assessed post vaccination and its association with both serum and mucosal immune responses is similar to findings by Wong et al who showed the seroconversion after influenza infection was dependent on the increase in intermediate monocytes in the blood and nasopharynx and more activated CD4+ T cells (Wong *et al.*, 2021).

Antibody responses are important in the protection against influenza infection. CD16 expressed on monocytes has the ability to mediate ADCC (Wong *et al.*, 2011) in virally infected cells, cancer cells (Yeap *et al.*, 2016) and parasitized red blood cells (Royo *et al.*, 2019). We observed an increase in intermediate monocytes post LAIV in Gambian children. This increase was statistically significant in the seroconverters but not the nonseroconverters, which was consistent with findings from a previous study on influenza infection (Wong *et al.*, 2021). In dengue infection, a similar increase in intermediate monocytes was noted. In this study, the authors further explored how intermediate monocytes contribute to the antibody responses to dengue infection in a series of experiments using monocytes isolated from PBMC in healthy donors. They showed that infection of CD14 monocytes led to a rapid differentiation of monocytes to intermediate monocytes. These monocytes had a high expression of markers including CXCR5, CD163 and CD169 and secreted chemokines and cytokines such as MCP-1, IP-10, IL-6, IL-8, and IL-10. Coculture of monocytes with resting CD19+B cells and IL-2 and CpG led to proliferation of B cells and within these CD27++CD38++ plasmablasts within 6 days

of culture. This increase in plasmablast led to a secretion of IgG and IgM but not IgA. BAFF and APRIL were two molecules associated with the mechanism through which intermediate monocytes stimulated plasmablast differentiation and this correlated with the magnitude of the intermediate monocytes in the blood (Kwissa *et al.*, 2014). Plasmablast differentiation and antibody secretion was reduced by blocking of these molecules, as well as IL-10, a cytokine produced by all monocyte subsets highlighting their role in the antibody response to dengue infection. Taken together, these results show that early intermediate monocyte expansion is key for an effective humoral immune response to influenza infection. Thus, the findings from this study may therefore explain how increase in frequency of intermediate monocytes contribute to the antibody responses post LAIV in Gambian children.

We also reported a reduced frequency of mDCs post LAIV, corroborating the findings from earlier studies showing a decrease in the frequency of mDCs in blood samples of patients with viral infections. In a study comparing the DC populations in influenza patients and controls, DC populations in influenza infected patients were reduced in blood and increased in the nasopharynx (Vangeti *et al.*, 2022). A reduction in frequency of myeloid DCs post IIV vaccination was also noted post vaccination with IIV vaccine. In these vaccinees, the magnitude of the reduction in the frequency of the myeloid DCs was associated with antibody responses measured a month post vaccination (Kobie, Treanor and Ritchlin, 2014). Reduction in frequency of DCs in other viral infections notably, dengue infection (Kwissa *et al.*, 2014) and RSV (Gill *et al.*, 2005) have also been reported. The reduced frequency of mDCs from the blood upon viral infections suggest that upon infection these cells move to the mucosal site of infection to carry out their effector functions and that they play a role in the antiviral immune responses.

6.5 Increase in frequency of CD4+T cells post LAIV in Gambian children

LAIV led to the induction of both IFN-γ and IL-2 positive Influenza-specific CD4+ T cells pre and post vaccination in Gambian children. The magnitude of CD8+ responses was higher at baseline but post LAIV, only the frequency of CD4+T cells was increased. The higher baseline influenza specific CD8+T cells we observed in our study participants, might have potentially led to more effective viral (LAIV) control, less viral replication and lower frequency of CD8+ T cells post LAIV. The low frequency of CD8+T cells induced post LAIV seen in our study is similar to findings from previous studies of LAIV in children, were an induction of CD4+T cells but not CD8+T cells was reported (Islam *et al.*, 2019).

In 2018, there was a change in the H1N1 portion of the vaccine which led to an enhanced CD4+T cell induction post LAIV. We found an increase in the CD4+ IFNγ-positive responses to the updated pH1 portion of the vaccine in those children given the 2017–18 LAIV, (50 of 109 [46%, 95% CI 36·3–55·7] compared to those that received the 2016–17 LAIV, 29 of 111 [26%, 18·2–35·3]. A similar increase was also noted for the CD4+ IL2-positive responses with children given the 2017–18 LAIV, 57 of 109 [52%, 42·5–61·9] showing a greater T cell response compared to those given the 2016-17 LAIV, 23 of 111 [20·7%, 13·6–29·5. The number of vaccinees that had CD4+ IFNγ-positive and/or CD4+ IL2-positive responses post LAIV was also impacted by the vaccine change with an increased number of children inducing CD4+T cell responses in 2017-18 vaccinees, 73 of 111 children (66%, 60·0–75·6) compared to those that were vaccinated with the 2016–17 LAIV, 45 of 111 children (41%, 95% CI 31·3–50·3). The switch from Cal09 to NY15, (H1 portion) led to a significant increase in replication and improved cellular and humoral immune responses suggesting that viral replicative fitness should be considered when selecting vaccine strains. This finding of reduced replicative fitness of the Cal09 gives a plausible explanation for the poor efficacy result seen with the LAIV vaccine in Senegal (Lindsey *et al.*, 2019).

Although not assessed in our studies, NK cell functionality was required for the induction of CD8+T cell responses in influenza both *in vivo* and *in vitro* (Kos and Engleman, 1996). Previous studies in Gambian children have shown that vaccination with influenza (using IIV) and DTPiP failed to induce NK cell responses (Darboe *et al.*, 2017) and this was attributed to the change in NK cell phenotype due to CMV infection early in life. Darboe et al found that Gambian children infected with HCMV had more NKG2C+ and CD57+ receptors and had a lower expression of CD25 and IFN- γ compared to UK adults. Thus, the lack of CD8 T cell induction post vaccination may have been due to the change in NK cell phenotype observed in Gambian children as the interaction between the two cell types is mediated partly by cytokine responses (Darboe *et al.*, 2017), which were reduced due to CMV infection. CMV is highly prevalent in The Gambia and by 1 year of age over 80% of children are infected (Kaye *et al.*, 2008).

6.6 Increased frequency of follicular helper T cells post LAIV

I further explored the role that these induced CD4+ T cells play in the immune response to LAIV. An increase in the frequency of circulating Tfh cells was noted in Gambian children post LAIV. The increased frequency of circulating Tfh cells in vaccine responders but not the nonresponders has been previously reported in healthy and HIV infected adults after the administration of a single

intramuscular dose of inactivated monovalent A/California/07/2009 H1N1 vaccine (Novartis Vaccines and Diagnostics) (Pallikkuth *et al.*, 2012). Tfh cells are essential for germinal center formation and contribute to the development of high affinity antibodies, plasma cells and memory B cells (Schmitt et al., 2013; Crotty et al., 2014). Analysis of the Tfh responses post vaccination showed that Tfh-1 cells expressing ICOS and PD1 are induced post vaccination and when assessed within seroconverters and not seroconverters, a significant increase in frequency of these cells was noted in those that seroconverted but not the nonseroconverters. Previous studies have showed that Tfh-1 cells increase post vaccination with trivalent inactivated influenza vaccine and the transient increase observed was associated with the generation of high avidity antibodies and CD19+CD27++ antibody secreting cells (ASC) (Bentebibel *et al.*, 2013, 2016; Koutsakos *et al.*, 2018). In influenza patients, increase in the number of activated Tfh-1 cells compared to Tfh-2 and Tfh-17 has also been noted. The authors further showed that these Tfh-1 were PD1+ and ICOS+ cells and emerged at the same time as antibody secreting cells with both peaking between day 7 and 10 post infection. A strong correlation between circulating Tfh-1 cells and ASC responses was noted during the acute stage of the infection (Nguyen *et al.*, 2021). The dominance of circulating Tfh-1 cells in influenza infection and vaccination may be as a result of its role in inducing IgG1, the most abundant immunoglobulin subclass, which is important in responses to viral infections and vaccination (Chi, Gu and Ma, 2022).

There is a significant reduction in frequency of ICOS+Tfh cells in all Tfh subsets in non-IgA responders that is not seen in the the IgA responders (Figure 32) at day 21, suggesting that maintenance of Tfh cells in the blood after the infection has resolved may be key in the mucosal antibody responses post LAIV. In our cohort, we detected Influenza-specific CD4+ IFNγ-positive, CD4+ IL2+, and CD8+ IFNγ-positive T-cell responses at baseline. Baseline seropositivity for pH1N1 was 33% in 2016-17 and 49% in 2017-18, for H3N2 it was 76% in 2016-17 and 56% in 2017-18 and for B/Vic it was 21% in 2016-17 and 43% in 2017-18. In 2016-17, 5% of children seroconverted to pH1N1 22% to H3N2 and 34% to BVic whilst in 2017-18, 19% of children seroconverted to pH1N1 28% to H3N2 and 44% to B/Vic (Lindsey *et al.*, 2019). Memory B cells can rapidly proliferate and differentiate into plasma cells upon re-exposure to antigens whilst memory T cells are rapidly activated by pro-inflammatory cytokines to kill virally infected cells and differentiate into effector cells (Woodland and Kohlmeier, 2009; Palm and Henry, 2019). It is therefore possible that in our cohort, the mechanism through which the development of both serum and mucosal antibody responses happened in LAIV vaccinated children is possibly via activation of memory B and T cells induced by natural exposure to seasonal influenza. Using the activation induced marker assay (AIM), activation of antigen specific circulating

Tfh cells could be detected using various combinations of the AIM markers (OX40+CD25+PDL1+) post LAIV although responses differed depending on the influenza antigen used for stimulation.

6.7 Conclusion

The data presented in this thesis characterizes the innate and adaptive immune cell changes post-LAIV and the relationship between the innate immune response with the later adaptive immune responses.

The main findings from this study are that there is an increase in the frequency of intermediate monocytes at day 2 and 7 post LAIV and this was associated with humoral and mucosal antibody responses to the vaccine. LAIV induced CD4+ and CD8+ T cells in Gambian children but increase in the frequency of cells post vaccination was only significant for CD4+T cells. Circulating follicular helper T cells are induced by LAIV and this was also associated with humoral antibody responses. The AIM assay used in this thesis allowed the identification of antigen specific Tfh cells in LAIV vaccinated children, using whole blood.

In 2018, the second year of the study, there was a strain change in the pH1 portion of the vaccine. When immune responses were assessed, enhanced shedding, improved cellular and humoral immune responses were observed in participants who received the vaccine in the second year compared to vaccinees in the first year. Further investigations within the wider NASIMMUNE study showed that there was lower replication and shedding of Cal09 HIN1 virus compared to the NY15 HIN1 strain when monovalent vaccine strains were cultured in primary human nasal epithelial cells. These ultimately led to the lower immune responses seen post LAIV in 2017 (Lindsey *et al.*, 2019). Although not assessed as part of this thesis, these were key findings from the wider NASIMMUNE study that need to be highlighted as it provides new information that could be important in future vaccine designs.

Taken together, our results show that vaccination of children with LAIV induces the increase in frequency of intermediate monocytes and reduction in frequency of classical monocytes. The frequency of myeloid dendritic cells which are antigen presenting cells were also shown to decrease post vaccination, which I hypothesise is due to trafficking of mDCs to a mucosal site. The increase in frequency of intermediate monocytes in blood may contribute to CD4+T cell activation and differentiation into follicular helper T cells, which can then help B cells to differentiate into plasmablast and antibody secreting cells post LAIV. A schematic diagram of the association between

the frequency of intermediate monocytes post vaccination and how it may relate with the subsequent humoral immune responses generated towards LAIV is illustrated below in Figure 45.

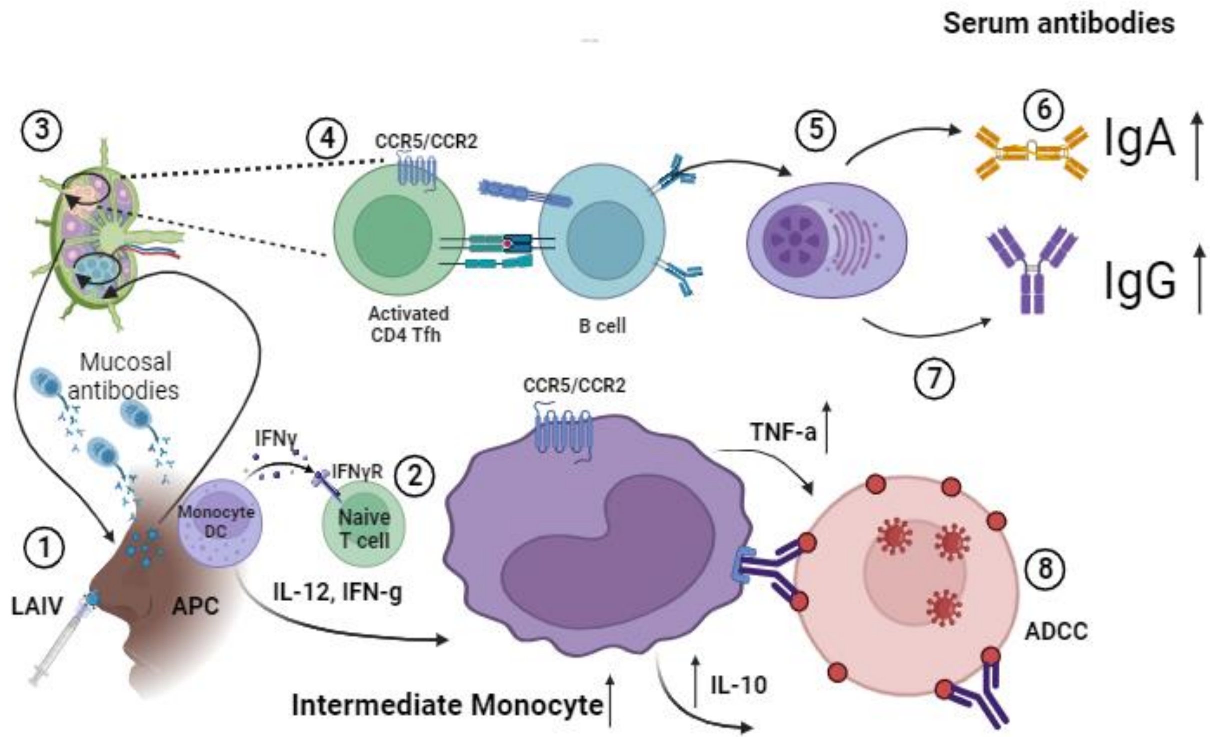


Figure 45: Intermediate monocytes mediate antibody responses post LAIV in Gambian children.

1) LAIV administered intranasally. Limited viral replication occurs within the nasopharynx leading to release of IFN-gamma from activated cells which can activate monocytes/DCs. 2) Monocyte maturation leads to increase in intermediate monocytes post LAIV both at the site of infection and in circulation. 3) Viral antigens transported to lymph node by monocytes/DCs lead to activation of T and B cells. Activated B cells will secrete antibodies and home to vaccination site/recirculate in blood. Intermediate monocytes in lymph nodes/vaccination site will interact with Tfh cells via CCR5. 4) Tfh cells will interact with B cells. 5) B cells will migrate to GCs. 6) IgA and 7) IgG antibodies will be produced and secreted both at the site of vaccination and in the blood 8) Intermediate monocytes initiate ADCC via CD16.

7 Chapter 7: Study outcome and future work

7.1 Study Limitations

One of the main challenges with working with young children is to avoid bleeding at multiple timepoints and the volume of blood to be drawn. In this study, we had to group the children in subgroups with some bled at day 0 and day 2 or day 7 to answer the question of how LAIV affected the innate immune responses. Due to the volume of blood collected, we could not carry out co-culture experiments to determine the functionality of the cells being assessed.

In terms of T cell responses measured, the peptide sets that we used for quantification of CD4+ and CD8+ T-cell responses were overlapping 15-18-mer peptide pools to MNP, HA1 and HA3. As previously reported, the length of the peptide dictates binding to MHC Class I or II (Madden, 1995). It is therefore possible that the overlapping peptides used are biased towards the activation of CD4+ as opposed to CD8+T cells.

Our study observation is limited to the robustness of the frequency we have used for our analysis of the cell populations for the flow data. We did not use BD true count beads to get the absolute cell counts of the different cell population of interest, which could have given us the true cell population count of our samples and will explain if the observed change in frequency post vaccination was absolute or relative to the gates used in our analysis.

Our study is also limited by the fact that global cytokine/chemokine production and the overall contribution of the different cell types identified was not assessed.

Finally, we did not use flow cytometry to analyse the immune cells in the nasal washes from the children which would have provided us with data to compare the responses in blood and those at the site of infection and their contribution to the immune response to LAIV.

7.2 Implication of the research

Despite the widespread use of vaccines to protect against influenza, very few African countries routinely offer them. Numerous efforts geared towards availability and use of influenza vaccines are underway. Among the few influenza studies in Africa, no studies of LAIV immunogenicity in children exist. This leaves us with many unanswered questions regarding immunity to LAIV in African children. What is the role of innate immune cells in the response to LAIV vaccination? Does LAIV induce robust CD4 and CD8 T cell responses? Does LAIV induce follicular helper T cells? Does induction of these follicular helper T cells correlate with antibody responses post LAIV? How does the magnitude of the innate response affect the subsequent adaptive immune response? Since LAIV is a mucosal vaccine, will the measurement of antibody responses in oral fluid samples be more indicative of immunological changes following LAIV, as opposed to serum antibody responses? Can these changes be used to predict the efficacy of LAIV rather than HAI titre alone? This study attempted to answer some of these questions by assessing the frequency and function of both innate and adaptive immune cells post LAIV and how they relate to the antibody responses post vaccination. The result from this study is the first report of immunogenicity data in African children vaccinated with LAIV. It by no means answers all the questions regarding immunogenicity to LAIV but provides novel data on the immune pathways induced by LAIV in children, and will serve as a benchmark for further studies to evalute the mechanisms around successful LAIV immunisation.

