

Vaccine design for global health pathogens

Thesis submitted for the degree of Doctor of Philosophy

Trinity Term 2019

Eriko Padron-Regalado

St Edmund Hall

Word count: ~40,200 (excluding Bibliography)

Vaccine design for global health pathogens

Eriko Padron-Regalado, St Edmund Hall, University of Oxford

Thesis submitted for the degree of Doctor of Philosophy, Trinity Term 2019

Abstract

MERS and malaria are two global infectious diseases with no commercial vaccines available. This thesis describes work undertaken to develop and improve adenovirus-based vaccines for these infectious diseases.

In the case of MERS coronavirus, a highly effective heterologous prime-boost vaccination based on a chimpanzee adenovirus was created (ChAdOx1). In order to further optimise its immunogenicity, previously described genetic designs modulating transgene expression were evaluated. It was found that ChAdOX1 encoding the MERS S glycoprotein induced high levels of antigen-specific immune responses. Such levels were further increased by boosting with an MVA encoding the same antigen. The inclusion of a tissue-plasminogen activator leader sequence (tPA-LS) had a positive impact on the levels of antigen-specific antibody responses, while the inclusion of the early promoter F11 in the MVA had a positive impact on the levels of antigen-specific cellular responses.

In the case of malaria, the immunological effects of recently discovered adjuvants were explored. Spermidine induced higher levels of antigen-specific $CD8^+$ T cell responses from spleens, induced by an adenovirus vaccine model

(AdHu5). Nevertheless, no influence of the spermidine was found on PBMCs. In the case of 2’3’-cGAMP, the adjuvant enhanced the immunogenicity of the capsid proteins of the adenovirus, but not the transgene antigens. An optimisation of the use of this adjuvant using bivalent adenoviruses resulted in a significant enhancement in transgene immunogenicity. Furthermore, it was found that IFN-β, induced during the activation of the cGAS/STING pathway, affected adenovirus transgene expression and immunogenicity.

It is hoped that the results presented in this thesis contribute to the advancement in the creation of safe and effective vaccines for MERS coronavirus and malaria.

Acknowledgements

I would like to thank Prof Adrian V.S. Hill and Prof Arturo Reyes for opening the doors of the University of Oxford so I could conduct this research. From all the people that could have been accepted, I like to think that they saw something on me that they considered of extreme value.

I would also like to thank Dr Alex Spencer for her patience and valuable day-to-day advice on the performance of these experiments. In addition, I would like to thank Dr Sandy Douglas for his valuable advice in the final stages of the present investigation.

Dr Naif Alharbi, Prof Sarah Gilbert, Dr Vincent Pavot and Marta Ulaszewska also helped greatly at different stages during this thesis. Especially, I would like to thank Dr Naif Alharbi for his input in the MERS-related investigation that resulted in a published paper (indicated in the section of **Papers and patents published from these studies**). The co-authors in the paper have given permission and are aware of the inclusion of information from this paper in this thesis.

Nevertheless, the people I would like to acknowledge the most is my family and my friends. I would like to confess that I tend to neglect their importance in my life. But I have realised during this PhD that they are the ones that matter the most. Life is meaningless without the presence of such people.

Table of Contents

Abstract 2

Acknowledgements 4

Table of Figures 13

Table of Tables 19

Papers and patents published from these studies 20

Chapter 1 Introduction 21

1.1 MERS coronavirus 21

1.1.1 Virus structure and genome 22

1.1.2 Epidemiology 26

1.1.3 Mechanism of transmission 28

1.1.4 MERS coronavirus life cycle 30

1.1.5 Status of vaccine development 31

1.1.6 Correlates of protection 37

1.2 Malaria 39

1.2.1 Symptomatology 39

1.2.2 Epidemiology 40

1.2.3 Mechanism of transmission and parasite life cycle 43

1.2.4 Naturally-acquired immunity to malaria and correlates of protection 47

1.2.5 Vaccine development for malaria 51

1.3 Viral vectors for global infectious diseases 57
1.3.1 Vaccinia virus 59
1.3.2 Adenovirus vectors 59
1.3.3 Cytomegalovirus 61
1.3.4 Vesicular Stomatitis Virus 61
1.3.5 Measles virus 62
1.3.6 Sendai virus 63
1.4 Recent improvements of adenovirus-based vaccines 66
1.4.1 Adjuvants for adenovirus-based vaccines 69
1.5 Thesis aims and outline 80
1.5.1 Aims 80
1.5.2 Outline 81
Chapter 2 Materials and Methods 82
2.1 Ethics statement 82
2.2 Construction of viral vectors 84
2.3 Other viral vectors and antigens used in the study 86
2.3.1 Virus-like particle R21 87
2.4 Adjuvant administration 87
2.4.1 Spermidine 87
2.4.2 2’3’-cGAMP 88
2.5 Mouse immunogenicity 89

2.5.1 ELISA 91

2.5.2 ELISpot 92

2.5.3 MERS pseudotyped neutralisation assay 94

2.6 Adjuvant *in-vitro* inhibition assays 95

2.7 Statistical analysis 96

Chapter 3 Pre-clinical assessment of genetic designs modulating transgene expression (MERS coronavirus) 98

3.1 Introduction 98

3.2 Construction of MVA-MERS-S-mH5, a benchmark MVA vaccine for comparison of immunogenicity 101

3.2.1 Vaccine design and production 101

3.2.2 Virus titration and quality control 105

3.2.3 Evaluation of integrity of the mH5 promoter and the MERS S glycoprotein DNA sequences 107

3.2.4 Evaluation of expression of the MERS S glycoprotein 108

3.3 Development of a pseudotype-based neutralisation assay 110

3.3.1 Pseudotyped virus construction 110

3.3.2 Selection of a reporter cell line 112

3.3.3 Development of the pseudotype-based neutralisation assay 113

3.3.4 Correlation with other serological assays 115

3.4 Effect on immunogenicity of the tissue plasminogen activator leader sequence (tPA-LS) in the chimpanzee adenovirus ChAdOx1 119

3.4.1 Experiment design 119

3.4.2 Humoral immunogenicity 120

3.5 Effect on antigen immunogenicity of an early/late promoter modulating transgene expression in MVA 121

3.5.1 Experiment design 121

3.5.2 Homologous prime-boost vaccination regimens 122

3.5.3 Heterologous prime-boost vaccination regimens 123

3.5.4 Vaccination immunogenicity comparison based on their induced neutralising antibodies 125

3.6 Discussion 126

Chapter 4 Pre-clinical assessment of spermidine as an adjuvant for viral-vectored vaccines (malaria) 129