7.3 Future work

In this thesis, I showed that LAIV induced the expansion of intermediate monocytes, CD4+T cells and Tfh cells post LAIV. It will be important to isolate and carry out coculture experiments for these immune cells with influenza virus to assess their function. Frequency of bleeding is a major hurdle in studies especially those involving children, therefore *in vitro* assays might provide answers as to how influenza infection affects innate cells, especially in the first few hours post infection.

LAIV is a mucosal vaccine and therefore analysis of Tfh in blood may not be reflective of the extent of the mucosal immune response induced by vaccination. A recent study in children vaccinated with LAIV showed that tonsillar Tfh cells are induced post LAIV and they correlate with systemic but not HAI antibody titres (Lartey *et al.*, 2020). Recent studies have shown that flow cytometry can be done on nasal swab samples to assess immune cells at the site of infection (Cosgrove *et al.*, 2021). Analysis of nasal swab samples to assess the different immune cells pre and post LAIV and the different chemokines and cytokines produced will be important in addressing the varying reports on the specific contribution of the different subsets of cells in the immune response to LAIV.

Future studies should also assess B cell responses as studies with IIV have shown that the induction of Tfh at day 7 post vaccination occurs at the same time as induction of plasmablasts and antibody secreting cells (Morita *et al.*, 2011; Bentebibel *et al.*, 2013).

In addition to the phenotype of Tfh cells, future studies could assess the functionality of these cells by measuring cytokine production using methods such as Luminex and assessing the ability of Tfh to provide help to B cell through co-culture experiments.

Finally, it will be important to compare Tfh induction in children vaccinated with LAIV versus IIV. This will allow us to assess the effect of the mode of delivery of the vaccine on induction of Tfh.

In all the studies that I came across during my write-up, the AIM assay was done on thawed PBMC samples. It will be important to compare the responses using whole blood and PBMC to ensure that the assay is able to detect optimal responses with both methods.

7.4 References

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Appendix 1: Scientific Coordinating Commitee

Dr Thushan de Silva
Visiting Scientist
Vaccines and Immunity Theme
MRC Unit The Gambia

17 November 2016

Dear Dr de Silva

L2016.67v1.1: Re SCC1502, A study of intranasal live attenuated influenza vaccine immunogenicity and associations with the nasopharyngeal microbiome among children in The Gambia – The NASIMMUNE Study.

Thank you for submitting your response letter dated 15 November 2016 addressing the query raised by the SCC at its meeting held on 7 November 2016.

I am happy to fully approve your request which will be forwarded to the Ethics Committee for further consideration at their next meeting on 25 November 2016.

With best wishes

Yours sincerely

Professor Umberto D'Alessandro
Chair, Scientific Coordinating Committee

Documents submitted for review:-

- Response letter – 15 November 2016
- Request letter – 24 October 2016
- Protocol, version 2.0 – 22 October 2016

Scientific Coordinating Committee
MRC Unit The Gambia
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- ICDs (groups A,B, C and D), version 2.0- 22 October 2016
- Assessment of understanding (Groups A, B, C and D), version 2.0 – 22 October 2016
- Protocol amendment sheet – 22 October 2016
- Appointment card, version 1.0 –16 October 2016
- Subject ID card, version 1.0 – 16 October 2016
- Study information and contact details document (Groups A, B, C and D) – 16 October 2016
- Priority information, version 1.0 – 16 October 2016

Appendix 2: Ethics Committee

The Gambia Government/MRC Joint
ETHICS COMMITTEE

C/o MRC Unit: The Gambia, Fajara
P.O. Box 273, Banjul
The Gambia, West Africa
Fax: +220 – 4495919 or 4496513
Tel: +220 – 4495442-6 Ext. 2308
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28 December 2016

Dr Thushan de Silva
Visiting Scientist
MRC Unit The Gambia
Vaccines and Immunity Theme

Dear Dr de Silva

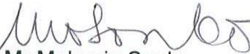
L2016.67v1.1, Re SCC1502: A study of intranasal live attenuated influenza vaccine immunogenicity and associations with the nasopharyngeal microbiome among children in The Gambia – The NASIMMUNE Study

Thank you for submitting your letters dated 24 October 2016 and 15 November 2016 for consideration by The Gambia Government/MRC Joint Ethics Committee at its meeting held on 16 December 2016.

We are pleased to approve your request.

With best wishes

Yours sincerely



Mr Malamin Sonko
Chairman, Gambia Government/MRC Joint Ethics Committee

Documents submitted for review:-

- SCC approval letter – 17 November 2016
- Response letter – 15 November 2016
- Request letter – 24 October 2016
- Protocol, version 2.0 – 22 October 2016
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The Gambia Government/MRC Joint Ethics Committee:

Mr Malamin Sonko, Chairman
Professor Ousman Nyan, Scientific Advisor
Ms Naffie Jobe, Secretary
Dr Roddie Cole
Dr Ahmadou Lamin Samateh
Mrs Tulai Jawara-Ceesay
Prof. Umberto D'Alessandro
Dr Ramatoulie Njie
Dr Kalifa Bojang
Dr Jane Achan
Dr Momodou L. Waggeh
Dr Siga Fatima Jagne

Appendix 3: Participant Information sheet and consent form

PARTICIPANT INFORMATION SHEET GROUP A,B, and C

Version 4.0 Date 16 February 2018

Study Title: A Study of Live Attenuated Influenza Vaccine Immunogenicity and Associations with the Nasopharyngeal Microbiome Among Children in The Gambia – The NASIMMUNE Study

SCC:	1502	Protocol:	

Sponsor: London School of Hygiene & Tropical Medicine

Funder: The Wellcome Trust

What is informed consent?

You are invited to let your child take part in a research study. Before you decide, you need to understand why the research study is being done and what it will involve. Please take time to read the following information or get the information explained to you in your language. Listen carefully and feel free to ask if there is anything that is not clear or you do not understand. You may also wish to consult your spouse, family members, friends or others before deciding to let your child take part in the study.

If you decide to allow your child to join the study, you will need to sign or put a thumbprint on a consent form saying you agree for your child to be in the study. You will receive a copy of this.

Why is this study being done?

Flu is a germ that can cause an illness with a fever, runny nose, sore throat and body aches. It can also cause more serious infections in the lung. Children are especially at risk of serious infections with flu. There are vaccines available to protect against flu, but these are not currently used in The Gambia, despite international recommendations to do so. More information is now available to show that flu is a problem in The Gambia and West Africa in general, responsible for serious lung infections in children that need to be admitted to hospital.

We would like to study how well a new vaccine against flu works in The Gambia. If it only works in some children but not others, we would also like to study why this happens so that this and similar vaccines can be improved.

What is the new vaccine?

'Nasovac-S', is a new vaccine that is given as a spray into a child's nose rather than as an injection. It contains weakened versions of the flu germ and can help your child's body build up protection against future flu infections. Similar vaccines have been used in other parts of the world for many years and have been shown to protect children from flu infections. This particular vaccine has been made so it would be affordable to use in countries such as those in Africa and Asia. Although it is a new vaccine, it has been shown to be safe in children aged 2 and over and has received a licence from the World Health Organization (WHO).

What does this study involve?

If you allow your child to take part in this study and sign or thumbprint the consent form, we will first ask you a few questions about your child's health and examine your child to see if they are suitable to take part. If suitability is confirmed, we will randomly assign your child to one of three groups. This means that you cannot choose which group your child is placed in. Two of the groups will receive the vaccine early in the study and have samples taken to see how well it works. The other group will receive the vaccine at the end of the study.

If your child is assigned to a group that receives the vaccine early:

You will be asked to bring your child to the Health Centre 3 more times (4 visits in total) over 21 days. During these visits, your child will have a total of 3 blood samples, 4 swabs from the nose and 2 swabs from the mouth taken.

1st visit: We will take one swab from your child's mouth, another from your child's nose and a blood sample. Your child will then receive the vaccine, as a spray given into each nostril. We will observe your child for 30 minutes after this to make sure that they are OK to go home after receiving the vaccine. We will make an appointment for you to attend with your child 2 days later.

2nd visit: When you return 2 days later, we will ask you some questions to see how well your child has been since he/she received the vaccine. We will then take a swab from your child's nose. Children assigned to *one* of the groups will also have a blood sample taken on this day. We will make an appointment for you to attend with your child 5 days later.

3rd visit: When you return 5 days later, we will ask you some questions to see how well your child has been since he/she was last seen. We will then take a swab from your child's nose. Children in the group that did not have a blood sample taken during the second visit will have a blood sample taken on this visit. We will make an appointment for you to attend with your child 14 days later.

4th visit: When you return 14 days later, we will ask you some questions to see how well your child has been since he/she was last seen. We will take one swab from your child's mouth, another from your child's nose and a blood sample.

We ask that until the end of October of this year, if your child develops a fever, along with a cough or a sore throat, that you bring your child to be assessed at the Health Centre. If this happens, once we ask you some questions about your child's recent illness, we may take a swab from your child's nose. This is to see if your child has developed influenza illness or not and therefore to see if the vaccine has worked. If necessary, your child will receive immediate care at the study site and then be referred to the appropriate health facility if this is considered to be necessary by the study staff.

We will contact you to arrange a final visit in November of this year, to ensure your child has been well during the course of the study. If it is easier, in some cases this could be by telephone to save you coming to the site.

<u>If your child is assigned to a group that receives the vaccine at the end of the study</u>:

You will be asked to bring your children to the Health Centre 2 more times (3 visits in total) over 21 days. During these visits, your child will have a total of 3 swabs from the nose.

1st visit: We will take a swab from your child's nose. We will make an appointment for you to attend with your child 7 days later.

2nd visit: When you return 7 days later, we will take a swab from your child's nose. We will make an appointment for you to attend with your child 14 days later.

3rd visit: When you return 14 days later, we will take one swab from your child's nose. Your child will then receive the vaccine, as a spray given into each nostril. We will observe your child for 30 minutes after this to make sure that they are OK to go home after receiving the vaccine.

We ask that until the end of October of this year, if your child develops a fever, along with a cough or a sore throat, that you bring your child to be assessed at the Health Centre. If this happens, once we ask you some questions about your child's recent illness, we may take a swab from your child's nose. This is to see if your child has developed influenza illness or not and therefore to see if the vaccine has worked. If necessary, your child will receive immediate care at the study site and then be referred to the appropriate health facility if this is considered to be necessary by the study staff.

We will contact you to arrange a final visit in November of this year, to ensure your child has been well during the course of the study. If it is easier, in some cases this could be by telephone to save you coming to the site.

In case the investigator discovers your child is sick and decides that he/she cannot participate in the study because of that, he/she will receive immediate care at the study site and then be referred to the appropriate health facility.

If the research study needs to be stopped, you will be informed and your child will have the normal medical care.

What will happen to the samples taken in this study?

The blood samples, nose and mouth swabs will be taken to the MRC laboratories in Fajara. These samples will be labelled with a study number and *not* your child's name, so your child will not be directly identifiable from these samples. Some of these samples will be used at the MRC to look at how well your child's body is responding to the vaccine.

Some of the samples will be sent to international laboratories for the same purpose, as well as for genetic tests used specifically to look at genes that may influence how well your child responds to the vaccine. Genes carry information about us, including how our bodies respond to illness and vaccines, and are passed on from parents to children.

Your child's sample will be stored and if new scientific information is discovered, other tests including genetic testing testing may be performed in the future. We will always ask for the approval from the Gambia Government/MRC Joint Ethics Committee to use the samples from this study for new research.

The results of the study will be made available to your community.

What harm or discomfort can you expect in the study?

Giving the vaccine itself will not cause any discomfort to your child. Your child may experience some minor side effects from the vaccine in the week afterwards such as a sore throat, a runny nose, cough and a fever. This occurs only in about one of five children given the vaccine. These will get better in a few days at most. As with some other medicine or vaccine, very rarely, severe and potentially life threatening reactions can occur. We will monitor your child immediately after receiving the vaccine to ensure they do not have such a reaction and provide medical care in the unlikely event that they do.

Your child will experience some minor discomfort and may develop a small bruise when blood samples are taken. The amount of blood that is being taken over the course of the study will *not* be harmful to your child's health. Your child may also experience some minor irritation when the nose swab is being taken.

What benefits can you expect in the study?

As your child will receive a vaccine against flu by participating in this study, we expect he/she will be protected from getting ill with flu over the next year.

As we would like to see if children develop flu infections despite the vaccine until the end of October this year, your child is eligible to be assessed and if appropriate, treated at the Health Centre if they develop a fever and either a cough or a sore throat. Your child may, therefore, benefit from available healthcare if this occurs.

There are no other direct benefits for you or your child. But by agreeing to allow your child to participate in this study, you will be helping scientists understand how to improve vaccines in the future.

Will you be compensated for your child's/ward's participation in the study?

You will not get paid for participation of your child in the study, but you will get either transport by MRC or get money for transport.

Are there other products or treatment?

No, the only products or treatment in this study are described above.

What happens if you refuse to participate in the study or change your mind later?

You are free to let your child participate or not in the study and you have the right to stop his/her participating at anytime without giving a reason. This will not affect the medical care that your child would normally receive.

In case you decide to withdraw your child's participation during the study we would like to continue to work on the samples we have already taken from your child. We would also like to use the information already generated from the samples until the time of withdrawal. The study doctor may also ask for tests for your child's safety.

Should any new information become available during the study that may affect your child's participation, you will be informed as soon as possible.

What compensation will be available if your child is injured during the study?

We will be responsible to provide for treatment caused by procedures of the research study through the LSHTM non-negligent harm insurance and medical malpractice insurance policy. If your child has an unwanted reaction, we will treat him/her or refer him/her as needed.

If medical treatment is required as an emergency, please refer to your health centre or clinic and contact the field worker who gave his/her telephone number to you or contact Dr Armitage on 7034731.

How your child's information will be kept and who will be allowed to see it?

All information that is collected about your child in the course of the study will be kept strictly confidential. Your child's personal information will only be available to the study team members and might be seen by some rightful persons from the Ethics Committee, Government authorities and MRC.

Who should you contact if you have questions?

If you have any queries or concerns you can contact Dr Armitage or Dr de Silva on 2657389 and you can always call the personal numbers of the study staff given to you. If you have any concerns you can also contact staff at your health centre or clinic.

Please feel free to ask any question you might have about the research study.

Who has reviewed this study?

This study has been reviewed and approved by a panel of scientists at the Medical Research Council and the Gambia Government/MRC Joint Ethics Committee, which consists of scientists and lay persons to protect your rights and wellbeing.

CONSENT FORM GROUP A,B, and C

Participant's Name: ____

Participant's Identification Number: |__|__|__|__|__|

Parent/Guardian's Name: ____________________________________

☐ I have read the written information OR

I agree to further research on my child's samples as described in the information sheet

Yes ☐ No ☐

Participant's parent/guardian signature/thumbprint

______________ ____________

Date Time

Printed name of impartial witness*

____

Signature of impartial witness* ____________

______________ ______________

Date Time

Printed Name of Person obtaining consent

____

I attest that I have explained the study information accurately in ______________ and that is was understood to the best of my knowledge by, the parent/guardian and that he/she has freely given consent to participate *in the presence of the above named impartial witness (where applicable).

______________ ______________________

Signature of Person obtaining consent

_______________ _______________
Date (dd/mmm/yyyy) Time (24hr)

☐ I have had the information explained to me by study personnel in a language that I understand

and I confirm that my choice to let my child participate is entirely voluntarily,

confirm that I have had the opportunity to ask questions about this study and I am satisfied with the answers and explanations that have been provided,

understand that I grant access to data about my child to authorised persons described in the information sheet,

have received sufficient time to consider to let my child take part in this study

agree to allow my child take part in this study.

Tick as appropriate

**Only required if the participant is unable to read or write.*

A copy of this informed consent document has been provided for the participant.

Assessment of Understanding – Informed Consent Group A,B and C

No	Question	Correct answer	1st attempt	2nd attempt
1	The study will examine a vaccine against the flu germ	T		
2	Children in The Gambia are not currently vaccinated against the flu germ	T		
3	If your child is eligible for the study, you will be able to choose which group of the study your child is allocated to	F		
4	The vaccine is given as an injection in to your child's arm	F		
5	Your child will have a minimum of 3 swabs taken from the nose over the course of the study	T		
6	Your child may be allocated into a group that requires 3 blood samples to be taken from your child over 21 days	T		
7	Samples taken during the study will not be sent out of the Gambia	F		
8	You will be paid to take part in the study	F		
9	You should contact the study team if your child gets ill or have any concerns while your child is in the study	T		
10	You are free to withdraw your child from the study at any time and do not have to give a reason for this	T		

PARENT

Subject Identification Number: |___|-|___|___|___|___|

Date of Assessment ___ ___ / ___ ___ ___ / ___ ___

(dd/mmm/yyyy)

Time of Assessment ___ ___ : ___ ___ (24 hour)

Ask the subject the following questions

Tick the box (1st attempt or 2nd attempt) if they get the answer correct and count the correct answers

The assessment of understanding will be completed by a research clinician or nurse prior to the completion of the informed consent form. A maximum of two attempts are allowed

Outcome			
All questions answered correctly ☐	1 error ☐	2 or more errors ☐	
↓	↓	↓	
CONSENT AND ENROL	Review missed or incorrect question and ensure subject understands the correct answer	Review missed or incorrect questions and ensure subject understands the correct answer	
	↓	Repeat entire informed consent process	
	CONSENT AND ENROL	↙	↘

		All questions answered correctly ☐	1 or more errors ☐
		↓	↓
		CONSENT AND ENROL	DO NOT ENROL

	__/___/____
Signature	Date

PARTICIPANT INFORMATION SHEET GROUP D

Version 4.0 Date 16 February 2018

Study Title: A Study of Live Attenuated Influenza Vaccine Immunogenicity and Associations with the Nasopharyngeal Microbiome Among Children in The Gambia – The NASIMMUNE Study

SCC:	1502	Protocol:	

Sponsor: London School of Hygiene & Tropical Medicine

Funder: The Wellcome Trust

What is informed consent?

You are invited to let your child take part in a research study. Before you decide, you need to understand why the research study is being done and what it will involve. Please take time to read the following information or get the information explained to you in your language. Listen carefully and feel free to ask if there is anything that is not clear or you do not understand. You may also wish to consult your spouse, family members, friends or others before deciding to let your child take part in the study.

If you decide to allow your child to join the study, you will need to sign or put a thumbprint on a consent form saying you agree for your child to be in the study. You will receive a copy of this.

Why is this study being done?

Flu is a germ that can cause an illness with a fever, runny nose, sore throat, cough and body aches. It can also cause more serious infections in the lung. Children are especially at risk of serious infections with flu. There are vaccines available to protect against flu, but these are not currently used in The Gambia, despite international recommendations to do so. More information is now available to show that flu is a problem in The Gambia and West Africa in general, and responsible for some serious lung infections in children that need to be admitted to hospital.

We would like to study how well a new vaccine against flu works in The Gambia. If it only works in some children but not others, we would also like to study why this happens so that this and similar vaccines can be improved.

What is the new vaccine?

'Nasovac-S', is a new vaccine that is given as a spray into a child's nose rather than as an injection. It contains weakened versions of the flu germ and can help your child's body build up protection against future flu infections. Similar vaccines have been used in other parts of the world for many years and have been shown to protect children from flu infections. This particular vaccine has been made so it would be affordable to use in countries such as those in Africa and Asia. Although it is a new vaccine, it has been shown to be safe in children aged 2 and over and has received a licence from the World Health Organization (WHO).

As part of the study, your child will also receive an antibiotic medicine called azithromycin prior to having the vaccine. This is given as a liquid as one dose. This antibiotic is widely used to treat many infections and is safe. In this study, this antibiotic is given to see whether it can change the way in which your child's body responds to the vaccine and not because your child is unwell.

What does this study involve?

If you allow your child to take part in this study and sign or thumbprint the consent form, we will first ask you a few questions about your child's health and examine your child to see if they are suitable to take part. If suitability is confirmed, you will be asked to bring your child to the Health Centre 4 more times (5 visits in total). During these visits, your child will have a total of 3 blood samples, 5 swabs from the nose and 2 swabs from the mouth taken.

1st visit: We will take a swab from your child's nose. Your child will then receive the antibiotic azithromycin as a liquid. We will make an appointment for you to attend with your child 4 weeks later.

2nd visit: We will take one swab from your child's mouth, another from your child's nose and a blood sample. Your child will then receive the vaccine, as a spray given into each nostril. We will observe your child for 30 minutes after this to make sure that they are OK to go home after receiving the vaccine. We will make an appointment for you to attend with your child 2 days later.

3rd visit: When you return 2 days later, we will ask you some questions to see how well your child has been since he/she received the vaccine. We will then take a swab from your child's nose. We will make an appointment for you to attend with your child 5 days later.

4th visit: When you return 5 days later, we will ask you some questions to see how well your child has been since he/she was last seen. We will then take a swab from your child's nose and a blood sample. We will make an appointment for you to attend with your child 14 days later.

5th visit: When you return 14 days later, we will ask you some questions to see how well your child has been since he/she was last seen. We will take one swab from your child's mouth, another from your child's nose and a blood sample.

We ask that until the end of October of this year, if your child develops a fever, along with a cough or a sore throat, that you bring your child to be assessed at the Health Centre. If this happens, once we ask you some questions about your child's recent illness, we may take a swab from your child's nose. This is to see if your child has developed influenza illness or not and therefore to see if the vaccine has worked. If necessary, your child will receive immediate care at the study site and then be referred to the appropriate health facility if this is considered to be necessary by the study staff.

We will contact you to arrange a final visit in November of this year, to ensure your child has been well during the course of the study. If it is easier, in some cases this could be by telephone to save you coming to the site.

What should I do if my child becomes unwell during the study or I have any concerns?

You should contact the study team or bring your child to the health centre if he/she becomes ill while in the study or if you have any concerns.

In case the investigator discovers your child is sick and decides that he/she cannot participate in the study because of that, he/she will receive immediate care at the study site and then be referred to the appropriate health facility.

If the research study needs to be stopped, you will be informed and your child will have the normal medical care.

What will happen to the samples taken in this study?

The blood samples, nose and mouth swabs will be taken to the MRC laboratories in Fajara. These samples will be labelled with a study number and *not* your child's name, so your child will not be directly identifiable from these samples. Some of these samples will be used at the MRC to look at how well your child's body is responding to the vaccine.

Some of the samples will be sent to international laboratories for the same purpose, as well as for genetic tests used specifically to look at genes that may influence how well your child responds to the vaccine. Genes

carry information about us, including how our bodies respond to illness and vaccines, and are passed on from parents to children.

Your child's sample will be stored and if new scientific information is discovered, other tests including genetic testing may be performed in the future if you agree to this in the consent form. In this case, we will always ask for the approval from the Gambia Government/MRC Joint Ethics Committee to use the samples from this study for new research.

The results of the study will be made available to your community.

What harm or discomfort can you expect in the study?

Giving the vaccine itself will not cause any discomfort to your child. Your child may experience some minor side effects from the vaccine in the week afterwards such as a sore throat, a runny nose, cough and a fever. This occurs only in about one of five children given the vaccine. These will get better in a few days at most. As with some other medicine or vaccine, very rarely, severe and potentially life threatening reactions can occur. We will monitor your child immediately after receiving the vaccine to ensure they do not have such a reaction and provide medical care in the unlikely event that they do.

Your child will experience some minor discomfort and may develop a small bruise when blood samples are taken. The amount of blood that is being taken over the course of the study will *not* be harmful to your child's health. Your child may also experience some minor irritation when the nose swab is being taken.

What benefits can you expect in the study?

As your child will receive a vaccine against flu by participating in this study, we expect he/she will be protected from getting ill with flu over the next year.

As we would like to see if children develop flu infections despite the vaccine until the end of October this year, your child is eligible to be assessed and if appropriate, treated at the Health Centre if they develop a fever and either a cough or a sore throat. Your child may, therefore, benefit from available healthcare if this occurs.

There are no other direct benefits for you or your child. But by agreeing to allow your child to participate in this study, you will be helping scientists understand how to improve vaccines in the future.

Will you be compensated for your child's/ward's participation in the study?

You will not get paid for participation of your child in the study, but you will get either transport by MRC or get money for the transport.

Are there other products or treatment?

No, the only products or treatment in this study are described above.

What happens if you refuse to participate in the study or change your mind later?

You are free to let your child participate or not in the study and you have the right to stop his/her participating at anytime without giving a reason. This will not affect the medical care that your child would normally receive.

In case you decide to withdraw your child's participation during the study we would like to continue to work on the samples we have already taken from your child. We would also like to use the information already generated from the samples until the time of withdrawal will also be used. The study doctor may also ask for tests for your child's safety.

Should any new information become available during the study that may affect your child's participation, you will be informed as soon as possible.

What compensation will be available if your child is injured during the study?

We will be responsible to provide for treatment caused by procedures of the research study through the LSHTM non-negligent harm insurance and medical malpractice insurance policy. If your child has an unwanted reaction, we will treat him/her or refer him/her as needed.

If medical treatment is required as an emergency, please refer to your health centre or clinic and contact the field worker who gave his/her telephone number to you or contact Dr Armitage on 7034731.

How your child's information will be kept and who will be allowed to see it?

All information that is collected about your child in the course of the study will be kept strictly confidential. Your child's personal information will only be available to the study team members and might be seen by some rightful persons from the Ethics Committee, Government authorities and MRC.

Who should you contact if you have questions?

If you have any queries or concerns you can contact Dr Armitage or Dr de Silva on 2657389 and you can always call the personal numbers of the study staff given to you. If you have any concerns you can also contact staff at your health centre or clinic.

Please feel free to ask any question you might have about the research study.

Who has reviewed this study?

This study has been reviewed and approved by a panel of scientists at the Medical Research Council and the Gambia Government/MRC Joint Ethics Committee, which consists of scientists and lay persons to protect your rights and wellbeing.

CONSENT FORM GROUP D

Participant's Name: ____

Participant's Identification Number: |__|-|__|__|__|__|

Parent/Guardian's Name: ____

☐ I have read the written information OR

☐ I have had the information explained to me by study personnel in a language that I understand

and I

confirm that my choice to let my child participate is entirely voluntarily,

confirm that I have had the opportunity to ask questions about this study and I am satisfied with the answers and explanations that have been provided,

understand that I grant access to data about my child to authorised persons described in the information sheet,

have received sufficient time to consider to let my child take part in this study

agree to allow my child take part in this study.

Tick as appropriate

I agree to further research on my child's samples as described in the information sheet

Yes ☐ No ☐

Participant's parent/guardian signature/thumbprint

Date

Time

Printed name of impartial witness*

Signature of impartial witness*

Date Time

Printed Name of Person obtaining consent

I attest that I have explained the study information accurately in ______________________ and that is was understood to the best of my knowledge by, the parent/guardian and that he/she has freely given consent to participate *in the presence of the above named impartial witness (where applicable).

Signature of Person obtaining consent

Date (dd/mmm/yyyy) Time (24hr)

**Only required if the participant is unable to read or write.*

A copy of this informed consent document has been provided for the participant.

Assessment of Understanding – Informed Consent Group D

PARENT

Subject Identification Number ___ ___ ___ ___ ___

Date of Assessment ___ ___ / ___ ___ ___ / ___ ___ ___ ___

(dd/mmm/yyyy)

Time of Assessment ___ ___ : ___ ___ (24 hour)

Ask the subject the following questions

Tick the box (1st attempt or 2nd attempt) if they get the answer correct and count the correct answers

No	Question	Correct answer	1st attempt	2nd attempt
1	The study will examine a vaccine against the flu germ	T		
2	Children in The Gambia are not currently vaccinated against the flu germ	T		
3	If your child is eligible for the study, he/she will receive the antibiotic azithromycin only if they are unwell	F		
4	The vaccine is given as an injection in to your child's arm	F		
5	Your child will have a minimum of 5 swabs taken from the nose over the course of the study	T		
6	During the course of the study, 3 blood samples will be taken from your child over 21 days	T		

7	Samples taken during the study will not be sent out of the Gambia	F		
8	You will be paid to take part in the study	F		
9	You should contact the study team if your child gets ill or have any concerns while your child is in the study	T		
10	You are free to withdraw your child from the study at any time and do not have to give a reason for this	T		

The assessment of understanding will be completed by a research clinician or nurse prior to the completion of the informed consent form.

A maximum of two attempts are allowed.

Outcome			
All questions answered correctly ☐	1 error ☐	2 or more errors ☐	
↓	↓	↓	
CONSENT AND ENROL	Review missed or incorrect question and ensure subject understands the correct answer	Review missed or incorrect questions and ensure subject understands the correct answer	
	↓	Repeat entire informed consent process	
	CONSENT AND ENROL	↙	↘

		All questions answered correctly ☐	1 or more errors ☐
		↓	↓
		CONSENT AND ENROL	DO NOT ENROL

	_ _ / _ _ _ / _ _ _ _
Signature	Date

Appendix 4: Eligibility of participants

Children must meet all of the inclusion criteria and none of the exclusion criteria to be eligible to participate in the trial.

Inclusion criteria

- Each subject being initiated on the study protocol must satisfy the following inclusion criteria at study entry:
- Healthy male or female child at least 24 months of age and less than 60 months of age at the time of study entry.
- Resident in the study area and with no plans to travel outside the study area during the period of subject participation.
- Informed consent for the study participation obtained from a parent (or guardian only if neither parent is alive or if guardianship has been legally transferred (see section 11.2).
- Willingness and capacity to comply with the study protocol as judged by a member of the clinical trial team.

Exclusion criteria

- No subject being initiated on the study protocol may have any of the following exclusion criteria at study entry:
- Serious, active, medical condition, including but not limited to:
- chronic disease of any body system
- severe protein-energy malnutrition (weight-for-height Z-score of less than -3)
- known genetic disorders, such as Down's syndrome or other cytogenetic disorders.
- Active wheezing
- History of documented hypersensitivity to eggs or other components of the vaccine (including gelatin, sorbitol, lactalbumin and chicken protein), or with life-threatening reactions to previous influenza vaccinations.
- History of documented hypersensitivity to macrolide antibiotics
- History of Guillain-Barré syndrome.
- Receipt of aspirin therapy or aspirin-containing therapy within the two weeks before planned study vaccination.
- Any suspected or confirmed congenital or acquired state of immune deficiency including but not limited to primary immunodeficiencies including thymus disorders, HIV/AIDS, hematological or lymphoid malignancies (blood tests will not be routinely undertaken with this regard as part of the study).

- Any current immunosuppressive/immunomodulatory treatment or receipt of any such treatment within the six months preceding trial enrolment (for corticosteroids this is defined as a dose of prednisolone (or equivalent) of greater than 2mg/kg/day for one week or 1mg/kg/day for one month. The use of topical corticosteroids is not an exclusion criterion.
- The use of inhaled corticosteroids within the last one month.
- Receipt of an influenza vaccine within the past 12 months.
- Has any condition determined by investigator as likely to interfere with evaluation of the vaccine or be a significant potential health risk to the child or make it unlikely that the child would complete the study.
- Any significant signs or symptoms of an acute illness or infection including:
- an axillary temperature of 38.0°C or above or documented fever of 38°C or above in the preceding 14 days.
- Any acute respiratory infection within 14 days of enrollment visit.
- If the reason for ineligibility is likely to be temporary (e.g. a fever of 38°C or above or acute respiratory infection) and either will or may resolve before the infant reaches 60 months, they will not be recorded as a screening failure but instead will be re-screened within an appropriate future time-window (e.g. at least 14 days after the last documented fever of 38°C or above or resolution of respiratory illness) and a decision made regarding eligibility at that point.
- If eligibility is confirmed, subjects will be recorded in an enrollment log.
- Those that are not eligible based on one or more exclusion factors will be noted as screening failures on the screening log, with reasons for exclusion documented.

Appendix 5: Screening and Enrolment Log Template

Date of screening (dd/mmm/yyyy)	**Participant's name**	**Participant ID number**[1]	**Screened eligible?** Yes/No/Temporary No[2]	**If ineligible, state reason**	**Randomized** Yes/No	**Group Allocation** A, B, C or D	**Vaccinated or azithromycin given?** Yes/No/NA[3]	**Staff ID**[4]
|__|__|-|__|__|__|-|__|__|__|__|		|__|-|__|__|__|__|				|__|		|__|__|

[1] First digit is blank if not randomized

[2] If temporarily ineligible rescreen in two weeks and complete new line of the log (keep same PID#)

[3] NA if in group C

[4] Of person completing log

Appendix 6: Sensitization Log Template

Date of sensitization (dd/mmm/yyyy)	**Screening/ Sensitization number**	**Name of Child**	**Age of Child** (years)	**Name of parent/guardian**	**Age of mother** (years)	**Contact number(s)**	**Address**	**Outcome of sensitization**[5]	**Staff ID and initials**

[5] Please indicate the relevant number in the column:

1. Interested in taking part in the study
2. Not interested in taking part in the study
3. Not eligible to take part in the study (e.g. maternal age, travel plans)

Appendix 7: Table of median responses for Tfh and AIM panel

Table 10: Median and 95%CI of CXCR3+ICOS+ and CXCR3+ICOS+PD1+Tfh cells pre and post LAIV

<table>
<tr><th>Parameter
(All Tfh)</th><th>Visit</th><th colspan="2">Median</th><th colspan="2">95%CI
Lower</th><th colspan="2">95%CI
Upper</th></tr>
<tr><td>CXCR3+ICOS+</td><td>0</td><td colspan="2">5.915</td><td colspan="2">5.44</td><td colspan="2">6.96</td></tr>
<tr><td>CXCR3+ICOS+</td><td>7</td><td colspan="2">7.235</td><td colspan="2">6.61</td><td colspan="2">8.79</td></tr>
<tr><td>CXCR3+ICOS+</td><td>21</td><td colspan="2">5.975</td><td colspan="2">5.56</td><td colspan="2">6.97</td></tr>
<tr><td>CXCR3+ICOS+PD1</td><td>0</td><td colspan="2">4.725</td><td colspan="2">4.33</td><td colspan="2">5.25</td></tr>
<tr><td>CXCR3+ICOS+PD1</td><td>7</td><td colspan="2">5.465</td><td colspan="2">4.92</td><td colspan="2">6.38</td></tr>
<tr><td>CXCR3+ICOS+PD1</td><td>21</td><td colspan="2">4.555</td><td colspan="2">4.32</td><td colspan="2">5.21</td></tr>
<tr><th colspan="5">Seroconverters n=68</th><th colspan="3">Nonseroconverters n=62</th></tr>
<tr><th>All Tfh</th><th>Visit</th><th>Median</th><th>95%CI
Lower</th><th>95%CI
Upper</th><th>Median</th><th>95%CI
Lower</th><th>95%CI
Upper</th></tr>
<tr><td>CXCR3+ICOS+</td><td>0</td><td>6.01</td><td>5.26</td><td>7.38</td><td>5.915</td><td>5.29</td><td>7.23</td></tr>
<tr><td>CXCR3+ICOS+</td><td>7</td><td>8.39</td><td>7.36</td><td>10.2</td><td>6.33</td><td>5.62</td><td>8.3</td></tr>
<tr><td>CXCR3+ICOS+</td><td>21</td><td>6</td><td>5.45</td><td>7.35</td><td>5.895</td><td>5.34</td><td>7.26</td></tr>
<tr><td>CXCR3+ICOS+PD1</td><td>0</td><td>4.84</td><td>4.02</td><td>5.8</td><td>4.65</td><td>4.23</td><td>5.27</td></tr>
<tr><td>CXCR3+ICOS+PD1</td><td>7</td><td>6.335</td><td>5.31</td><td>7.61</td><td>4.915</td><td>4.42</td><td>6.1</td></tr>
<tr><td>CXCR3+ICOS+PD1</td><td>21</td><td>4.595</td><td>4.18</td><td>5.47</td><td>4.505</td><td>4.21</td><td>5.68</td></tr>
<tr><th colspan="5">IgA Responders n=44</th><th colspan="3">IgA nonresponders n=70</th></tr>
<tr><th>All Tfh</th><th>Visit</th><th>Median</th><th>95%CI
Lower</th><th>95%CI
Upper</th><th>Median</th><th>95%CI
Lower</th><th>95%CI
Upper</th></tr>
<tr><td>CXCR3+ICOS+</td><td>0</td><td>5.67</td><td>5.18</td><td>6.47</td><td>6.365</td><td>5.4</td><td>7.39</td></tr>
<tr><td>CXCR3+ICOS+</td><td>7</td><td>6.93</td><td>5.91</td><td>9.62</td><td>6.915</td><td>6.1</td><td>9.56</td></tr>
<tr><td>CXCR3+ICOS+</td><td>21</td><td>6.365</td><td>5.27</td><td>8.31</td><td>5.755</td><td>5.34</td><td>7.03</td></tr>
<tr><td>CXCR3+ICOS+PD1</td><td>0</td><td>4.605</td><td>4.13</td><td>5.11</td><td>4.86</td><td>4.12</td><td>5.75</td></tr>
<tr><td>CXCR3+ICOS+PD1</td><td>7</td><td>5.335</td><td>4.62</td><td>7.05</td><td>5.265</td><td>4.53</td><td>6.31</td></tr>
<tr><td>CXCR3+ICOS+PD1</td><td>21</td><td>4.735</td><td>3.94</td><td>6.43</td><td>4.395</td><td>4.08</td><td>4.86</td></tr>
</table>