4.1 Introduction 129

4.2 Effect of spermidine on effector IFNγ$^+$ CD8$^+$ T cell responses from viral vectors 134

4.2.1 Experiment design 134

4.2.2 Spermidine enhances the frequency of effector Pb9-specific IFNγ$^+$ CD8$^+$ T cells from spleens at day 14 136

4.2.3 Spermidine does not influence the persistence of effector IFNγ$^+$ CD8$^+$ T cells from PBMCs 139

4.3 Spermidine does not influence the re-activation of memory CD8$^+$ T cell responses 142

4.4 Spermidine does not influence the antibody responses induced by viral vectors 145

4.5 Spermidine does not influence the immunogenicity of the virus-like particle R21 147

4.6 Effect of the intramuscular administration of spermidine on the immunogenicity of viral-vectored vaccines 150

4.6.1 Spermidine injected intramuscularly does not influence the IFNγ^{+} CD8^{+} T cell responses induced by viral vectors 152

4.7 Effect of spermidine in older mice 155

4.8 Discussion 158

Chapter 5 Preclinical assessment of 2'3'-cGAMP as an adjuvant for viral-vectored vaccines (malaria) 161

5.1 Introduction 161

5.2 Effect of 2'3'-cGAMP on the immunogenicity of virus-encoded antigens 166

5.2.1 Experiment design 166

5.2.2 2'3'-cGAMP diminishes the immunogenicity of AdHu5-encoded antigens when administered intramuscularly 167

5.2.3 2'3'-cGAMP does not enhance the immunogenicity of MVA-encoded antigens by the intramuscular route of vaccination 172

5.2.4 The intradermal route of vaccination does not help in increasing the immunogenicity against transgene-encoded antigens 175

5.3 Effect of 2’3’-cGAMP on the immunogenicity of the structural proteins of AdHu5 178

5.3.1 Study design..... 178

5.3.2 2’3’-cGAMP enhances the humoral and cellular immunogenicity against the AdHu5 hexon..... 179

5.4 Effect of 2’3’-cGAMP on the immunogenicity of R21, a highly immunogenic virus-like particle 181

5.4.1 Study design..... 181

5.4.2 2’3’-cGAMP enhances the cellular immunogenicity of R21 182

5.5 Discussion 187

Chapter 6 Mechanism of action of 2’3’-cGAMP for viral-vectored vaccines. 190

6.1 Introduction 190

6.2 Effect of the adjuvant 2’3’-cGAMP on the immunogenicity of AdHu5 in STING-deficient mice 193

6.2.1 Study design..... 193

6.2.2 The enhancement effect of the adjuvant 2’3’-cGAMP on the AdHu5 capsid immunogenicity is dependent on the cGAS/STING pathway..... 194

6.2.3 The detrimental effect of 2’3’-cGAMP on the AdHu5 transgene immunogenicity is not apparent in STING-deficient C57BL/6 mice..... 196

6.2.4 Use of an inactive analogue of 2’3’-cGAMP suggests that its detrimental effect upon transgene immunogenicity in BALB/c mice is due to cGAS-STING activation 198

6.3 Cell biology mechanisms behind the detrimental effect of 2'3'-cGAMP on the AdHu5 transgene immunogenicity 203

6.3.1 Study design 203

6.3.2 The production of IFN-β triggered during the activation of the cGAS/STING pathway correlates with the blocking of AdHu5 transgene expression *in vitro* 204

6.4 Effect of the co-expression of cGAS on the transgene immunogenicity of AdHu5 209

6.4.1 Study design 209

1.1.1 Construction of an AdHu5 expressing a catalytic cGAS 211

6.4.2 The activation of cGAS/STING pathway at a similar time frame to the AdHu5 transgene expression enhances the AdHu5 transgene immunogenicity 215

6.4.3 Viral vector expression of cGAS enhances the immunogenicity of the highly immunogenic virus-like particle R21 221

6.5 Discussion 224

Chapter 7 Concluding remarks 229

7.1 Overview 229

7.2 Conclusions and further directions 230

7.2.1 Generation of a viral-vectored vaccination regimen for MERS coronavirus based on adenovirus 230

7.2.2 Spermidine as a chemical adjuvant for an adenovirus-based vaccination regimen 232

7.2.3 2’3’-cGAMP as a chemical adjuvant for an adenovirus-based vaccine 233

7.2.4 Final remarks 235

References 236

Table of Figures

Figure 1.1. Structure of the MERS coronavirus and genomic organisation. 26

Figure 1.2. Frequency of reported MERS cases from 2012. 27

Figure 1.3. Life cycle of the MERS coronavirus. 31

Figure 1.4. Distribution of reported indigenous malaria cases per country. 41

Figure 1.5. Distribution of estimated malaria cases per country reported by the WHO. 42

Figure 1.6. Distribution of estimated malaria deaths per country reported by the WHO. 43

Figure 1.7. Life cycle of Plasmodium spp. 46

Figure 3.1. Cloning strategy for the construction of the shuttle vector. .. 102

Figure 3.2. Transient-dominant selection (TDS) strategy for the construction of the recombinant MVA. 103

Figure 3.3. Microscopy of plaques used to isolate the recombinant MVA. 104

Figure 3.4. Confirmation of the identity and purity of the MVA-MERS-S-mH5. 106

Figure 3.5. Sanger sequencing read showing a point mutation. 107

Figure 3.6. Confirmation of the expression of the MERS S glycoprotein by fluorescence immunostaining. 108

Figure 3.7. Confirmation of the expression of the MERS S glycoprotein by whole-cell ELISA. 109

Figure 3.8. Construction of the MERS S glycoprotein-pseudotyped viruses. 110

Figure 3.9. Confirmation of the formation of the MERS S glycoprotein pseudotypes by indirect ELISA. 111

Figure 3.10. Luciferase activity dependent on MERS pseudotype concentration in different cell lines. 112

Figure 3.11. Development of the pseudotype neutralisation assay. 113

Figure 3.12. Luciferase activity dependent on neutralising serum concentration. 114

Figure 3.13. Correlation between the pseudotype neutralisation assay and a microneutralisation assay. 116

Figure 3.14. Correlation between the pseudotype neutralisation assay and an endpoint dilution ELISA. 117

Figure 3.15. Correlation between the pseudotype neutralisation assay and an endpoint dilution ELISA for camel samples. 118

Figure 3.16. Schematic representation of the ChAdOx1 MERS vaccines. 119

Figure 3.17. Effect on neutralising antibodies of the use of the tPA-LS in the ChAdOx1 vaccine. 120