Table 11: Median and 95CI% of bulk Tfh and Tfh subsets pre and post LAIV

Parameter		Median	95CI% Lower	95%CI Upper
All Tfh	V0	8.275	7.41	9.97
All Tfh	V7	8.445	7.83	9.27
All Tfh	V21	8.86	8.15	9.77
Tfh-1	V0	36.1	34.8	39.6
Tfh-1	V7	36.35	32.9	38.7
Tfh-1	V21	35.75	34.6	38.7
Tfh-2	V0	33.25	31.2	35.5
Tfh-2	V7	33.7	32.2	35
Tfh-2	V21	33.15	31.7	36.3
Tfh-17	V0	22.1	21.2	23.8
Tfh-17	V7	22.2	20.5	24.7
Tfh-17	V21	22.35	20.8	23.6
Tfh-1-ICOS+	V0	9.53	7.74	11.4
Tfh-1-ICOS+	V7	10.25	8.96	14.1
Tfh-1-ICOS+	V21	8.605	7.06	9.45
Tfh-2-ICOS+	V0	8.29	6.87	10.4
Tfh-2-ICOS+	V7	8.425	7.45	11
Tfh-2-ICOS+	V21	7.66	6.84	9.19
Tfh-17-ICOS+	V0	7.765	6.82	9.9
Tfh-17-ICOS+	V7	8.455	5.97	10.9
Tfh-17-ICOS+	V21	7.355	6.42	8.32

Seroconverters n=32					Nonseroconverters n=26		
Tfh subsets	**Visit**	**Median**	**95%CI Lower**	**95%CI Upper**	**Median**	**95%CI Lower**	**95%CI Upper**
Tfh-1-ICOS+	0	9.615	7.49	13.6	9.42	6.82	11.7
Tfh-1-ICOS+	7	10.8	9.08	16.2	9.355	7.48	14.1
Tfh-1-ICOS+	21	8.14	7.06	10.3	8.835	5.41	10.3
Tfh-2-ICOS+	0	8.74	6.68	11.3	8.09	6.61	11.3
Tfh-2-ICOS+	7	9.35	7.45	11.7	8.09	4.46	11.7
Tfh-2-ICOS+	21	7.66	6.81	10.3	7.695	4.51	9.19
Tfh-17-ICOS+	0	7.65	6.82	12.8	7.875	6.28	11.4
Tfh-17-ICOS+	7	9.175	6.18	11.6	7.27	4.9	13.7
Tfh-17-ICOS+	21	7.45	5.54	8.4	7.125	5.12	9.34

IgA Responders n=22					IgA nonresponders n=23		
Tfh subsets	**Visit**	**Median**	**95%CI Lower**	**95%CI Upper**	**Median**	**95%CI Lower**	**95%CI Upper**
Tfh-1-ICOS+	0	8.635	5.13	10.7	9.54	7.19	13.6
Tfh-1-ICOS+	7	10.6	7.32	16.2	8.96	8.22	14.2
Tfh-1-ICOS+	21	9.72	5.46	13.1	7.1	6.03	8.97
Tfh-2-ICOS+	0	8.29	5.49	11.3	8.03	6.68	10.1
Tfh-2-ICOS+	7	8.89	4.46	12.9	7.67	6.41	11
Tfh-2-ICOS+	21	9.13	5.09	12.2	6.81	4.41	8.43
Tfh-17-ICOS+	0	8.62	4.15	12.5	7.55	6.7	12.8
Tfh-17-ICOS+	7	8.605	5.37	15	7.9	5.8	10.9
Tfh-17-ICOS+	21	8.055	4.63	12.3	6.42	4.98	8.03

Table 12: Median and 95% CI of antigen specific CD45R0+CXCR5+ Tfh cells pre and post LAIV.

Parameter	Visit	Condition	Median	(95%CI) Lower	(95%CI) Upper
CD25	0	H1	0.375	0.250	0.850
CD25	0	H3	0.300	0.280	0.730
CD25	0	B	0.445	0.340	0.910
CD25	7	H1	0.640	0.550	1.560
CD25	7	H3	0.715	0.580	1.190
CD25	7	B	0.640	0.580	1.370
CD25	21	H1	1.115	0.970	1.950
CD25	21	H3	0.885	0.730	1.500
CD25	21	B	0.660	0.420	1.580
CD25PDL1	0	H1	0.002	0.002	0.072
CD25PDL1	0	H3	0.002	0.002	0.033
CD25PDL1	0	B	0.010	0.002	0.071
CD25PDL1	7	H1	0.071	0.050	0.167
CD25PDL1	7	H3	0.042	0.026	0.150
CD25PDL1	7	B	0.021	0.002	0.120
CD25PDL1	21	H1	0.061	0.044	0.210
CD25PDL1	21	H3	0.043	0.021	0.147
CD25PDL1	21	B	0.028	0.010	0.130
OX40CD25	0	H1	0.055	0.040	0.174
OX40CD25	0	H3	0.037	0.020	0.120
OX40CD25	0	B	0.057	0.049	0.180
OX40CD25	7	H1	0.185	0.140	0.350
OX40CD25	7	H3	0.110	0.090	0.200
OX40CD25	7	B	0.060	0.010	0.254
OX40CD25	21	H1	0.145	0.120	0.356
OX40CD25	21	H3	0.175	0.130	0.380
OX40CD25	21	B	0.096	0.039	0.280
OX40PDL1	0	H1	0.008	0.002	0.046
OX40PDL1	0	H3	0.002	0.002	0.060
OX40PDL1	0	B	0.009	0.002	0.102
OX40PDL1	7	H1	0.002	0.002	0.092
OX40PDL1	7	H3	0.010	0.002	0.100
OX40PDL1	7	B	0.008	0.002	0.140
OX40PDL1	21	H1	0.012	0.002	0.130
OX40PDL1	21	H3	0.023	0.002	0.090
OX40PDL1	21	B	0.002	0.002	0.080
CD25OX40PDL1	0	H1	0.001	0.001	0.001
CD25OX40PDL1	0	H3	0.001	0.001	0.001
CD25OX40PDL1	0	B	0.001	0.001	0.020
CD25OX40PDL1	7	H1	0.001	0.001	0.039
CD25OX40PDL1	7	H3	0.001	0.001	0.050
CD25OX40PDL1	7	B	0.001	0.001	0.037
CD25OX40PDL1	21	H1	0.001	0.001	0.056
CD25OX40PDL1	21	H3	0.001	0.001	0.041
CD25OX40PDL1	21	B	0.001	0.001	0.033

Table 13: Median and 95%CI of antigen specific CD45R0+CXCR5+ Tfh cells pre and post LAIV in seroconverters and nonseroconverters

			Seroconverters n= 32			Nonseroconverters n= 36		
Parameter	**Visit**	**Condition**	**Median**	**95% CI Lower**	**95%CI Upper**	**Median**	**95%CI Lower**	**95%CI Upper**
CD25	0	H1	0.355	0.250	0.990	0.425	0.010	1.060
CD25	0	H3	0.445	0.290	0.990	0.235	0.050	0.740
CD25	0	B	0.445	0.340	1.650	0.395	0.020	0.910
CD25	7	H1	1.035	0.630	3.120	0.535	0.200	1.320
CD25	7	H3	0.880	0.680	1.990	0.445	0.220	1.070
CD25	7	B	0.560	0.270	1.410	0.700	0.470	1.500
CD25	21	H1	1.380	1.140	3.170	0.970	0.360	2.170
CD25	21	H3	0.810	0.590	4.740	0.885	0.510	1.710
CD25	21	B	0.795	0.560	2.420	0.535	0.220	1.270
CD25PDL1	0	H1	0.002	0.002	0.110	0.002	0.002	0.145
CD25PDL1	0	H3	0.002	0.002	0.041	0.002	0.002	0.053
CD25PDL1	0	B	0.005	0.002	0.130	0.011	0.002	0.120
CD25PDL1	7	H1	0.097	0.068	0.390	0.052	0.002	0.113
CD25PDL1	7	H3	0.062	0.020	0.340	0.040	0.002	0.069
CD25PDL1	7	B	0.035	0.020	0.240	0.002	0.002	0.100
CD25PDL1	21	H1	0.078	0.024	0.510	0.058	0.024	0.200
CD25PDL1	21	H3	0.034	0.020	0.350	0.050	0.007	0.140
CD25PDL1	21	B	0.018	0.002	0.270	0.030	0.002	0.160
OX40CD25	0	H1	0.083	0.060	0.310	0.042	0.003	0.174
OX40CD25	0	H3	0.048	0.033	0.230	0.018	0.003	0.120
OX40CD25	0	B	0.059	0.051	0.320	0.020	0.003	0.210
OX40CD25	7	H1	0.261	0.202	0.414	0.085	0.003	0.270
OX40CD25	7	H3	0.110	0.090	0.610	0.095	0.032	0.200
OX40CD25	7	B	0.031	0.003	0.306	0.065	0.003	0.330
OX40CD25	21	H1	0.215	0.121	0.630	0.125	0.048	0.300
OX40CD25	21	H3	0.121	0.067	0.610	0.185	0.130	0.420
OX40CD25	21	B	0.114	0.003	0.530	0.066	0.003	0.330
OX40PDL1	0	H1	0.002	0.002	0.100	0.010	0.002	0.048
OX40PDL1	0	H3	0.002	0.002	0.071	0.003	0.002	0.089
OX40PDL1	0	B	0.015	0.002	0.120	0.002	0.002	0.120
OX40PDL1	7	H1	0.002	0.002	0.120	0.006	0.002	0.100
OX40PDL1	7	H3	0.002	0.002	0.210	0.032	0.002	0.100
OX40PDL1	7	B	0.002	0.002	0.200	0.010	0.002	0.141
OX40PDL1	21	H1	0.011	0.002	0.160	0.014	0.002	0.140
OX40PDL1	21	H3	0.002	0.002	0.160	0.051	0.007	0.100
OX40PDL1	21	B	0.002	0.002	0.170	0.002	0.002	0.080
CD25OX40PDL1	0	H1	0.001	0.001	0.058	0.001	0.001	0.001
CD25OX40PDL1	0	H3	0.001	0.001	0.028	0.001	0.001	0.006
CD25OX40PDL1	0	B	0.001	0.001	0.062	0.001	0.001	0.036
CD25OX40PDL1	7	H1	0.001	0.001	0.070	0.001	0.001	0.043
CD25OX40PDL1	7	H3	0.001	0.001	0.096	0.001	0.001	0.050
CD25OX40PDL1	7	B	0.001	0.001	0.110	0.001	0.001	0.037
CD25OX40PDL1	21	H1	0.001	0.001	0.096	0.001	0.001	0.067
CD25OX40PDL1	21	H3	0.001	0.001	0.078	0.012	0.001	0.050
CD25OX40PDL1	21	B	0.001	0.001	0.055	0.001	0.001	0.050

Table 14: Median and 95%CI of antigen specific CD45R0+CXCR5+ Tfh cells pre and post LAIV in IgA responders and nonresponders

			IgA responder n=21			IgA nonresponder n=43		
Parameter	**Visit**	**Condition**	**Median**	**95% CI Lower**	**95%CI Upper**	**Median**	**95% CI Lower**	**95%CI Upper**
CD25	0	H1	0.010	0.010	0.950	0.390	0.220	1.030
CD25	0	H3	0.310	0.220	1.170	0.290	0.200	0.740
CD25	0	B	0.320	0.190	0.860	0.440	0.260	0.910
CD25	7	H1	1.040	0.550	1.980	0.520	0.410	1.560
CD25	7	H3	0.530	0.260	1.390	0.750	0.580	1.190
CD25	7	B	0.470	0.010	1.380	0.700	0.570	1.410
CD25	21	H1	0.750	0.710	1.920	1.190	0.930	2.630
CD25	21	H3	0.730	0.460	1.910	0.930	0.680	1.830
CD25	21	B	0.650	0.230	2.170	1.060	0.290	1.790
CD25PDL1	0	H1	0.002	0.002	0.150	0.004	0.002	0.110
CD25PDL1	0	H3	0.002	0.002	0.085	0.002	0.002	0.041
CD25PDL1	0	B	0.002	0.002	0.071	0.014	0.002	0.120
CD25PDL1	7	H1	0.091	0.002	0.246	0.070	0.042	0.150
CD25PDL1	7	H3	0.044	0.025	0.200	0.040	0.002	0.154
CD25PDL1	7	B	0.010	0.002	0.100	0.022	0.002	0.153
CD25PDL1	21	H1	0.062	0.025	0.452	0.050	0.020	0.220
CD25PDL1	21	H3	0.020	0.002	0.147	0.057	0.021	0.170
CD25PDL1	21	B	0.040	0.015	0.181	0.026	0.003	0.145
OX40CD25	0	H1	0.030	0.003	0.190	0.080	0.040	0.230
OX40CD25	0	H3	0.077	0.016	0.196	0.030	0.003	0.117
OX40CD25	0	B	0.040	0.013	0.180	0.065	0.050	0.320
OX40CD25	7	H1	0.230	0.071	0.420	0.140	0.080	0.310
OX40CD25	7	H3	0.090	0.066	0.350	0.110	0.080	0.200
OX40CD25	7	B	0.003	0.003	0.250	0.060	0.003	0.320
OX40CD25	21	H1	0.110	0.035	0.440	0.220	0.149	0.440
OX40CD25	21	H3	0.130	0.003	0.380	0.230	0.130	0.540
OX40CD25	21	B	0.040	0.003	0.660	0.140	0.020	0.380
OX40PDL1	0	H1	0.002	0.002	0.130	0.010	0.002	0.048
OX40PDL1	0	H3	0.005	0.002	0.120	0.002	0.002	0.071
OX40PDL1	0	B	0.002	0.002	0.120	0.020	0.002	0.110
OX40PDL1	7	H1	0.002	0.002	0.107	0.002	0.002	0.100
OX40PDL1	7	H3	0.010	0.002	0.125	0.020	0.002	0.100
OX40PDL1	7	B	0.010	0.002	0.170	0.002	0.002	0.141
OX40PDL1	21	H1	0.003	0.002	0.161	0.064	0.002	0.140
OX40PDL1	21	H3	0.020	0.002	0.147	0.036	0.002	0.140
OX40PDL1	21	B	0.002	0.002	0.110	0.029	0.002	0.130
CD25OX40PDL1	0	H1	0.001	0.001	0.002	0.001	0.001	0.028
CD25OX40PDL1	0	H3	0.001	0.001	0.031	0.001	0.001	0.006
CD25OX40PDL1	0	B	0.001	0.001	0.001	0.001	0.001	0.049
CD25OX40PDL1	7	H1	0.001	0.001	0.072	0.001	0.001	0.039
CD25OX40PDL1	7	H3	0.001	0.001	0.073	0.001	0.001	0.053
CD25OX40PDL1	7	B	0.001	0.001	0.037	0.001	0.001	0.048
CD25OX40PDL1	21	H1	0.001	0.001	0.060	0.001	0.001	0.088
CD25OX40PDL1	21	H3	0.024	0.001	0.060	0.001	0.001	0.054
CD25OX40PDL1	21	B	0.001	0.001	0.071	0.001	0.001	0.050

Table 15: Median and 95%CI of antigen specific CD45R0+CXCR5+ Tfh cells pre and post LAIV in children with or without pre-existing responses to H1, H3 and B HA influenza antigens

Parameter	Visit	Condition	Median	95% CI Lower	95%CI Upper	Median	95% CI Lower	95%CI Upper
CD25	0	H1	0.705	0.410	1.130	0.090	0.010	0.880
CD25	0	H3	0.410	0.200	0.740	0.280	0.230	1.660
CD25	0	B	0.620	0.450	1.290	0.240	0.120	0.860
CD25	7	H1	0.750	0.520	1.990	0.565	0.380	1.570
CD25	7	H3	0.780	0.380	1.190	0.580	0.480	1.990
CD25	7	B	0.725	0.580	1.500	0.510	0.220	1.380
CD25	21	H1	0.660	0.210	1.950	1.470	1.140	2.710
CD25	21	H3	0.810	0.540	1.830	0.920	0.870	4.740
CD25	21	B	0.255	0.010	1.290	1.020	0.560	2.320
CD25PDL1	0	H1	0.032	0.002	0.150	0.002	0.002	0.053
CD25PDL1	0	H3	0.002	0.002	0.041	0.002	0.002	0.085
CD25PDL1	0	B	0.030	0.002	0.159	0.002	0.002	0.091
CD25PDL1	7	H1	0.086	0.042	0.220	0.049	0.002	0.236
CD25PDL1	7	H3	0.044	0.020	0.123	0.026	0.002	0.540
CD25PDL1	7	B	0.046	0.002	0.176	0.002	0.002	0.200
CD25PDL1	21	H1	0.038	0.020	0.210	0.103	0.059	0.452
CD25PDL1	21	H3	0.035	0.002	0.125	0.051	0.029	0.650
CD25PDL1	21	B	0.004	0.002	0.160	0.036	0.015	0.181
OX40CD25	0	H1	0.096	0.043	0.310	0.020	0.003	0.150
OX40CD25	0	H3	0.033	0.003	0.120	0.056	0.030	0.490
OX40CD25	0	B	0.083	0.013	0.405	0.045	0.003	0.177
OX40CD25	7	H1	0.196	0.080	0.420	0.174	0.030	0.360
OX40CD25	7	H3	0.090	0.032	0.200	0.126	0.108	0.530
OX40CD25	7	B	0.112	0.020	0.350	0.003	0.003	0.254
OX40CD25	21	H1	0.135	0.067	0.356	0.185	0.120	1.120
OX40CD25	21	H3	0.230	0.119	0.380	0.130	0.020	1.187
OX40CD25	21	B	0.056	0.003	0.280	0.129	0.039	0.446
OX40PDL1	0	H1	0.015	0.002	0.100	0.002	0.002	0.046
OX40PDL1	0	H3	0.002	0.002	0.054	0.002	0.002	0.140
OX40PDL1	0	B	0.011	0.002	0.120	0.002	0.002	0.120
OX40PDL1	7	H1	0.011	0.002	0.150	0.002	0.002	0.100
OX40PDL1	7	H3	0.030	0.002	0.087	0.002	0.002	0.210
OX40PDL1	7	B	0.023	0.002	0.160	0.002	0.002	0.170
OX40PDL1	21	H1	0.022	0.002	0.130	0.002	0.002	0.156
OX40PDL1	21	H3	0.020	0.002	0.072	0.025	0.002	0.220
OX40PDL1	21	B	0.002	0.002	0.090	0.033	0.002	0.130
CD25OX40PDL1	0	H1	0.001	0.001	0.058	0.001	0.001	0.001
CD25OX40PDL1	0	H3	0.001	0.001	0.001	0.001	0.001	0.028
CD25OX40PDL1	0	B	0.001	0.001	0.088	0.001	0.001	0.025
CD25OX40PDL1	7	H1	0.001	0.001	0.057	0.001	0.001	0.050
CD25OX40PDL1	7	H3	0.001	0.001	0.050	0.001	0.001	0.100
CD25OX40PDL1	7	B	0.001	0.001	0.063	0.001	0.001	0.049
CD25OX40PDL1	21	H1	0.001	0.001	0.059	0.001	0.001	0.130
CD25OX40PDL1	21	H3	0.001	0.001	0.045	0.001	0.001	0.140
CD25OX40PDL1	21	B	0.001	0.001	0.027	0.001	0.001	0.055

Table 16: Median and 95%CI of antigen specific CD45R0+CXCR5+ CXCR3+ Tfh cells pre and post LAIV

Parameter	Visit	Condition	Median	(95%CI) Lower	(95%CI) Upper
CD25	0	H1	0.150	0.090	0.660
CD25	0	H3	0.020	0.015	0.450
CD25	0	B	0.060	0.020	0.670
CD25	7	H1	0.380	0.210	0.940
CD25	7	H3	0.260	0.140	0.630
CD25	7	B	0.280	0.170	0.800
CD25	21	H1	0.690	0.500	1.270
CD25	21	H3	0.460	0.350	0.850
CD25	21	B	0.090	0.050	0.670
CD25PDL1	0	H1	0.002	0.002	0.002
CD25PDL1	0	H3	0.002	0.002	0.011
CD25PDL1	0	B	0.002	0.002	0.070
CD25PDL1	7	H1	0.002	0.002	0.120
CD25PDL1	7	H3	0.002	0.002	0.042
CD25PDL1	7	B	0.002	0.002	0.002
CD25PDL1	21	H1	0.002	0.002	0.130
CD25PDL1	21	H3	0.002	0.002	0.040
CD25PDL1	21	B	0.002	0.002	0.077
OX40CD25	0	H1	0.021	0.003	0.140
OX40CD25	0	H3	0.003	0.003	0.037
OX40CD25	0	B	0.003	0.003	0.110
OX40CD25	7	H1	0.013	0.003	0.240
OX40CD25	7	H3	0.050	0.003	0.180
OX40CD25	7	B	0.003	0.003	0.180
OX40CD25	21	H1	0.088	0.061	0.320
OX40CD25	21	H3	0.091	0.040	0.260
OX40CD25	21	B	0.016	0.003	0.230
OX40PDL1	0	H1	0.003	0.003	0.060
OX40PDL1	0	H3	0.003	0.003	0.070
OX40PDL1	0	B	0.003	0.003	0.083
OX40PDL1	7	H1	0.003	0.003	0.120
OX40PDL1	7	H3	0.003	0.003	0.086
OX40PDL1	7	B	0.003	0.003	0.050
OX40PDL1	21	H1	0.003	0.003	0.120
OX40PDL1	21	H3	0.003	0.003	0.100
OX40PDL1	21	B	0.003	0.003	0.050

Table 17: Median and 95%CI of antigen specific CD45R0+CXCR5+ CXCR3+ Tfh cells pre and post LAIV in seroconverters and non seroconverters

			Seroconverters n=31			Nonseroconverters n=33		
Parameter	Visit	Condition	Median	(95%CI) Lower	(95%CI) Upper	Median	(95%CI) Lower	(95%CI) Upper
CD25	0	H1	0.190	0.080	0.920	0.115	0.015	0.660
CD25	0	H3	0.130	0.015	0.770	0.018	0.015	0.350
CD25	0	B	0.300	0.020	1.050	0.045	0.015	0.750
CD25	7	H1	0.790	0.200	2.370	0.250	0.050	0.610
CD25	7	H3	0.170	0.100	1.610	0.290	0.070	0.580
CD25	7	B	0.170	0.070	1.010	0.290	0.160	0.980
CD25	21	H1	0.880	0.480	2.810	0.590	0.240	1.110
CD25	21	H3	0.460	0.300	1.830	0.445	0.270	0.770
CD25	21	B	0.290	0.090	1.790	0.023	0.015	0.370
CD25PDL1	0	H1	0.002	0.002	0.120	0.002	0.002	0.030
CD25PDL1	0	H3	0.002	0.002	0.170	0.002	0.002	0.002
CD25PDL1	0	B	0.002	0.002	0.140	0.002	0.002	0.051
CD25PDL1	7	H1	0.002	0.002	0.320	0.002	0.002	0.094
CD25PDL1	7	H3	0.002	0.002	0.290	0.002	0.002	0.073
CD25PDL1	7	B	0.002	0.002	0.013	0.002	0.002	0.032
CD25PDL1	21	H1	0.002	0.002	0.330	0.034	0.002	0.139
CD25PDL1	21	H3	0.002	0.002	0.280	0.002	0.002	0.020
CD25PDL1	21	B	0.002	0.002	0.230	0.002	0.002	0.076
OX40CD25	0	H1	0.060	0.003	0.270	0.003	0.003	0.140
OX40CD25	0	H3	0.003	0.003	0.120	0.003	0.003	0.007
OX40CD25	0	B	0.038	0.003	0.260	0.003	0.003	0.060
OX40CD25	7	H1	0.065	0.003	0.550	0.008	0.003	0.200
OX40CD25	7	H3	0.020	0.003	0.300	0.064	0.003	0.200
OX40CD25	7	B	0.003	0.003	0.210	0.081	0.003	0.270
OX40CD25	21	H1	0.090	0.020	0.621	0.075	0.003	0.330
OX40CD25	21	H3	0.091	0.003	0.360	0.085	0.003	0.230
OX40CD25	21	B	0.120	0.003	0.430	0.003	0.003	0.150
OX40PDL1	0	H1	0.003	0.003	0.230	0.003	0.003	0.053
OX40PDL1	0	H3	0.003	0.003	0.133	0.003	0.003	0.069
OX40PDL1	0	B	0.003	0.003	0.160	0.003	0.003	0.083
OX40PDL1	7	H1	0.065	0.003	0.297	0.003	0.003	0.060
OX40PDL1	7	H3	0.003	0.003	0.180	0.003	0.003	0.098
OX40PDL1	7	B	0.003	0.003	0.200	0.003	0.003	0.003
OX40PDL1	21	H1	0.003	0.003	0.210	0.003	0.003	0.130
OX40PDL1	21	H3	0.003	0.003	0.204	0.003	0.003	0.076
OX40PDL1	21	B	0.003	0.003	0.130	0.003	0.003	0.104

Table 18: Median and 95%CI of antigen specific CD45R0+CXCR5+ CXCR3+ Tfh cells pre and post LAIV in IgA responders and nonresponders

			IgA responder n=20			IgA nonresponder n=41		
Parameter	**Visit**	**Condition**	**IgA responder Median**	**(95%CI) Lower**	**(95%CI) Upper**	**IgA nonresponder Median**	**(95%CI) Lower**	**(95%CI) Upper**
CD25	0	H1	0.030	0.015	0.800	0.170	0.080	0.690
CD25	0	H3	0.290	0.120	0.800	0.015	0.015	0.450
CD25	0	B	0.020	0.015	1.050	0.300	0.015	0.670
CD25	7	H1	0.230	0.050	1.190	0.360	0.160	1.030
CD25	7	H3	0.140	0.050	0.950	0.270	0.140	0.730
CD25	7	B	0.190	0.015	1.010	0.300	0.070	0.980
CD25	21	H1	0.560	0.100	1.540	0.690	0.480	1.520
CD25	21	H3	0.410	0.240	1.270	0.520	0.350	0.850
CD25	21	B	0.090	0.015	1.160	0.070	0.015	0.890
CD25PDL1	0	H1	0.002	0.002	0.063	0.002	0.002	0.053
CD25PDL1	0	H3	0.002	0.002	0.018	0.002	0.002	0.011
CD25PDL1	0	B	0.002	0.002	0.070	0.002	0.002	0.095
CD25PDL1	7	H1	0.002	0.002	0.170	0.002	0.002	0.134
CD25PDL1	7	H3	0.002	0.002	0.099	0.002	0.002	0.074
CD25PDL1	7	B	0.002	0.002	0.110	0.002	0.002	0.002
CD25PDL1	21	H1	0.002	0.002	0.139	0.030	0.002	0.190
CD25PDL1	21	H3	0.002	0.002	0.034	0.002	0.002	0.140
CD25PDL1	21	B	0.002	0.002	0.170	0.002	0.002	0.149
OX40CD25	0	H1	0.003	0.003	0.140	0.060	0.003	0.170
OX40CD25	0	H3	0.003	0.003	0.120	0.003	0.003	0.007
OX40CD25	0	B	0.003	0.003	0.098	0.020	0.003	0.180
OX40CD25	7	H1	0.003	0.003	0.200	0.012	0.003	0.280
OX40CD25	7	H3	0.068	0.003	0.260	0.050	0.003	0.150
OX40CD25	7	B	0.003	0.003	0.270	0.003	0.003	0.180
OX40CD25	21	H1	0.090	0.003	0.480	0.088	0.020	0.460
OX40CD25	21	H3	0.083	0.003	0.310	0.091	0.003	0.270
OX40CD25	21	B	0.029	0.003	0.340	0.003	0.003	0.230
OX40PDL1	0	H1	0.003	0.003	0.160	0.003	0.003	0.053
OX40PDL1	0	H3	0.003	0.003	0.110	0.003	0.003	0.081
OX40PDL1	0	B	0.003	0.003	0.240	0.003	0.003	0.083
OX40PDL1	7	H1	0.003	0.003	0.120	0.050	0.003	0.140
OX40PDL1	7	H3	0.003	0.003	0.190	0.003	0.003	0.083
OX40PDL1	7	B	0.003	0.003	0.040	0.003	0.003	0.080
OX40PDL1	21	H1	0.003	0.003	0.230	0.003	0.003	0.140
OX40PDL1	21	H3	0.003	0.003	0.240	0.003	0.003	0.134
OX40PDL1	21	B	0.009	0.003	0.370	0.003	0.003	0.023

Table 19: Median and 95%CI of antigen specific CD45R0+CXCR5+ CXCR3+ Tfh cells pre and post LAIV in baseline responders and nonresponders

Parameter	Visit	Condition	Median Baseline responders	(95% CI) Lower	(95% CI) Upper	Median Baseline nonresponders	(95%CI) Lower	(95%CI) Upper
CD251	0	H1	0.315	0.090	0.820	0.030	0.015	0.670
CD252	0	H3	0.020	0.015	0.350	0.073	0.015	0.860
CD253	0	B	0.340	0.020	0.800	0.028	0.015	0.730
CD251	7	H1	0.455	0.230	1.250	0.130	0.015	0.990
CD252	7	H3	0.300	0.130	0.730	0.120	0.050	0.750
CD253	7	B	0.280	0.190	1.000	0.100	0.015	0.980
CD251	21	H1	0.440	0.120	1.540	0.840	0.690	1.520
CD252	21	H3	0.410	0.240	0.740	0.470	0.390	1.410
CD253	21	B	0.015	0.015	0.520	0.250	0.090	1.240
CD25PDL11	0	H1	0.002	0.002	0.120	0.002	0.002	0.002
CD25PDL12	0	H3	0.002	0.002	0.007	0.002	0.002	0.130
CD25PDL13	0	B	0.002	0.002	0.032	0.002	0.002	0.100
CD25PDL11	7	H1	0.002	0.002	0.150	0.002	0.002	0.140
CD25PDL12	7	H3	0.002	0.002	0.006	0.002	0.002	0.360
CD25PDL13	7	B	0.002	0.002	0.072	0.002	0.002	0.002
CD25PDL11	21	H1	0.002	0.002	0.130	0.002	0.002	0.240
CD25PDL12	21	H3	0.002	0.002	0.020	0.002	0.002	0.260
CD25PDL13	21	B	0.002	0.002	0.149	0.002	0.002	0.160
OX40CD251	0	H1	0.058	0.003	0.220	0.003	0.003	0.140
OX40CD252	0	H3	0.003	0.003	0.030	0.003	0.003	0.170
OX40CD253	0	B	0.020	0.003	0.200	0.003	0.003	0.100
OX40CD251	7	H1	0.107	0.003	0.320	0.003	0.003	0.240
OX40CD252	7	H3	0.100	0.003	0.210	0.003	0.003	0.300
OX40CD253	7	B	0.020	0.003	0.240	0.003	0.003	0.190
OX40CD251	21	H1	0.069	0.003	0.240	0.110	0.020	0.650
OX40CD252	21	H3	0.083	0.003	0.230	0.133	0.040	0.360
OX40CD253	21	B	0.003	0.003	0.160	0.090	0.003	0.390
OX40PDL11	0	H1	0.003	0.003	0.140	0.003	0.003	0.077
OX40PDL12	0	H3	0.003	0.003	0.069	0.003	0.003	0.170
OX40PDL13	0	B	0.003	0.003	0.036	0.003	0.003	0.180
OX40PDL11	7	H1	0.003	0.003	0.120	0.003	0.003	0.160
OX40PDL12	7	H3	0.003	0.003	0.086	0.003	0.003	0.300
OX40PDL13	7	B	0.003	0.003	0.082	0.003	0.003	0.108
OX40PDL11	21	H1	0.003	0.003	0.130	0.003	0.003	0.210
OX40PDL12	21	H3	0.003	0.003	0.095	0.003	0.003	0.204
OX40PDL13	21	B	0.003	0.003	0.023	0.003	0.003	0.130

Appendix 8: Publication

Effect of a Russian-backbone live-attenuated influenza vaccine with an updated pandemic H1N1 strain on shedding and immunogenicity among children in The Gambia: an open-label, observational, phase 4 study

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Summary

Background The efficacy and effectiveness of the pandemic H1N1 (pH1N1) component in live attenuated influenza vaccine (LAIV) is poor. The reasons for this paucity are unclear but could be due to impaired replicative fitness of pH1N1 A/California/07/2009-like (Cal09) strains. We assessed whether an updated pH1N1 strain in the Russian-backbone trivalent LAIV resulted in greater shedding and immunogenicity compared with LAIV with Cal09.

Methods We did an open-label, prospective, observational, phase 4 study in Sukuta, a periurban area in The Gambia. We enrolled children aged 24–59 months who were clinically well. Children received one dose of the WHO pre-qualified Russian-backbone trivalent LAIV containing either A/17/California/2009/38 (Cal09) or A/17/New York/15/5364 (NY15) based on their year of enrolment. Primary outcomes were the percentage of children with LAIV strain shedding at day 2 and day 7, haemagglutinin inhibition seroconversion, and an increase in influenza haemagglutinin-specific IgA and T-cell responses at day 21 after LAIV. This study is nested within a randomised controlled trial investigating LAIV–microbiome interactions (NCT02972957).