Figure 3.18. Schematic representation of the MVA vaccines used to test the effect of a strong early promoter (F11). 121

Figure 3.19. Effect on immunogenicity of the use of the strong early poxvirus promoter F11 compared to the benchmark promoter mH5 in a homologous prime-boost vaccination regimen. 122

Figure 3.20. Effect on immunogenicity of the use of the strong early poxvirus promoter F11 compared to the benchmark promoter mH5 in a heterologous prime-boost vaccination regimen. 124

Figure 3.21. Comparison of the neutralising antibody titres from the vaccination regimens. 125

Figure 4.1. Biosynthesis of spermidine from putrescine. 132

Figure 4.2. Autophagy induction by spermidine. 132

Figure 4.3. Scheme of the transgene peptides encoded by the viral vectors. 135

Figure 4.4. Effect of spermidine on the frequency of IFNγ$^+$ T cells at day 14 from spleens. A. 138

Figure 4.5. Effect of spermidine on the IFNγ$^+$ CD8$^+$ T cell responses against Pb9 from PBMCs. 140

Figure 4.6. Effect of spermidine of the Pb9-specific memory CD8$^+$ T cells. 144

Figure 4.7. Effect of spermidine on the antibody responses induced by viral vectors. 146

Figure 4.8. Schematic representation of the vaccine R21, a highly immunogenic virus-like particle for malaria. 147

Figure 4.9. Effect of spermidine on the R21 immunogenicity. A. 149

Figure 4.10. pH of spermidine at different concentrations in PBS as measured by a pH-meter. 151

Figure 4.11. Effect of spermidine on the antigen-specific T cell and B cell responses induced by AdHu5 injected intramuscularly. A. 155

Figure 4.12. Effect of spermidine on the T and B cell responses against viral vectors using older BALB/c mice...... 157

Figure 5.1. Scheme of the cGAS-STING pathway. 162

Figure 5.2. Effect of 2'3'-cGAMP on the cellular immunogenicity against the transgene antigens encoded by AdHu5 by the intramuscular route of vaccination. A. BALB/c mice groups were injected intramuscularly with 10^7 IU of AdHu5 expressing TIPeGFP with or without 10 µg of 2'3'-cGAMP per mouse. Spleens were harvested at day 14 after vaccination. 168

Figure 5.3. Dose-dependent effect of 2'3'-cGAMP on the transgene immunogenicity of AdHu5...... 170

Figure 5.4. Effect of 2'3'-cGAMP using increasing doses of AdHu5. 171

Figure 5.5. Effect of 2'3'-cGAMP on the cellular immunogenicity against the transgene-encoded antigens of MVA...... 173

Figure 5.6. Effect on the $CD8^+$ T cell responses against the murine malaria protective peptide Pb9 encoded by AdHu5 and MVA by different routes of vaccination. 177

Figure 5.7. Effect of 2'3'-cGAMP on the immunogenicity of the AdHu5 hexon proteins. 180

Figure 5.8. Effect of 2'3'-cGAMP on the immunogenicity of R21 and its comparison to the adjuvant Matrix M1...... 184

Figure 5.9. Interactive effect of 2'3'-cGAMP and Matrix M1 on the R21 immunogenicity. 185

Figure 5.10. Effect of 2'3'-cGAMP on the T cell immunogenicity of R21 from PBMCs. 186

Figure 6.1. Effect of 2’3’-cGAMP on the AdHu5 capsid immunogenicity (hexon) in wild type and STING knockout C57BL/6 mice. 195

Figure 6.2. Effect of the adjuvant 2’3’-cGAMP on the AdHu5 transgene immunogenicity in wild type and STING-knockout C57BL/6 (B6) mice. .. 198

Figure 6.3. Effect of the adjuvant 2’3’-cGAMP on the transgene immunogenicity of AdHu5 compared to its biologically inactive analogue 2’5’-GpAp. 202

Figure 6.4. IFN-β but not IFN-α (α2) affects AdHu5 transgene expression. 205

Figure 6.5. Effect of 2’3’-cGAMP on AdHu5 transgene expression in vitro. 207

Figure 6.6. Effect of 2’3’-cGAMP on the AdHu5 transgene expression. ... 208

Figure 6.7. Effect of the time of administration of 2’3’-cGAMP on the AdHu5 transgene immunogenicity. 210

Figure 6.8. Scheme of the molecular cloning for the construction of the AdHu5 expressing the mouse cGAS. 211

Figure 6.9. Evaluation of cGAS and TIP-eGFP expression from the bicistronic AdHu5s. 214

Figure 6.10. Effect on the AdHu5 transgene immunogenicity of the activation of the cGAS/STING pathway in a time-frame similar to the TIPeGFP antigen expression. 217

Figure 6.11. Effect on the AdHu5 transgene immunogenicity of the expression of cGAS by the intradermal route of vaccination. 219

Figure 6.12. Effect of the cGAS/STING pathway activation on the AdHu5 transgene immunogenicity at different doses of the viral vectors. 220

Figure 6.13. Effect of the viral vector expression of cGAS on the R21 immunogenicity. 223

Table of Tables

Table 1.1 Structural proteins of the MERS coronavirus. 23

Table 1.2. Accessory proteins and non-structural proteins of the MERS coronavirus. 24

Table 1.3. Number of laboratory-confirmed MERS cases reported by countries since 2012..... 27

Table 1.4. Passive and active immunisation strategies for MERS...... 35

Table 1.5. Vaccines under development for pre-erythrocytic malaria...... 55

Table 1.6. Viral-vectored vaccines for global infectious diseases. 64

Table 1.7. Chemical adjuvants successfully tested with adenovirus-based vaccines...... 76

Table 1.8. Genetically-encoded adjuvants successfully tested for adenovirus-based vaccines 78

Table 2.1. ELISA coating antigens..... 91

Table 2.2. Peptides used to re-stimulate cells in ELISpot 93

Table 6.1. Computationally-predicted physicochemical properties of 2'3'-cGAMP and its biologically inactive analogue 2'5'-GpAp. 199

Papers and patents published from these studies

Papers:

Alharbi, N. K., **Padron-Regalado, E.**, Thompson, C. P., A., Wells, D., Sloan, M. A., … Gilbert, S. C. (2017). ChAdOx1 and MVA based vaccine candidates against MERS-CoV elicit neutralising antibodies and cellular immune responses in mice. *Vaccine*, *35*(30), 3780–3788. doi:10.1016/ j.vaccine.2017.05.032

Patent:

Hill, A. V. S. (inventor), Gilbert, S. C. (inventor), Alharbi, N. (contributor) … **Eriko Padron-Regalado** (contributor)

Non-GMP compliant seedbank for ChAdOx1-MERS

Patent application: PCT/GB2018/051399

Chapter 1 Introduction

Middle East Respiratory Syndrome (MERS) and malaria are two infectious diseases with important on global health. Despite this, no effective vaccines are commercially available. Recent investigations have produced a range of vaccine platforms for these diseases but that need further development. One of these vaccine platforms, replication-deficient adenoviruses, represent a promising tool for the control and eradication of these global infectious diseases.