Findings Between Feb 8, 2017, and April 12, 2017, 118 children were enrolled and received one dose of the Cal09 LAIV from 2016–17. Between Jan 15, 2018, and March 28, 2018, a separate cohort of 135 children were enrolled and received one dose of the NY15 LAIV from 2017–18, of whom 126 children completed the study. Cal09 showed impaired pH1N1 nasopharyngeal shedding (16 of 118 children [14%, 95% CI 8·0–21·1] with shedding at day 2 after administration of LAIV) compared with H3N2 (54 of 118 [46%, 36·6–55·2]; p<0·0001) and influenza B (95 of 118 [81%, 72·2–87·2]; p<0·0001), along with suboptimal serum antibody (seroconversion in six of 118 [5%, 1·9–10·7]) and T-cell responses (CD4+ interferon γ-positive and/or CD4+ interleukin 2-positive responses in 45 of 111 [41%, 31·3–50·3]). After the switch to NY15, a significant increase in pH1N1 shedding was seen (80 of 126 children [63%, 95% CI 54·4–71·9]; p<0·0001 compared with Cal09), along with improvements in seroconversion (24 of 126 [19%, 13·2–26·8]; p=0·011) and influenza-specific CD4+ T-cell responses (73 of 111 [66%, 60·0–75·6; p=0·00028]). The improvement in pH1N1 seroconversion with NY15 was even greater in children who were seronegative at baseline (24 of 64 children [38%, 95% CI 26·7–49·8] *vs* six of 79 children with Cal09 [8%, 2·8–15·8]; p<0·0001). Persistent shedding to day 7 was independently associated with both seroconversion (odds ratio 12·69, 95% CI 4·1–43·6; p<0·0001) and CD4+ T-cell responses (odds ratio 7·83, 95% CI 2·99–23·5; p<0·0001) by multivariable logistic regression.

Interpretation The pH1N1 component switch that took place between 2016 and 2018 might have overcome the poor efficacy and effectiveness reported with previous LAIV formulations. LAIV effectiveness against pH1N1 should, therefore, improve in upcoming influenza seasons. Our data highlight the importance of assessing replicative fitness, in addition to antigenicity, when selecting annual LAIV components.

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See **Comment** page 641

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Introduction

Live attenuated influenza vaccine (LAIV) has been highly efficacious against prepandemic seasonal H1N1 viruses in children, with a meta-analysis[1] estimating a pooled efficacy of 85% from several randomised controlled trials. However, concerns have been expressed about protection against pandemic H1N1 (pH1N1) influenza using LAIV. Since 2009, when pH1N1 viruses have circulated as the main seasonal H1N1 strain, vaccine effectiveness of the Ann Arbor-backbone LAIV against

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Research in context

Evidence before this study

We searched Embase, MEDLINE, Global health, and Web of Science databases up to May 9, 2018, with the terms: ("influenza" OR "flu") AND ("vaccin*" OR "immuni#ation" OR "Influenza Vaccines [Subject heading]") AND ("effic*" OR "effect*" OR "immune*" OR "respons*" OR "protect*") AND ("Africa" OR "Africa [Subject heading]" OR each African country [defined by the UN]). This search strategy identified no live attenuated influenza vaccine (LAIV) immunogenicity studies and only two LAIV efficacy studies in African children. The first study was of the Ann-Arbor LAIV in 2001–02, when pre-pandemic H1N1 was circulating. This study was a multicentre randomised placebo-controlled trial that included 277 children aged 6–36 months from South Africa. The efficacy of LAIV in this subset of children was 87% (95% CI 64–95). The second study was a single-centre, randomised, placebo-controlled trial of Russian-backbone LAIV in children aged 2–5 years in Senegal. The study was done in 2013 and, therefore, included pandemic H1N1 (pH1N1). The efficacy against vaccine-matched isolates was −6·1% (95% CI −50·0 to 25·0), and pH1N1 was the predominant circulating strain. In the 2017–18 season, the pH1N1 component in LAIV was updated for the first time in both the Ann Arbor and Russian-backbone LAIVs, with A/Michigan/45/2015-like strains. No studies have been published about whether this change has affected the shedding and immunogenicity of pH1N1 in LAIV.

Added value of this study

Our findings show that A/17/California/2009/38 pH1N1 strain shedding and immunogenicity is less than that of H3N2 and influenza B in the Russian-backbone LAIV, providing an explanation for the lack of efficacy seen in the randomised controlled trial in Senegal. Our data suggest this observation is not attributable to reduced pH1N1 vaccine take secondary to pre-existing immune responses. Our findings show for the first time that updating the Russian-backbone LAIV pH1N1 component has resulted in a vaccine with significantly greater nasopharyngeal shedding, seroconversion, and influenza-specific T-cell induction to pH1N1. We are able to model this difference in replicative ability of old and new pH1N1 LAIV components in vitro.

Implications of all the available evidence

Impaired replicative ability of pH1N1 components in LAIV might have caused recent low efficacy and effectiveness of LAIV. An improvement in protection against pH1N1 can be expected in the future. These data highlight the importance of assessing viral replicative fitness in addition to antigenicity when selecting vaccine formulations. Studies are needed to ascertain whether improved shedding and immunogenicity translates into improved efficacy and effectiveness. Further research is needed to understand the genetic factors that underlie these phenotypes in vaccine strains to design more rational choices of vaccine antigens for LAIV.

pH1N1 in the USA has been low, ranging from −21% in 2015–16 to 17% in 2013–14.[2] This reduction in effectiveness resulted in the Advisory Committee on Immunisation Practices removing their recommendation for LAIV use in 2016.[3]

A randomised controlled trial of Russian-backbone LAIV (Nasovac-S; Serum Institute of India Pvt, Pune, India) among children aged 2–5 years in Senegal did not show efficacy (0·0%, 95% CI −26·4 to 20·9) in 2013, when pH1N1 was the predominant circulating vaccine-matched virus.[4] Both LAIV formulations in these studies contained haemagglutinin and neuraminidase from pH1N1 A/California/07/2009-like (Cal09) viruses. It is unclear why protection conferred by the pH1N1 component in LAIV has been suboptimal. Potential reasons include pre-existing immunity, poor viral replicative fitness, or competition from other co-formulated strains—all limiting pH1N1 take and immunogenicity.[3]

The Russian-backbone LAIV (Nasovac-S) was granted a WHO prequalification certificate in 2015, opening up the potential for use in low-income and middle-income countries. The findings in Senegal with this vaccine are especially pertinent because the burden of influenza in Africa is high; influenza-related admissions to hospital in children younger than 5 years are approximately threefold higher than in Europe.[5] Superior efficacy of LAIV over inactivated influenza vaccine in young children (predominantly in high-income settings),[1] needle-free delivery, and lower manufacturing costs make LAIV an attractive option to tackle this burden in Africa.[1] However, few LAIV studies have been done in African cohorts and no published immunogenicity data are available from African children to date.[6] In particular, the absence of immunological endpoints from the randomised controlled trial in Senegal makes it difficult to understand the reasons for the lack of efficacy recorded.[4]

In 2017–18, the pH1N1 Cal09 strain (A/17/California/2009/38) was updated according to WHO recommendations to an A/Michigan/45/2015-like strain (A/17/New York/15/5364 [NY15]), following antigenic drift. This first-ever recommended update to pH1N1 provided a unique opportunity to compare replicative ability and immunogenicity of these two pH1N1 strains. To understand how differences in strain shedding and immunogenicity might account for the findings of the Senegal trial, we compared one cohort of influenza vaccine-naive Gambian children vaccinated with the Russian-backbone Cal09 LAIV formulation from 2016–17 with a second cohort vaccinated with the NY15 LAIV formulation from 2017–18.

Methods

Study design and participants

We did an open-label, prospective, observational, phase 4 immunogenicity study in Sukuta, a periurban area in The Gambia. Our study is nested within a larger randomised trial comparing microbiome changes in children assigned LAIV with changes in unvaccinated children (NCT02972957; appendix pp 3, 4). Data in our study are from all children enrolled in the randomised trial who were given LAIV. After community sensitisation, parents expressing an interest in the randomised study were invited for consent discussions. Eligible children had to be aged 24–59 months and clinically well, with no history of respiratory illness within the past 14 days (appendix p 2).

This study was approved by The Gambia Government and UK Medical Research Council (MRC) joint ethics committee and the Medicines Control Agency of The Gambia, and it was done according to International Conference on Harmonisation Good Clinical Practice standards. A parent provided written or thumbprinted informed consent for their child or children to participate. If parents were not English literate, an impartial witness was present throughout the informed consent discussion undertaken in a local language, who signed to confirm completeness of the consent provided.

Procedures

When LAIV was updated in 2017–18, haemagglutinin and neuraminidase from pH1N1 Cal09 were replaced with those from NY15, whereas identical H3N2 (A/17/Hong Kong/2014/8296) and B/Vic (B/Texas/02/2013 [Victoria lineage]) strains were used. Vaccine titres per dose (50% egg infectious dose equivalents [EID50eq] per mL) were $1 \times 10^{8\cdot0}$ for pH1N1, $1 \times 10^{7\cdot5}$ for H3N2, $1 \times 10^{7\cdot2}$ for B/Vic in the 2016–17 LAIV and $1 \times 10^{7\cdot7}$ for pH1N1, $1 \times 10^{7\cdot6}$ for H3N2, $1 \times 10^{7\cdot3}$ for B/Vic in the 2017–18 LAIV.

The study was done outside the peak influenza transmission season (June to October) based on surveillance data from Senegal and unpublished data from studies in The Gambia.[7] Children received one dose of intranasal trivalent Russian-backbone LAIV (Nasovac-S; northern hemisphere formulation) in either 2017 (the Cal09 strain from 2016–17) or 2018 (the NY15 strain from 2017–18 formulation). Children received the vaccine formulation that corresponded with their year of enrolment.

Nasopharyngeal swabs were taken before vaccination (day 0), on day 2, and on day 7 using flocked swabs (FLOQSwabs; Copan, Murrieta, CA, USA). We obtained buccal cavity oral fluid with swabs (Oracol Plus; Malvern Medical Development, Worcester, UK) on day 0 and day 21. Whole blood samples were obtained for flow cytometry and serum separation on day 0 and day 21. We chose day 21 to measure vaccine response, in line with previous work.[8–10] Nasopharyngeal swabs, oral fluid, and serum samples were stored at −70°C before further processing.

Haemagglutinin inhibition assays were done according to standard methods,[11] using vaccine haemagglutinin-matched and neuraminidase-matched viruses. Seroconversion was defined as a fourfold or greater titre increase (to ≥1:40) from day 0 to day 21. Total and influenza haemagglutinin-specific IgA in oral fluid was detected using a previously described ELISA,[12] using recombinant vaccine-matched haemagglutinin. Samples were assayed at dilutions ranging from 1:1000 to 1:20000 for total IgA and from undiluted to 1:16 for influenza-specific IgA, and samples were quantified using an IgA standard curve. Undiluted samples with influenza-specific IgA below the limit of quantitation (LOQ) were assigned LOQ values. We calculated the fold change in the proportion of influenza-specific IgA to total IgA from day 0 to day 21. A twofold increase was considered a significant response.[13]

See Online for appendix

T-cell responses were quantified by stimulating fresh whole blood (200 µL) on the day of collection for 18 h with overlapping 15–18-mer peptide pools (2 µg/mL) covering vaccine-matched whole haemagglutinin, matrix and nucleoprotein, and co-stimulatory antibodies (antiCD28 and antiCD49; BD Biosciences, Franklin Lakes, NJ, USA). Influenza B responses were measured in 2018 only. We did intracellular cytokine staining for interferon (IFN)γ and interleukin (IL)2 and analysed cells with a flow cytometer (LSR Fortessa; BD Biosciences; appendix pp 5, 6).[14] Responses in negative controls (antiCD28 and antiCD49) were subtracted from peptide-stimulated conditions before further analysis; negative values were set to zero. To avoid systematic bias in adjusting for negative values alone, we set a threshold (based on the distribution of negative values; appendix p 7) below which all positive values were also considered a non-response, as described previously.[14,15] In analyses calculating the fold change from day 0 to day 21, null responses were assigned a value halfway between zero and this threshold. A twofold increase after LAIV was considered a significant response.

Vaccine shedding from nasopharyngeal swabs was assessed with monoplex reverse-transcriptase PCR (RT-PCR) using haemagglutinin-specific primers and probes (appendix p 8). In 2018, fully quantitative RT-PCR results were obtained by inclusion of a standard curve with known vaccine titres ($\log_{10}$EID50eq per mL; appendix p 9). RT-PCR assays with primers and probes mapping to internal genes were used to distinguish LAIV from seasonal influenza viruses (appendix p 8).[16] Despite optimisation of assay conditions, maximum LAIV dilutions detected by LAIV-specific RT-PCR were at least one $\log_{10}$ lower than those detected by haemagglutinin-specific RT-PCR (appendix p 10). Therefore, only samples with cycle threshold (ct) values of 30 or lower in seasonal influenza assays were tested

with LAIV-specific assays, with 100% confirmed as LAIV strains.

Primary human nasal epithelial cell cultures (MucilAir; Epithelix Sàrl, Geneva, Switzerland) were used for in vitro viral replication experiments. Madin-Darby Canine Kidney (MDCK) cells (ATCC, Manassas, VA, USA) and MDCK-SIAT cells (WHO Collaborating Centre for Reference and Research on Influenza, London, UK) were maintained at 37°C with 5% CO_2 in Dulbecco's modified Eagle's Medium (DMEM; Gibco-Life Technologies, Waltham, MA, USA) supplemented with 10% fetal bovine serum, 1% penicillin–streptomycin, and 1% non-essential amino acids. We also added 1 mg/mL G418 (Gibco-Life Technologies) for MDCK-SIAT cells. Viral stocks of Nasovac-S monovalent forms were titrated by plaque assay at 32°C on MDCK cells (for pH1N1 and influenza B) or MDCK-SIAT cells (for H3N2). Apical surfaces of human nasal epithelial cells were inoculated with each monovalent virus (multiplicity of infection 0·01 plaque-forming units per cell) for 1 h at 32°C and 5% CO_2 in triplicate. The inoculum was removed and the apical surface of the human nasal epithelial cells was washed with DMEM before incubation at 32°C. At indicated timepoints (days 1–6 after inoculation), DMEM was added to the human nasal epithelial cells and incubated for 30 min, then it was removed and stored; the stored DMEM—containing virions from the epithelial cell cultures—was titrated by plaque assay. Experiments were done on two separate occasions using cells from different donors.

For more on SciPy see http://www.scipy.org/

To assess the acid stability of pH1N1 strains, Cal09 or NY15 were mixed with pH-adjusted MES (2-[N-morpholino] ethanesulphonic acid) buffer (100 mmol/L MES, 150 mol/L sodium chloride, 0·9 mol/L calcium chloride, 0·5 mol/L magnesium chloride) in triplicate (1:10 dilution) and the mixture was incubated for 15 min at room temperature. The buffer was inactivated with DMEM and infectious virus was titrated by plaque assay.

For more on R see https://www.r-project.org/

Outcomes

Primary shedding and immunogenicity outcomes were the percentage of children with LAIV strain shedding at day 2 and day 7, haemagglutinin inhibition seroconversion, and an increase in influenza haemagglutinin-specific IgA and T-cell responses at day 21 after LAIV.

Statistical analysis

The sample size calculation was based on LAIV microbiome endpoints not presented here (appendix p 3). Differences in unpaired proportions (shedding, seroconversion, IgA responses, and T-cell responses) between years (2017 and 2018) were assessed with either the χ^2 test or Fisher's exact test. Differences in continuous variables (ct value, log_{10}EID50eq per mL, and geometric mean fold change in haemagglutinin inhibition) between years (2017 and 2018) or serostatus (positive or negative) were assessed with either the unpaired *t* test or Mann-Whitney *U* test. Differences in viral load (log_{10}EID50eq per mL) between strains (NY15, H3N2, and influenza B) within the same visit (paired data) were assessed with the Friedman test (with Dunn's post-test for multiple comparisons). Pairwise viral load correlations were assessed using Spearman's rank-order correlation (r_s). Correlation coefficients were interpreted as low (r_s=0·30–0·49), moderate (r_s=0·50–0·69), high (r_s=0·70–0·89), or very high (r_s=0·90–1·00). The Wilcoxon signed-rank test was used to compare T-cell responses before and after vaccination. Separate logistic regression analyses were done for dependent variables (shedding, seroconversion, T-cell responses, and IgA responses). Independent variables were selected for multivariable logistic regression models if biologically relevant and the p value from univariable regression was less than 0·2. Each multivariable logistic regression model always included year, age, and sex as potential confounders. Viral loads were ascertained from standard curves using Python version 3.6 (SciPy package). In vitro viral replication was quantified using the area under the curve function in GraphPad Prism 5.0d (GraphPad Software, San Diego, CA, USA) and compared between NY15 and Cal09 strains using an unpaired *t* test. The proportion of monofunctional and dual-functional T-cell responses were estimated using Boolean gating on FlowJo 10.4 (FlowJo LLC, Ashland, OR, USA) and statistical significance between timepoints tested with the Permutation test in SPICE (version 6.0).[15] Proportions are displayed with 95% CIs. All tests were two-sided at the 5% significance level and were Bonferroni-adjusted for multiple comparisons within each set of analyses. Statistical analyses were done using R version 3.5.1, Stata release 12 (StataCorp, College Station, TX, USA), and GraphPad Prism 5.0d.

Role of the funding source

The funder had no role in study design, data collection, data analysis, data interpretation, or writing of the report. BBL, TIdS, EPA, YJJ, NIM, DJ, KH, and AS had access to raw data. The corresponding author had full access to all data in the study and had final responsibility for the decision to submit for publication.

Results

Between Feb 8, 2017, and April 12, 2017, 118 children were enrolled and received one dose of the 2016–17 northern hemisphere formulation LAIV (Cal09 pH1N1; figure 1A). Between Jan 15, 2018, and March 28, 2018, a separate cohort of 135 children were enrolled and received one dose of the 2017–18 northern hemisphere formulation LAIV (NY15 pH1N1; figure 1B). 118 children in 2017 and 126 children in 2018 completed the study. All study visits were within protocol-defined windows (+1 day for day 2 visit, +7 days for day 7 visits, and +7 days for day 21 visits). In the 2017 cohort, 118 (100%) of 118 day 2 visits were 2 days after LAIV, 115 (97%) of 118 day 7 visits were 7 days after LAIV (three visits were 8 days, 12 days, and 14 days after LAIV), and 112 (95%) of

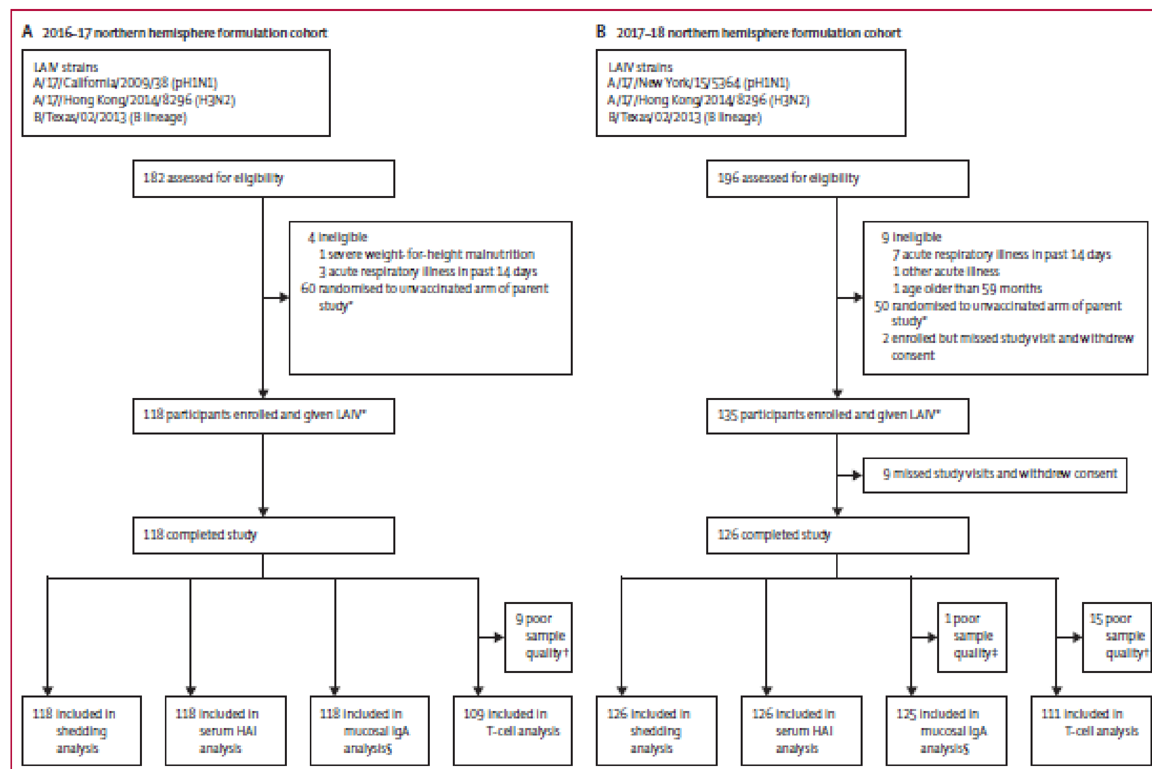

Figure 1: Study profile

Overview of participants who received (A) the 2016–17 northern hemisphere Russian-backbone LAIV formulation and (B) the 2017–18 northern hemisphere Russian-backbone LAIV formulation. LAIV=live attenuated influenza vaccine. pH1N1=pandemic H1N1. HAI=haemagglutinin inhibition. *The study was nested within a larger randomised controlled trial (NCT02972957; appendix pp 3, 4). †Sparse cell populations seen on flow cytometry. ‡Total IgA not detected in sample. §No pH1N1 data for one sample in 2016–17 cohort and no pH1N1 data for four samples and H3N2 data for three samples in 2017–18 cohort because of inadequate sample volume.

118 day 21 visits were 21 days after LAIV (five visits were 22 days after LAIV and one was 25 days after LAIV). In the 2018 cohort, 122 (97%) of 126 day 2 visits were 2 days after LAIV (four visits were 3 days after LAIV), 119 (94%) of 126 day 7 visits were 7 days after LAIV (seven visits were 8 days after LAIV), and 117 (93%) of 126 day 21 visits were 21 days after LAIV (eight visits were 22 days after LAIV and one visit was 26 days after LAIV). Baseline demographics did not differ significantly between the two cohorts with the exception of baseline haemagglutinin inhibition titres (table).

No influenza strains were detected from nasopharyngeal swabs taken immediately before vaccination in any children. After administration of the 2016–17 LAIV, pH1N1 Cal09 shedding was seen in significantly fewer children (16 of 118 [14%, 95% CI 8·0–21·1]) at day 2 compared with H3N2 (54 of 118 [46%, 36·6–55·2]; $p<0·0001$) and B/Vic (95 of 118 [81%, 72·2–87·2]; $p<0·0001$; figure 2). No pH1N1 Cal09 shedding was recorded at day 7 with the 2016–17 LAIV, with H3N2 shedding noted in 21 of 118 children (18%, 95% CI 11·4–25·9) and B/Vic shedding in 70 of 118 children (59%, 49·9–68·3). Administration of the 2017–18 LAIV resulted in significantly more children shedding pH1N1 NY15 at day 2 (80 of 126 [63%, 95% CI 54·4–71·9]; $p<0·0001$ compared with pH1N1 Cal09; figure 2A), with shedding of H3N2 seen in 82 of 126 children (65%, 95% CI 56·1–73·4) and shedding of B/Vic reported in 91 of 126 children (72%, 63·5–79·8). Shedding of pH1N1 NY15 was detected at day 7 with the 2017–18 LAIV (65 of 126 children [52%, 42·5–60·6]), with shedding of H3N2 recorded in 40 of 126 children (32%, 95% CI 23·7–40·6) and shedding of B/Vic noted in 60 of 126 children (48%, 38·7–56·7).

Significantly higher pH1N1 nasopharyngeal viral loads (lower ct values) were also seen with the 2017–18 LAIV compared with the 2016–17 LAIV at day 2 ($p=0·0026$; figure 2B). Quantitative RT-PCR data showed that pH1N1

	2016–17 LAIV (n=118)	2017–18 LAIV (n=126)	p value
Age (months)	35·1 (28·3–44·9)	35·3 (28·0–40·5)	0·44
Sex	..	..	0·61
Female	57 (48%)	56 (44%)	..
Male	61 (52%)	70 (56%)	..
Height (cm)	92·9 (7·4)	91·8 (6·3)	0·23
Weight (kg)	12·9 (2·1)	12·6 (1·7)	0·30
Weight-for-height malnutrition*	..	..	0·93
None	76 (64%)	82 (65%)	..
Mild	33 (28%)	36 (29%)	..
Moderate	9 (8%)	8 (6%)	..
Tribe	..	..	0·27
Mandinka	96 (81%)	99 (79%)	..
Wolof	5 (4%)	7 (6%)	..
Fula	3 (3%)	5 (4%)	..
Jola	6 (5%)	4 (3%)	..
Serehule	2 (2%)	5 (4%)	..
Serere	5 (4%)	1 (1%)	..
Other	1 (1%)	5 (4%)	..
History of ever being admitted to hospital with a respiratory infection	6 (5%)	3 (2%)	0·32
History of more than one respiratory infection needing medication in the past year	8 (7%)	13 (10%)	0·37
Age when stopped breastfeeding (months)	20 (18–24)	20 (18–24)†	0·80
Baseline seropositive (haemagglutinin inhibition titre ≥1:10)			
pH1N1‡	39 (33%)	62 (49%)	0·013
H3N2	90 (76%)	70 (56%)	0·00070
B/Vic	25 (21%)	54 (43%)	0·00040
Haemagglutinin inhibition titre in children seropositive at baseline			
pH1N1‡	160 (80–160)	226 (160–320)	0·00050
H3N2	160 (80–160)	160 (80–320)	0·16
B/Vic	160 (80–226·3)	226 (160–320)	0·015

Data are n (%), median (IQR), or mean (SD). pH1N1=pandemic H1N1. *Malnutrition was categorised based on weight-for-height SD (Z score): none (≥–1), mild (–2 to <–1), moderate (–3 to <–2). Children with severe malnutrition (weight-for-height SD <–3) were excluded. †Missing data for two children. ‡pH1N1 virus used for serum haemagglutinin inhibition assays was changed for the cohort given 2017–18 LAIV to reflect the update from Cal09 to NY15.

Table: Demographic characteristics and baseline influenza serological data

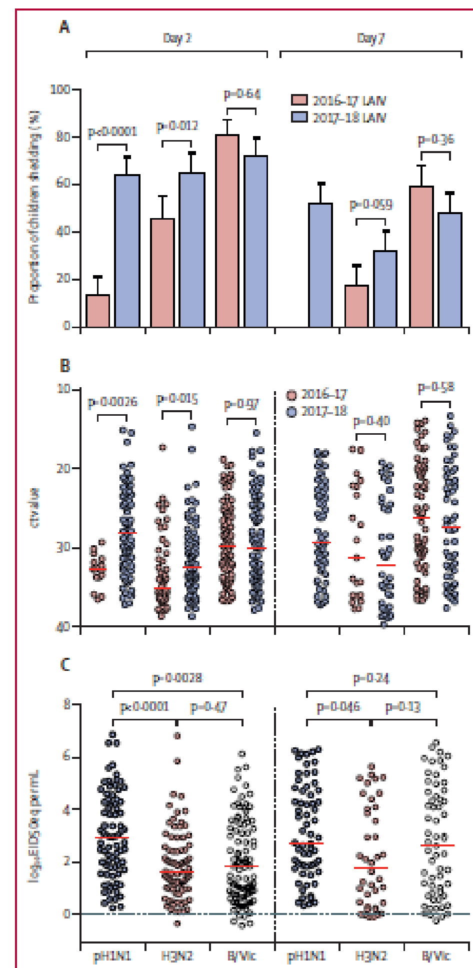

Figure 2: Shedding of strains in the nasopharynx after vaccination
(A) Percentage of children shedding vaccine virus with 2016–17 LAIV formulation compared with the 2017–18 LAIV formulation, at day 2 and day 7. Error bars represent the upper 95% CI. (B) Viral load in the nasopharynx is indicated by ct values from RT-PCR. Red bars indicate median ct values. Lower ct values indicate higher viral loads. (C) Quantitative RT-PCR viral load in children from the 2018 cohort for each strain. Red bars indicate median values. p values are Bonferroni-adjusted for multiplicity within each group of analyses. LAIV=live attenuated influenza vaccine. pH1N1=pandemic H1N1. H3N2=A/17/Hong Kong/2014/8296. B/Vic=B/Texas/02/2013 (Victoria lineage). ct=cycle threshold. RT-PCR=reverse-transcriptase PCR. EID50eq=50% egg infectious dose equivalents.

NY15 viral loads from the 2017–18 LAIV were significantly higher than H3N2 ($p<0·0001$) and B/Vic ($p=0·0028$) at day 2 (figure 2C). To investigate whether the improved replication of pH1N1 with the 2017–18 LAIV resulted in greater competition with H3N2 and B/Vic and, therefore, lower viral loads of these strains, viral loads were compared in co-shedders of each strain. No significant negative effect on H3N2 and B/Vic replication was reported, with a significant positive correlation between pH1N1 and H3N2 shedding noted at day 2 (low correlation, $r_s=0·40$; $p=0·0012$) and day 7 (moderate correlation, $r_s=0·51$; $p=0·0032$; appendix p 11).

To ascertain whether pre-existing adaptive immunity accounted for poor pH1N1 Cal09 shedding, univariable logistic regression was done to calculate the predicted probability of shedding at each baseline haemagglutinin inhibition titre (figure 3), adjusting for year in the H3N2 and B/Vic models. Although an inverse relation was evident for H3N2 (figure 3C) and B/Vic (figure 3D) between baseline haemagglutinin inhibition titre and shedding, this relation was not evident for Cal09 (figure 3A), for which low shedding was predicted even

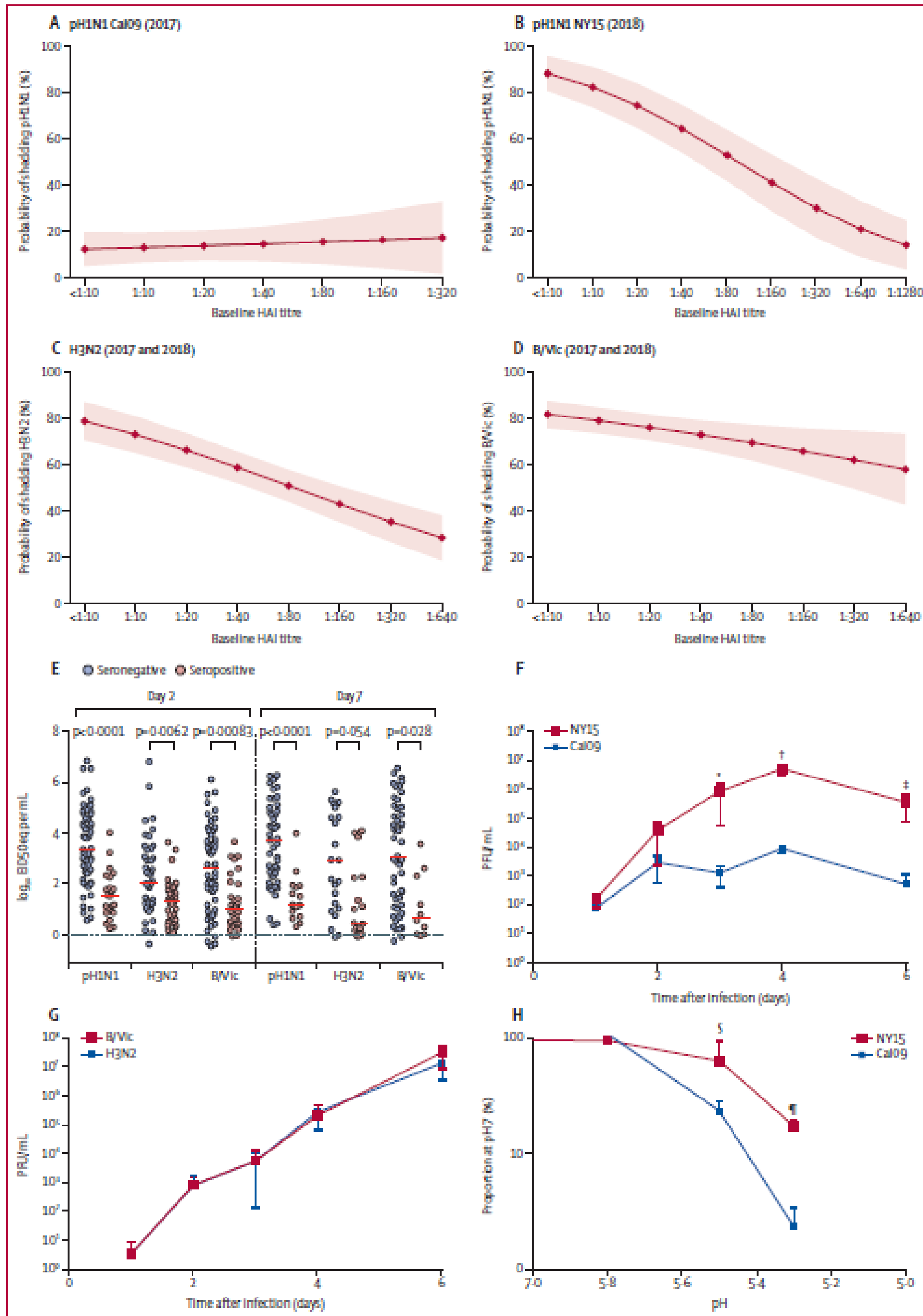

Figure 3: Effect of baseline serum antibody on LAIV strain shedding in the nasopharynx and replicative ability of viruses in primary epithelial cell cultures

(A–D) Predicted probability from logistic regression of vaccine strain shedding at day 2 after LAIV at a given baseline serum HAI titre to each matched strain. Dots show predicted proportions and shaded areas show 95% CIs. Data shown for Cal09 pH1N1 (A), NY15 pH1N1 (B), H3N2 (C), and B/Vic (D). Upper limit is based on maximum observed HAI titre in the dataset. When data from 2017 and 2018 were combined for H3N2 and B/Vic, results were adjusted for year (appendix p 12). (E) Nasopharyngeal viral load at day 2 and day 7 after 2017–18 LAIV, with participants stratified by baseline serostatus to vaccine haemagglutinin-matched and neuraminidase-matched influenza strains. Red bars indicate median values. (F and G) Replication of pH1N1 (F) or H3N2 and B/Vic (G) vaccine strains in primary nasal epithelium. Dots denote mean values and errors bars the SD. In (F), p<0·0001 comparing area under the curve. (H) Effect of pH on vaccine strain growth in vitro. Dots denote mean values and errors bars the SD. The y axis is a logarithmic scale. LAIV=live attenuated influenza vaccine. HAI=haemagglutinin inhibition. pH1N1=pandemic H1N1. Cal09=A/17/California/2009/38. NY15=A/17/New York/15/5364. H3N2=A/17/Hong Kong/2014/8296. B/Vic=B/Texas/02/2013 (Victoria lineage). EID50eq=50% egg infectious dose equivalents. PFU=plaque-forming units. p values for specific timepoints are *p=0·047, †p=0·0019, ‡p=0·029, §p=0·013, and ¶p<0·0001.