1.1 MERS coronavirus

In September 2012 the world experienced the emergence of a new strain of coronavirus, the Middle East Respiratory Syndrome coronavirus (MERS-CoV). The virus was isolated for the first time from a 60-year-old patient in Saudi Arabia by Egyptian virologist Dr Ali Mohamed Zaki [1].

The virus was found to be closely related to the Severe Acute Respiratory Syndrome (SARS) coronavirus. Both belong to the *Betacoronavirus* genus and these are enveloped viruses with a positive-sense single-stranded RNA genome [2]. There are two clades of the MERS-CoV strain, A and B. The first cases correspond to the clade A, while subsequent transmitted isolated viruses corresponded to clade B. The strain EMC/2012 was the first virus isolated for the disease and it is also used as the virus genome model. This strain corresponds to clade A [3].

Symptoms of MERS vary in severity. Severe disease cases are usually found in elders and people with chronic diseases such as renal disease, diabetes, and chronic lung disease [4]. In these patients, the disease might progress rapidly to

respiratory failure and acute kidney injury. In mild disease cases, coronavirus infection causes fever, cough and/or shortness of breath. Pneumonia is common, however, some people infected with the MERS virus have been reported to be asymptomatic. Gastrointestinal symptoms, including diarrhoea, have also been reported [5].

MERS has a high mortality rate of 30% [5]. Since the first identification of the virus, cases have been continuously reported in different parts of the world. Due to these characteristics, MERS-CoV represents an infectious disease of global health importance. Therefore, the research and development of an effective vaccine towards this disease are highly necessary to stop the spread of the disease.

1.1.1 Virus structure and genome

The RNA genome of the MERS coronavirus has at least ten predicted open reading frames (ORFs) [6], [7]. The ORFs for the structural proteins are arranged as 5'-ORF1a/b-S-E-M-N-poly(A)-3' and have been investigated for the development of vaccines, diagnostic tools and therapeutic approaches [8]–[10] (**Table 1.1**).

Table 1.1 Structural proteins of the MERS coronavirus. The table summarises the functions of the structural proteins of the MERS coronavirus (S, M, E, N) and their corresponding open-reading frames (ORFs). Source: https://www.sciencedirect.com/ science/article/pii/ S2095809918307598

Proteins	Open Reading Frame (ORF)	Function
Spike (S) protein	ORF2	Viral entry, receptor binding, membrane fusion
Envelope (E) protein	ORF6	Virion assembly; putative ion channel activity and it is involved in viral budding and release
Membrane (M) protein	ORF7	Virion assembly (the formation of the viral envelope and viral core) by interacting with the N protein; IFN antagonism
Nucleocapsid (N) protein	ORF8a	Main component of the nucleocapsid structure. It is essential for viral replication and assembly, and post-translational modifications. It modulates the host's initial innate immune response

In addition to the structural proteins, the MERS coronavirus genome also encodes accessory proteins (APs; ORFs 3, 4a/b, 5) and non-structural proteins (nsps). Accessory proteins antagonise antiviral responses (usually type I IFNs) and contribute to the virulence and pathogenicity of the MERS coronavirus [11]–[15]. On the other hand, non-structural proteins are encoded by ORF1a and ORF1b at the 5'-terminus of the MERS coronavirus genome. ORF1a and ORF1b encode polyprotein 1a and 1b, respectively. These proteins, in turn, are cleaved into 16 non-structural proteins. Both accessory proteins and nsps are essential for viral replication and transcription [16]–[21]. The functions and

corresponding open-reading frames of the accessory proteins and non-structural proteins are described in (**Table 1.2**).

Table 1.2. Accessory proteins and non-structural proteins of the MERS coronavirus. *The table summarises the functions of the accessory proteins and non-structural proteins of the MERS coronavirus. Source: https://www.sciencedirect.com/science/article/pii/S2095809918307598*

Proteins	**Open Reading Frame (ORF)**	**Function**
AP 3	ORF3	Viral replication and pathogenesis
AP 4a	ORF4a	Viral replication, IFN antagonism, protein kinase R antagonism
AP 4b	ORF4b	IFN antagonism, NF-κB inhibition
AP 5	ORF5	IFN antagonist, modulation of NF-κB-mediated inflammation
Nsp1-coding region	nsp1	Specific recognition of viral RNA that is required for efficient viral replication
Papain-like protease (PLpro)	nsp3	Responsible for the cleavage at positions 1–3 to develop three non-structural proteins; two selected sites (G720 and R911) were detected in the protease domain; viral replication; membrane proliferation; IFN antagonist; deubiquitination; putative dephosphorylation of Appr-1″-p, a side-product of cellular tRNA splicing, to ADP-ribose
Transmembrane domain	nsp4	Viral replication; membrane proliferation
Main, chymotrypsin-like, or 3C-like protease (3CLpro)	nsp5	Viral survival—proteolytic processing of the replicative polyprotein at specific sites and 3CLpro cleaves the remaining positions 4–16

		key functional enzymes, such as replicase and helicase
Transmembrane domain	nsp6	Membrane proliferation; interaction with nsp3 and nsp4
Primase	nsp8	Primase activity
Unknown	nsp9	Nsp9 is an essential protein dimer with RNA/DN-binding activity in SARS-CoV
Unknown	nsp10	Membrane proliferation—regulating 2′-*O*-MTase activity
RNA-dependent RNA polymerase	nsp12	Viral replication and transcription
Superfamily 1 helicase	nsp13	Viral replication; affects tropism and virulence
3′-to-5′exonuclease	nsp14	Viral replication (exoribonuclease activity)
N7-methyltransferase Nidoviral endoribonuclease specific for U	nsp15	Viral replication (exoribonuclease activity)
S-adenosylmethionine-dependent ribose 2′-*O*-methyltransferase	nsp16	Methyltransferase inhibition; viral replication; IFN antagonism

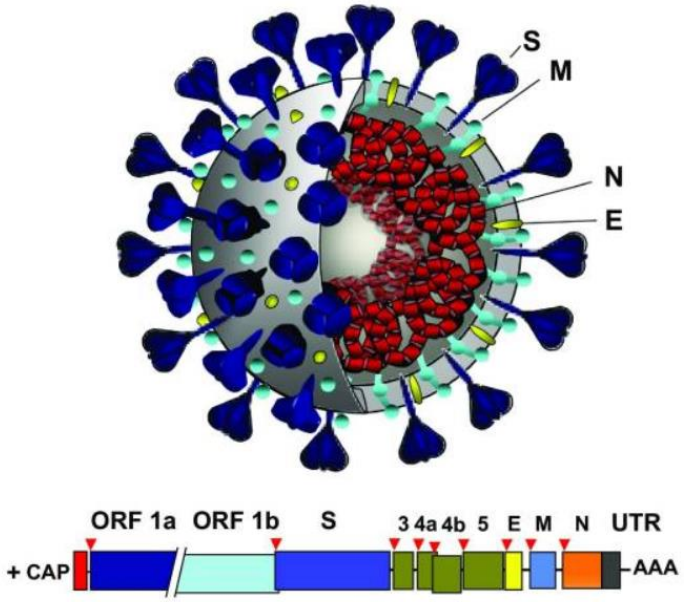


Figure 1.1. Structure of the MERS coronavirus and genomic organisation*. The MERS coronavirus virion particle is an enveloped virus and it is approximately 120-160 nm in diameter. The single-stranded positive RNA-based genome of the MERS coronavirus (~30 kb) encodes four structural proteins (S: spike, E: envelope, M: matrix and N: nucleocapsid). Accessory proteins and non-structural proteins correspond to ORF1a, 1b, 3, 4a, 4b, 5 in the image. ORF: Open-reading frame, UTR: untranslated region, CAP: RNA capping. Source: https://www.sciencedirect.com/ science/article/pii/S2095809918307598*

1.1.2 Epidemiology

According to the World Health Organisation (WHO), outbreaks since the virus' emergence in September 2012 have been reported in 27 countries, resulting in 2,428 laboratory-confirmed cases and 838 deaths as of July 2019 [5]. The majority of these countries are located in the Middle East, especially Saudi Arabia, where the virus emerged and the first disease cases were reported. Nevertheless, the capacity of the virus to produce a considerable number of disease cases in other countries was exemplified by the outbreak in South Korea in 2015 causing 185 reported cases (**Figure 1.2**).

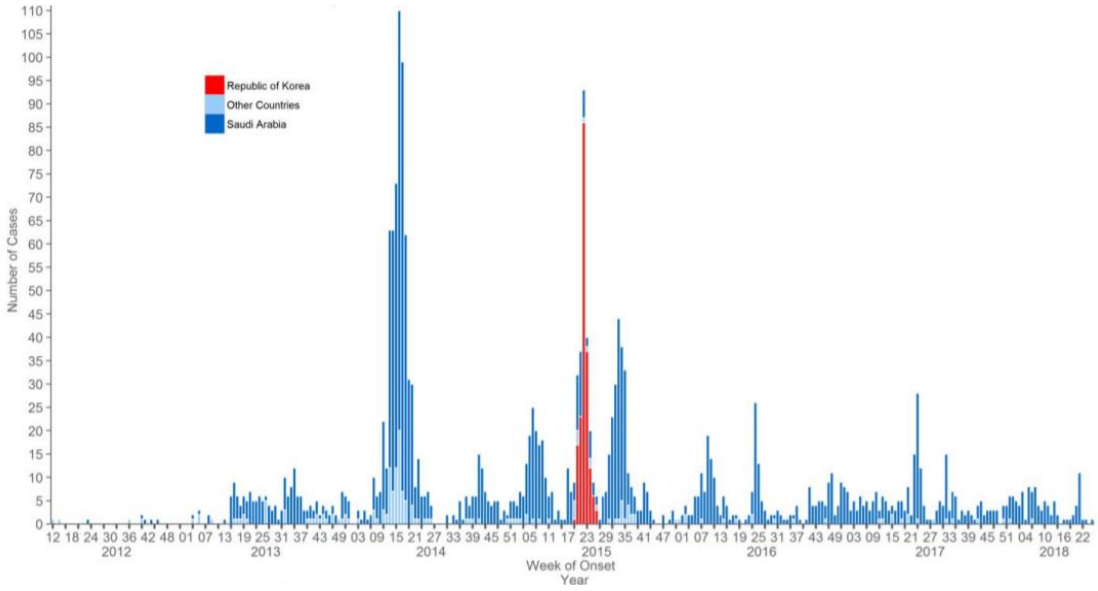


Figure 1.2. Frequency of reported MERS cases from 2012. *From the emergence of the disease in 2012, 2,428 laboratory-confirmed cases have been reported (as of June 2018). Most of the cases have been reported in Saudi Arabia, where the disease was first identified. Nevertheless, the disease has proved to represent a threat to global health as exemplified by the outbreak in South Korea in 2015 (red bar distribution). Source: https://www.who.int/emergencies/mers-cov/en/*

Table 1.3. Number of laboratory-confirmed MERS cases reported by countries since 2012*. The table shows the number of reported MERS cases per country (as of June 2018). Most of the cases belong to Saudi Arabia where the disease was first identified. Source: https://www.who.int/emergencies/mers-cov/en/*

Country	Number of reported cases
Algeria	2
Austria	2
Bahrain	1
China	1
Egypt	1

France	2
Germany	3
Greece	1
Iran	6
Italy	1
Jordan	28
Kuwait	4
Lebanon	2
Malaysia	2
Netherlands	2
Oman	11
Philippines	2
Qatar	19
Republic of Korea	185
Saudi Arabia	**1,854**
Thailand	3
Tunisia	3
Turkey	1
United Kingdom	4
United Arab Emirates	86
United States of America	2
Yemen	1
Total	2,229

1.1.3 Mechanism of transmission

The mechanism of transmission of the MERS-CoV is poorly understood. From seroepidemiological studies, it is known that the coronavirus has been circulating among camels since the early 80s, mainly in juveniles [22]–[27]. Due to the characteristic of the virus to not cause visible symptoms in camels, it is plausible that the coronavirus was circulating among camels for decades without being noticed and before jumping into humans. It is currently recognised that dromedary camels play important roles in sustaining the virus and may act as

amplifiers of the virus by secreting it in their body fluids, especially in nasal and rectal discharges (a type 2 intermediate host) [28], [29].