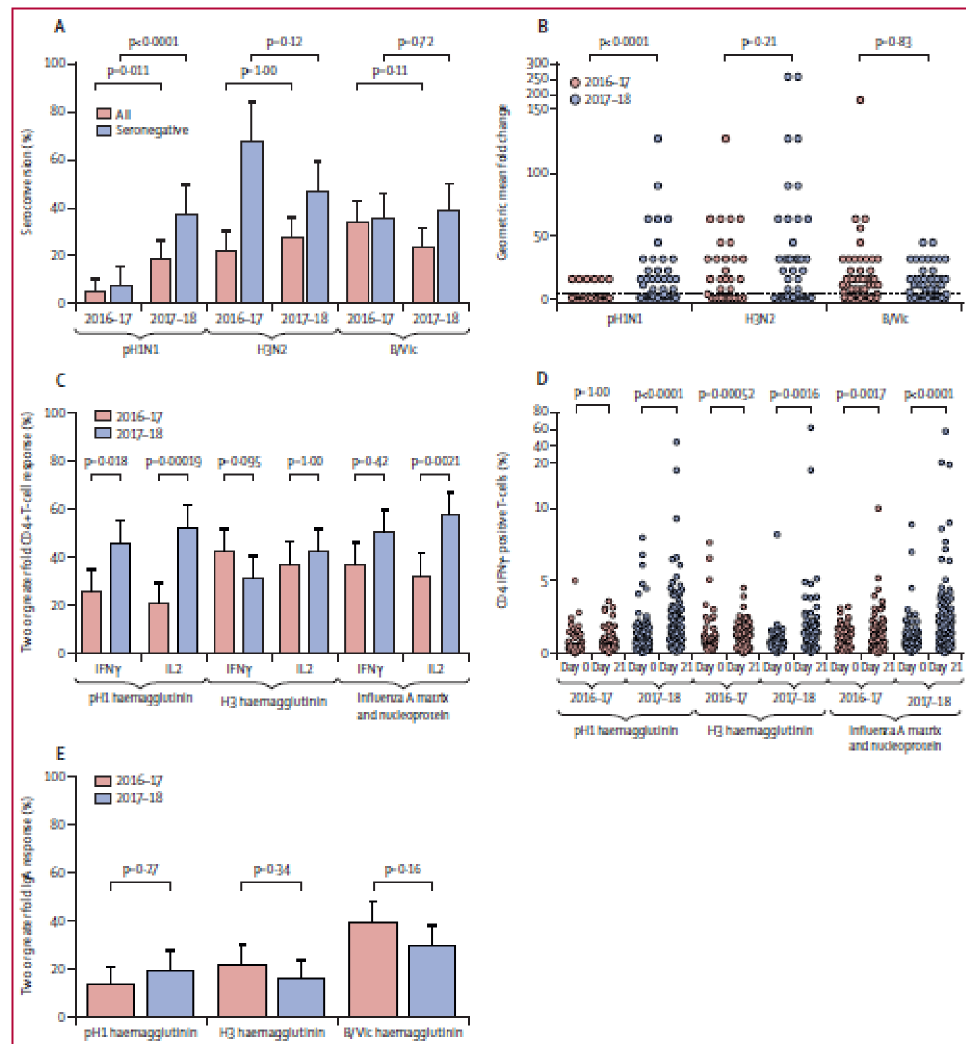

Figure 4: Immunogenicity to pH1N1 with the 2016–17 and 2017–18 LAIV formulations

p values are Bonferroni-adjusted for multiplicity within each group of analyses. (A) Percentage of children seroconverting to each LAIV strain, comparing 2016–17 and 2017–18 formulations. Error bars represent the upper 95% CI. (B) Geometric mean fold change in serum haemagglutinin inhibition titre from baseline to day 21, comparing children seronegative at baseline given 2016–17 and 2017–18 LAIVs. Dotted line depicts a fold change of four. y axis is a logarithmic scale. (C) Influenza-specific CD4+ T-cell responses to vaccine strain-matched pH1 haemagglutinin (Cal09 in 2016–17 or NY15 in 2017–18), H3 haemagglutinin, influenza A matrix and nucleoprotein (both matched to LAIV backbone) peptide pools, comparing 2016–17 and 2017–18 LAIVs. Error bars represent the upper 95% CI. (D) Percentage of children with a twofold rise in influenza-specific CD4+ T-cell responses at day 21 after 2016–17 and 2017–18 LAIVs. y axis is a logarithmic scale. (E) Percentage of influenza-specific mucosal IgA responders given the 2016–17 and 2017–18 LAIVs. Error bars represent the upper 95% CI. pH1N1=pandemic H1N1. LAIV=live attenuated influenza vaccine. Cal09=A/17/California/2009/38. NY15=A/17/New York/15/5364. H3N2=A/17/Hong Kong/2014/8296. B/Vic=B/Texas/02/2013 (Victoria lineage). IFNγ=interferon γ.

in seronegative children. By contrast, NY15 shedding was inversely related to the magnitude of the baseline haemagglutinin inhibition titre (figure 3B). Logistic regression also showed no associations between shedding and prevaccination T-cell responses or haemagglutinin-specific mucosal IgA responses for Cal09 (appendix p 12). Similarly, no association was seen between T-cell responses or IgA responses and shedding for H3N2 or B/Vic strains, after adjusting for baseline haemagglutinin inhibition titre (appendix p 12).

Significantly lower nasopharyngeal viral loads were recorded at day 2 and day 7 for all three strains in baseline seropositive children compared with baseline seronegative children who received the 2017–18 LAIV formulation (figure 3E), further emphasising the importance of serum antibody in this process.

In the seronegative population, shedding of pH1N1 Cal09 at day 2 was reported in ten of 79 children (13%, 95% CI 7·0–21·8) whereas shedding of pH1N1 NY15 was noted in 58 of 64 children (91%, 81·0–95·6; p<0·0001). Shedding of H3N2 with the 2016–17 LAIV was seen in 21 of 28 seronegative children (75·0%, 95% CI 56·6–87·3) and with the 2017–18 LAIV, shedding of H3N2 was recorded in 46 of 56 seronegative children (82%, 70·2–90·0; p=0·63). In the same seronegative population, shedding of B/Vic with the 2016–17 LAIV was detected in 78 of 93 children (84%, 95% CI 75·1–90·0) and, with the 2017–18 LAIV, shedding of B/Vic was recorded in 57 of 72 children (79%, 68·4–86·9; p=0·57). Comparisons of ct values between 2016–17 and 2017–18 LAIV strains in seronegative children showed a lower ct value (higher viral load) at day 2 with pH1N1 NY15 compared with pH1N1 Cal09 (p<0·0001; appendix p 13).

Monovalent vaccine strain replication was tested in primary human nasal epithelial cells cultured at an air–liquid interface to see whether in-vitro kinetics (in the absence of adaptive immune responses) reflected Cal09 and NY15 pH1N1 shedding in children. NY15 replication was greater than Cal09 replication (figure 3F), whereas H3N2 and B/Vic growth was equivalent (figure 3G). Since stability in acidic environments in the upper respiratory tract could be important for replicative ability, Cal09 and NY15 were quantified after exposure to varying pH levels. Greater stability of NY15 was seen in acidic environments compared with Cal09 (figure 3H).

The 2016–17 LAIV resulted in significantly fewer children seroconverting to pH1N1 Cal09 (six of 118 [5%, 95% CI 1·9–10·7]) compared with H3N2 (26 of 118 [22%, 14·9–30·6]; p=0·00030) and B/Vic (40 of 118 [34%, 25·4–43·2]; p<0·0001). A significant increase was recorded in pH1N1 NY15 seroconversion with the 2017–18 LAIV compared with Cal09 (24 of 126 [19%, 95% CI 13·2–26·8]; p=0·011; figure 4A), with no difference in H3N2 (35 of 126 [28%, 20·7–36·2]; p=1·00) or B/Vic (30 of 126 [24%, 17·2–32·0]; p=0·11). The improved seroconversion to pH1N1 with NY15 compared with Cal09 was especially evident in seronegative children (24 of 64 [38%, 95% CI 26·7–49·8] *vs* six of 79 [8%, 2·8–15·8]; p<0·0001; figure 4A), with a significant difference in geometric mean fold change in haemagglutinin inhibition for pH1N1 NY15 in 2017–18 compared with pH1N1 Cal09 in 2016–17 (p<0·0001; figure 4B).

Influenza-specific CD4+ IFNγ-positive, CD4+ IL2-positive, and CD8+ IFNγ-positive T-cell responses were detected at baseline and after vaccination. Although the magnitude of CD8+ responses was generally higher, LAIV-induced responses were predominantly CD4+ (figures 4C and 4D; appendix pp 14, 15). The 2016–17 LAIV did not induce significant pH1 haemagglutinin-specific CD4+ IFNγ-positive or CD4+ IL2-positive responses, whereas H3 haemagglutinin-positive and influenza A matrix and nucleoprotein-specific responses were significantly increased from baseline (figure 4D; appendix p 14). By contrast, the 2017–18 LAIV induced significant pH1 haemagglutinin-specific CD4+ T-cells at day 21. Accordingly, a twofold or greater rise in pH1 haemagglutinin-specific CD4+ T-cell responses was noted in more children given the 2017–18 LAIV than in those given the 2016–17 LAIV (50 of 109 [46%, 95% CI 36·3–55·7] *vs* 29 of 111 [26%, 18·2–35·3] for CD4+ IFNγ-positive responses; and 57 of 109 [52%, 42·5–61·9] *vs* 23 of 111 [20·7%, 13·6–29·5] for CD4+ IL2-positive responses; figure 4C). A twofold or greater rise in CD4+ IFNγ-positive and/or CD4+ IL2-positive responses was recorded in 45 of 111 children (41%, 95% CI 31·3–50·3) given the 2016–17 LAIV and in 73 of 111 children (66%, 60·0–75·6) given the 2017–18 LAIV. B/Vic haemagglutinin-specific and influenza B matrix and nucleoprotein-specific CD4+ responses were also induced (appendix p 15). No significant change in the proportion of monofunctional or dual-functional CD4+ T-cell responses was seen after vaccination (appendix p 15). Influenza-specific mucosal IgA responses to pH1N1 did not differ between the 2016–17 LAIV (16 of 117 children [14%, 95% CI 8·0–21·3]) and the 2017–18 LAIV (24 of 121 children [20%, 13·1–28·1]; p=0·27; figure 4E).

The effect of shedding on immunogenicity was investigated using H3N2 data (the largest sample of participants immunised with the same antigen and with T-cell data available). Seroconversion and T-cell responses were highest in children with shedding at both days 2 and 7 (appendix pp 16, 17). Multivariable logistic regression showed a significant effect of this prolonged shedding on the odds of seroconversion (odds ratio [OR] 12·69, 95% CI 4·1–43·6; p<0·0001) and CD4+ T-cell responses (7·83, 2·99–23·5; p<0·0001; appendix pp 16, 17). No such relation was seen with IgA responses (appendix pp 16–18). The odds of seroconversion were also reduced by higher baseline haemagglutinin inhibition titre (OR 0·11, 95% CI 0·04–0·27; p<0·0001) and increased by induction of an H3 haemagglutinin-specific CD4+ IL2-positive response (2·42, 1·05–5·62; p=0·037). Similar findings were seen in B/Vic and NY15 pH1N1 datasets, albeit with smaller sample sizes (appendix pp 18–20).

Discussion

The findings of our study showed limited shedding, in vitro Cal09 replication, and low immunogenicity after administration of the 2016–17 LAIV in Gambian children, providing an explanation for the scant efficacy of this vaccine that was reported in a randomised controlled trial from neighbouring Senegal.[4] After the switch to NY15, a significant increase in replication was seen, along with improved serum humoral and cellular

immunogenicity. No competitive inhibitory effect of enhanced pH1N1 replication was recorded with H3N2 or B/Vic replication or immunogenicity. Our data also showed that shedding for a longer duration is important for immunogenicity and that viral replicative fitness should be considered alongside antigenicity when selecting vaccine strains. Our findings represent the first reported LAIV immunogenicity data from African children and make a case for further studies of LAIV efficacy in Africa. They are also of relevance to the use of LAIV in other settings.

In a study of Ann Arbor-backbone LAIV, improved shedding and haemagglutinin inhibition seroconversion was reported with an updated A/Slovenia/2015 pH1N1 strain.[17] Parallel findings in two distinct cohorts of children—using two different LAIVs—provide strong support for Cal09 replicative fitness being culpable for the suboptimum pH1N1 LAIV effectiveness seen in recent years.[2] Our finding that limited Cal09 shedding is unlikely to be attributable to pre-existing immunity further supports this result and argues against the notion that reduced LAIV effectiveness in the USA might have been due to repeated vaccination in previous years.[18]

In an earlier study using the Ann Arbor-backbone LAIV, pre-pandemic seasonal H1N1 shedding was found to be higher than for H3N2 or influenza B.[19] Why pH1N1 Cal09 replication is impaired is uncertain. Haemagglutinin or neuraminidase residues must be the reason because the remaining six viral gene segments in LAIV are consistent between Cal09, NY15, and H3N2 strains. Differences in Cal09 haemagglutinin thermostability, sialic acid receptor binding, or pH sensitivity are potential explanations for the lower replication noted.[3] These properties are important for replication in the human upper respiratory tract. In particular, the pH of the upper respiratory tract in children might be lower than that of adults[20] and have a deleterious effect on replication of viruses with labile haemagglutinin. The pH1N1 virus first crossed into human beings in 2009 and has subsequently circulated as a human seasonal virus. During this time, changes in haemagglutinin stability and receptor binding properties might have adapted the virus to replicate better in the human upper respiratory tract.[21] Thus, the more recent haemagglutinin from A/Michigan/45/2015-like viruses of 2015 could have conferred enhanced shedding to LAIV pH1N1 components.

We were able to mirror our findings in a primary human nasal epithelial cell model. These cells have a mildy acidic apical surface environment akin to that of the human upper respiratory tract. This strategy could be a practical method for assessing vaccine virus replication before strain choice. Because cell lines traditionally used to culture influenza viruses (eg, MDCK) might not truly reflect replication in the upper respiratory tract, these subtleties were previously underappreciated.[22] Ultimately, a greater understanding of the viral genetic determinants of LAIV replicative fitness will be needed to select the best vaccine formulations.

Our study also emphasises the multifaceted nature of LAIV-induced immunity. Although seroresponse (the traditional correlate of protection after inactivated influenza vaccine) is modest, LAIV also induces mucosal IgA and T-cell responses. In our cohort, T-cell responses were elicited in a larger proportion of children than were mucosal or serum antibodies, showing the importance of assessing cellular immunity in LAIV studies. Using the 2017–18 LAIV formulation, a CD4+ IFNγ-positive or CD4+ IL2-positive T-cell response was seen in 55–68% of children to the influenza antigens tested, with approximately 80% of children showing a response to haemagglutinin or matrix and nucleoprotein (appendix p 18). LAIV provides protection in the absence of humoral immunity[23] and T-cell-mediated immunity is thought to have an important role.[24]

Unlike serum antibody and T-cell responses, we did not see an increase in mucosal IgA responses with NY15. This finding is in keeping with results reported after one dose of the updated Ann Arbor-backbone LAIV,[17] although a better response was seen after two doses. In a recent immunogenicity study of Nasovac-S in Bangladesh,[25] unlike serum antibody, nasal pH1N1-specific IgA was induced despite scant Cal09 shedding. Furthermore, by contrast to seroconversion and T-cell responses, we noted no association between shedding and IgA responses. Taken together, these data suggest the mechanisms and requirements for serum antibody and mucosal IgA induction by LAIV could be distinct.

Our study has several limitations. Although the association between shedding and immunogenicity was a predefined exploratory objective in the larger randomised controlled trial our study was a part of, comparison of formulations containing Cal09 and NY15 was a post-hoc analysis made possible only because of the WHO-recommended update to pH1N1 in the 2017–18 formulation. Because we show an improvement in several shedding and immunogenicity endpoints with NY15, we are confident that our main conclusions are justified. Nevertheless, since our sample size was based on endpoints not reported here, the negative findings reported in some subanalyses should be interpreted with caution. Also, participants were vaccinated with one LAIV dose, in keeping with the prequalification license from WHO and the randomised controlled trials in Senegal[4] and Bangladesh.[25] Our findings, therefore, might not be generalisable to children in high-income countries who receive booster doses and yearly influenza vaccination. We were also unable to confirm viral shedding with an LAIV-specific RT-PCR in all participants because of lower sensitivity compared with the haemagglutinin-specific RT-PCR, which is important to fully exclude interference from wild-type circulating strains. However, by doing the study outside of the peak influenza season,[7] undertaking clinical review at enrolment, and doing baseline RT-PCR

screening for influenza virus, it is unlikely that our results were affected by wild-type influenza infections. Our shedding data at day 2 are also similar to those reported from Senegal using Nasovac-S (Cal09 19%, H3N2 48%, and influenza B 66%).[4] Because we measured shedding with RT-PCR and not culture, we are unable to confirm to what degree shedding reflected viable viruses. Finally, an important unanswered question from our study is whether NY15 and related pH1N1 strains will result in improved LAIV effectiveness. Data from the 2017–18 UK season estimates the vaccine effectiveness (Ann Arbor-backbone LAIV) to be 90·3% against pH1N1 in children aged 2–17 years.[36] However, owing to low-level circulation of pH1N1, the precision around this estimate was low (95% CI 16·4–98·9). Our findings suggest improved effectiveness can indeed be expected with the updated LAIV and, if so, would support wider use of LAIV in the prevention of influenza.

Contributors

TIdS, EC, BK, EPA, and DJ contributed to the clinical study design. TIdS, BBL, YJJ, HJS, KH, JST, AM, TD, AS, and WB contributed to design of laboratory experiments. BBL, EPA, AS, WB, and TIdS contributed to the literature search. BBL, EPA, YJJ, HJS, SD, ES, KH, and AS contributed to data collection. BBL, TIdS, EPA, YJJ, NIM, DJ, KH, and AS contributed to data analysis. TIdS, BBL, EPA, AS, WB, EC, BK, TD, KH, AM, and JST contributed to data interpretation. BBL, TIdS, BK, EC, AS, and WB wrote the report. All authors reviewed the final report.

Declaration of interests

WB reports personal fees from AstraZeneca for contributing to a virtual advisory board in 2018, outside of the submitted work. BBL, YJJ, EPA, AS, HJS, SD, ES, NIM, DJ, KH, JST, AM, EC, TD, BK, and TIdS declare no competing interests.

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