Camel to human transmission has not been directly observed, but several cases in humans have been associated with direct human-camel contact, especially among farmers handling camels on a daily basis [30]–[32]. It is also known that MERS coronavirus genomes found in camels and humans are extremely similar, if not indistinguishable. Therefore, camels are considered to be the intermediate host of the disease in the transmission to humans [22]–[27]. This is supported by serology and inoculation experiments suggesting that other livestock animals such as sheep, goats, cows, chicken, and horses, are not susceptible to the virus [24], [33]–[36].

Human to human transmission has been linked to hospital settings, and therefore health-care workers are considered in danger of contracting the diseases. However, sustained transmission between humans has not been directly proven and it is therefore considered to be of low probability [37].

Virology and evolutionary studies could shed light on the possible mechanisms of transmission of the disease. For example, it is known that virus entry of human host cells is mediated by the spike glycoprotein of the virus. This glycoprotein binds to the dipeptidyl peptidase 4 (DPP4) receptor. This receptor is especially enriched in the respiratory tract cells where the virus is thought to be found during host invasion. Recent studies *in vitro* have proven that the MERS S glycoprotein has the capacity to mutate and adapt to a sub-optimal DPP4 receptor [38]. This finding is of special importance since this might

support the idea of mutations that enabled the transmission of MERS coronavirus from camels to humans.

1.1.4 MERS coronavirus life cycle

The entry of the MERS coronavirus to the host cells is mediated by the spike (S) glycoprotein, which is a type-I transmembrane protein [39]. The S glycoprotein has a receptor-binding domain (RBD), which plays a crucial role in the binding of this glycoprotein to the host cell receptor DPP4 (dipeptidyl peptidase 4) [40]. After this interaction, DPP4 cleaves the S glycoprotein into two functional domains: subunits S1 and S2 [41]. This cleavage triggers a conformational rearrangement exposing the fusion peptides found in two heptad repeats HR2 and HR1 (subunit S2). The newly formed structure, called 6HB (six-helix bundle), is responsible for the membrane fusion (between the enveloped virus and the host cell) [21]. The membrane fusion can happen either on the cell membrane or after internalisation by an endosome. This process releases the genomic RNA enclosed in the nucleocapsid. Once in the cytoplasm, the RNA goes to replication, transcription and translation steps for the formation of the new virus particles. Due to the presence of a 5'-cap structure and a 3'-poly (A) tail, the virus genomic RNA can be translated into replicase proteins during the first stages of infection. Furthermore, the endoplasmic reticulum helps in the assembling and packaging of the new virus particles by the formation of the envelope membrane and the posterior exocytosis of the virus particles by the formation of vesicles [42].

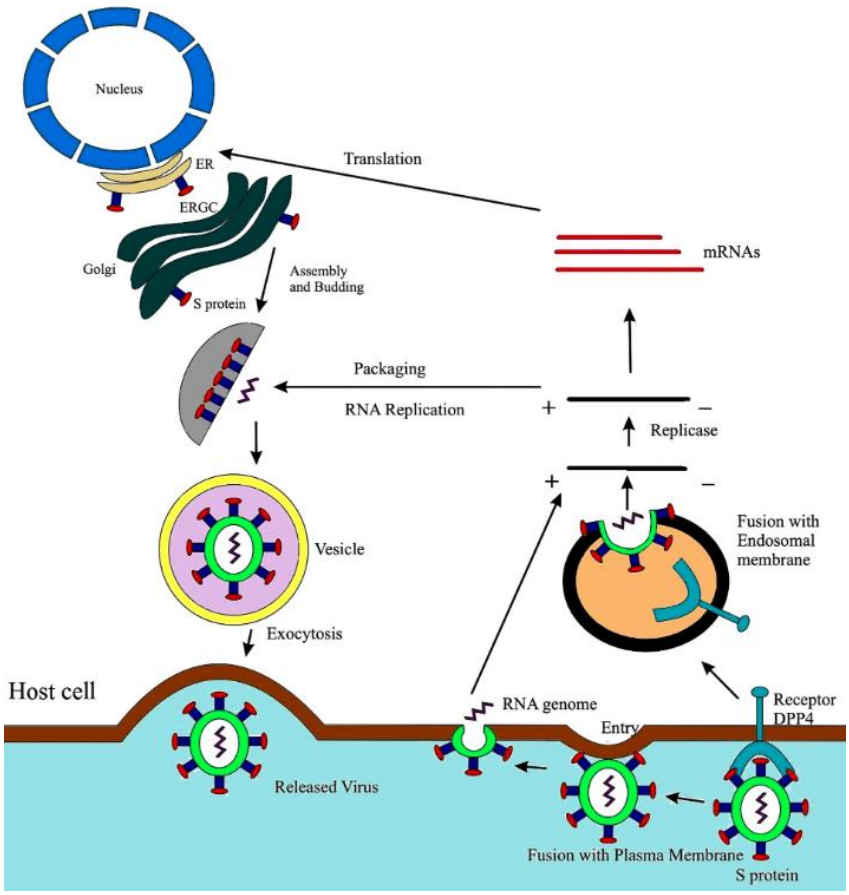


Figure 1.3. Life cycle of the MERS coronavirus. *During infection, the MERS coronavirus spike (S) glycoprotein binds to the host cell receptor DPP4 (dipeptidyl peptidase 4). DPP4 cleaves the S glycoprotein in two subdomains (S1 and S2). This interaction triggers a conformational rearrangement that culminates with the fusion of the virus envelope and the cell membrane. Once in the cytoplasm, the genomic RNA is replicated, transcribed and translated. The endoplasmic reticulum helps in the formation of the new virion particles with the subsequent release of vesicles for virus release. Source: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00569/full*

1.1.5 Status of vaccine development

Several vaccines have been developed for MERS since its emergence in 2012. Most of these vaccines are based on the MERS spike (S) glycoprotein, which

mediates virus entry of host cells. Therefore, most of these vaccines aim to induce antibodies to bind to this protein, neutralising the virus' ability to infect host cells. One of the major drawbacks in the development of a vaccine against MERS has been the lack of a mouse model that could sustain MERS-CoV infection as mice express a DPP4 receptor that does not support MERS S protein binding and virus entry [43]. This has changed recently due to the creation of humanised mice expressing the human DPP4 receptor that enables MERS-CoV infection, therefore enabling the assessment of protection from the vaccines under development.

The different vaccines against the MERS coronavirus that are under development are described in **Table 1.4**. The vaccine-type approaches were subdivided into passive and active immunisation strategies. Passive immunisation strategies have been investigated trying to produce *in vitro* or *in vivo* neutralising antibodies targeting the MERS S glycoprotein and then transfused into humans or camels. This *in vitro* approach has been explored using phage and yeast display technologies [44]–[48]. In this technique, a wide collection of fragment antigen-binding (Fab) regions of antibodies is tested to bind the MERS S glycoprotein, after being displayed on the surface of phages or yeasts. The selected Fab genes are then isolated and inserted in manufacturing cells such as mammalian cells, yeast or bacteria. This allows the production of high titres of the antibody and their subsequent purification. *In vivo* techniques of producing neutralising antibodies have also been explored. In this case, serum coming from infected humans and camels are subjected to purification of IgG antibodies and tested for the ability to neutralise the virus infection [49], [50]. These passive immunisation techniques have certain

advantages and disadvantages. Among the advantages, there is the rapid development of such antibodies to be transfused. This is of special interest when a relatively new infectious disease has emerged. On the other hand, among the disadvantages, there are the multiple administration requirements that a person must go through in order to sustain efficient protection against the virus. Also, the passive immunisations are by no means sustained long-term, but only for weeks to months, and therefore, they only serve for emergencies.

In the case of active immunisation strategies for MERS, these include the use of viral-vectored vaccines, subunit vaccines, inactivated virus, live attenuated viruses, nanoparticles, and DNA vaccines. Non-replicating viral vectors based on adenovirus, poxvirus, and measles have been engineered expressing the MERS S glycoprotein or the S1 subdomain (carrying the receptor-binding domain of the S glycoprotein). These approaches have been shown to induce high titres of neutralising antibodies and T cell responses [51]–[55]. DNA vaccines and subunit vaccines based on the S glycoprotein or the S1 subdomain have also been investigated resulting in high levels of humoral and cellular responses in non-human primates [56], [17], [18]. Combination vaccines using more than one vaccine platform have also been considered. Prime-boost immunisation regimens using protein-based and DNA vaccines of the S glycoprotein have been tested resulting in the induction of protection in non-human primates [57].

Active immunisation strategies have advantages and disadvantages compared to passive immunisation strategies. Among the advantages, there is the possibility of induction of long sustained protection against the MERS virus and the reduced number of immunisations required to achieve this protection.

Among the disadvantages, there is the relatively long time necessary to develop such vaccines and the differences in vaccine results from mice and humans/camels that might obstruct the development of the vaccines.

In summary, immunisation strategies mostly focus on the induction of antibody responses against the S glycoprotein of MERS, as a mechanism to neutralise the infection of cells by viruses. Some active immunisation strategies, particularly viral-vector vaccines, also focus on inducing T cell responses. In this case, these T cell responses might protect once the virus has infected host cells. Infected cells would present in an MHC Class I or II complex the virus antigens (peptides). Antigen-specific effector T cells would recognise such peptide-MHC complexes and kill the infected cells by producing cytotoxic molecules such as granzyme B and perforin [58]. In addition, effector T cells would produce IFN-γ that would inhibit viral replication [59]. The relative importance of both arms of the immunological system in the case of MERS is discussed in the next section.

Table 1.4. Passive and active immunisation strategies for MERS. *Passive and active immunisation strategies have been developed for MERS since its emergence in 2012. The table described these immunisation strategies detailing key information as of June 2019.*

Vaccine	Target	Use	Advantages	Problems	Status
Anti-MERS-CoV monoclonal antibodies [44]–[48]	Spike (S) glycoprotein	Passive immunization	High production yield	Short-lived protection, necessity of multiple administrations	Pre-clinical
Polyclonal anti-MERS-CoV antibodies from infected patients/reservoirs [49], [50]	Virus structural proteins including S glycoprotein	Passive immunization	Predicted better efficacy than monoclonal antibodies	Short-lived protection, necessity of multiple administrations	Pre-clinical
Live attenuated virus (E envelope-deleted) [60]	All virus genome components	Active immunization	(Not yet tested *in vivo*)	Safety concerns due to potential recombination with circulating coronavirus, potentially not safe for immunocompromised individuals	*In vitro*
Chemically-inactivated virus (rabies virus) [61]	S glycoprotein and S1 subunit	Active immunization	Induce high neutralising antibodies, protective in mouse models	Response might not be long term	Pre-clinical
Viral-vectored vaccines (poxvirus, adenovirus, measles) [51]–[55]	S glycoprotein or S1 subdomain (containing the receptor-binding protein)	Active immunization	Safe (non-replicating), induce antibody and T cell responses, long term immunity	Potential of pre-existing immunity in the population	Phase I [62]
Subunit vaccines (e.g. receptor-binding domain) [17], [18]	S glycoprotein	Active immunization	Safe, induce high antibody titres and may also induce T	Duration of response not known	Pre-clinical

Vaccine	Target	Use	Advantages	Problems	Status
			cell responses		
Nanoparticles [65], [66]	S glycoprotein	Active immunisation	Safe, usually induce higher antibody titres than protein-based vaccines		Pre-clinical
DNA vaccines [56]	S glycoprotein and subunits	Active immunization	Safe, induce high antibody titres and T cell responses.	Problems in delivery methods, anti-DNA immunogenicity	Phase I
Combination vaccines (protein and DNA) [57]	S glycoprotein and subunits	Active immunization	Safe, induce higher antibody titres and T cell responses Avoidance of anti-vector immunity when using a different vaccine platform for boosting. Protective in non-human primates	Necessity for multiple vaccinations	Pre-clinical

1.1.6 Correlates of protection

Knowledge about correlates of protection for MERS-CoV is limited, due to the relatively recent emergence of the disease, the need for high biosafety infrastructure to conduct the studies, and high rates of mortality precluding challenge trials in humans. Nevertheless, information about correlates of protection can be backed up by information from other coronaviruses, including SARS. Different strains of coronavirus infect mammals and birds, primarily the respiratory and gastrointestinal tract (e.g. SARS, TGE, MHV) [67].

It is generally accepted that protective immune responses to coronaviruses involve a combination of antibodies and T-cell responses [67]. Neutralising antibodies are directed against the spike (S) glycoprotein of the virus, especially the receptor-binding domain located at the S1 subunit. According to pre-clinical studies using the mouse hepatitis coronavirus (MHV), virus clearance might require the development of effective $CD8^+$ T and $CD4^+$ T cell responses as well as neutralising antibodies. Furthermore, the induction of neutralising antibodies is considered to be the most important feature for protection against coronavirus challenge [68], [69]. Nevertheless, this induction of protection might be species-dependant. In the case of the human coronaviruses HCoV-229E and HCoV-OC43, these respiratory-infecting viruses do not cause long-lived protection as challenge experiments suggest, even though systemic antibody responses are detected [70], [71].

Due to the severity of MERS, challenge studies are thwarted, and indirect evidence of protection comes from the monitoring of naturally-infected individuals. From these studies, it has been found that antibody and T cell

responses of the infected individuals diminish with the time, suggesting protection might be short-lived [72]–[74]. Nevertheless, studies about memory responses were not performed and these might potentially provide protection. These results could potentially be useful when assessing active immunisation strategies.

In summary, information about the correlates of protection in the case MERS is scarce and this is due to the high biosafety profile of the disease and its relatively recent emergence. Knowledge from other coronavirus points out that the mechanisms of protection might be different between coronavirus species. It is most likely that neutralising antibodies against the MERS S glycoprotein play a central role in the induction of protection, but the importance of T cell responses is not clearly defined, and more studies are needed in this regard.

1.2 Malaria

Malaria is a life-threatening global infectious disease caused by parasites of the genus *Plasmodium.* These parasites are transmitted to humans by the bite of the female mosquito *Anopheles* while blood-feeding. According to the WHO, nearly half a million people die each year from this disease, mainly in sub-Saharan Africa. Despite this disease burden, no highly effective vaccine is yet available. The following sections expand on the general features of this disease, including a description of the mechanisms of natural-acquired immunity against the parasite. A section about vaccine development efforts is included, focusing on pre-erythrocytic malaria, which will be the focus of this thesis research.

1.2.1 Symptomatology

Malaria is caused by the infection of different species of *Plasmodium* parasites. *P. falciparum* and *P. vivax* are the most frequent parasites causing the disease. It is known that *P. falciparum* causes most of the cases of malaria worldwide, including most of the severe and fatal cases of malaria [75].

Malaria is characterised by acute febrile illness. In the case of *P. falciparum*, when encountered for the first time, symptoms will appear 10-15 days after the *Anopheles* mosquito bite. The first symptoms: fever, headache, and chills, may be mild. Nevertheless, if the disease is not treated, *P. falciparum* malaria can progress to severe illness and often death, mainly in children and immunocompromised individuals [75].

Children with severe malaria often develop severe anaemia, respiratory distress or cerebral malaria. In adults, multi-organ failure is frequent. This multi-organ

failure refers to the dysfunction of peripheral organs, such as the lungs (acute respiratory distress syndrome) and kidneys (acute kidney injury) [76]. Nevertheless, in regions where the disease is endemic, people might develop partial immunity becoming asymptomatic rather than symptomatic on infection, but allowing the disease to be transmitted [75].

In the case of *P. vixax*, the parasite can stay dormant in a liver-stage form without causing visible symptoms of the disease. Nevertheless, the parasite might continue its development at certain times causing relapse episodes. Relapses have been observed when individuals are infected by malaria or other serious infectious diseases [77]. These relapses are characterised by a febrile illness that, in healthy individuals, rarely develop into severe disease [78].

1.2.2 Epidemiology

According to the World Health Organisation (WHO) in its last malaria report, in 2017 there were 219 million estimated cases of malaria in the world. Among these, the disease caused an estimated 435,000 deaths [79]. Most of the cases and deaths occurred in sub-Saharan Africa, accounting for 92% of cases and 93% of deaths. Nevertheless, regions such as South-East Asia, Eastern Mediterranean, Western Pacific, and the Americas are also at risk. In 2017, 87 countries from these regions reported malaria cases.

Children under 5 years old are the most vulnerable group affected by malaria. They accounted for 61% of all the malaria deaths (266,000 estimated deaths). The other vulnerable groups are immunocompromised and immunologically naive individuals. These groups are represented by pregnant women, migrants, mobile populations and travellers.

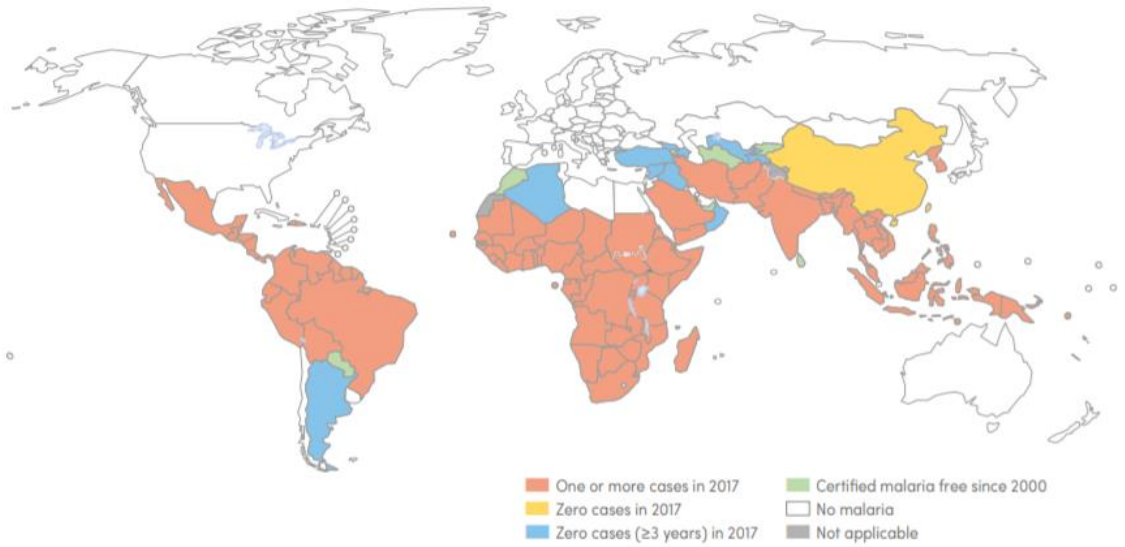


Figure 1.4. Distribution of reported indigenous malaria cases per country. *In 2017, malaria indigenous cases were reported in 87 countries and regions around the globe (World Health Organization, WHO). Most of the cases are in sub-Saharan Africa where the P. falciparum strain is more common (red colour). The Americas and South-East Asia are also represented where the P. vivax is more common (red colour). China reported zero cases this year (yellow), while countries such as Algeria, Argentina, Oman, and Turkey have reported no malaria cases for at least 3 years (blue colour). North America, Europe and Oceania are considered malaria-free regions since indigenous malaria cases have not been reported historically (white). Source: WHO Malaria Report 2018.*

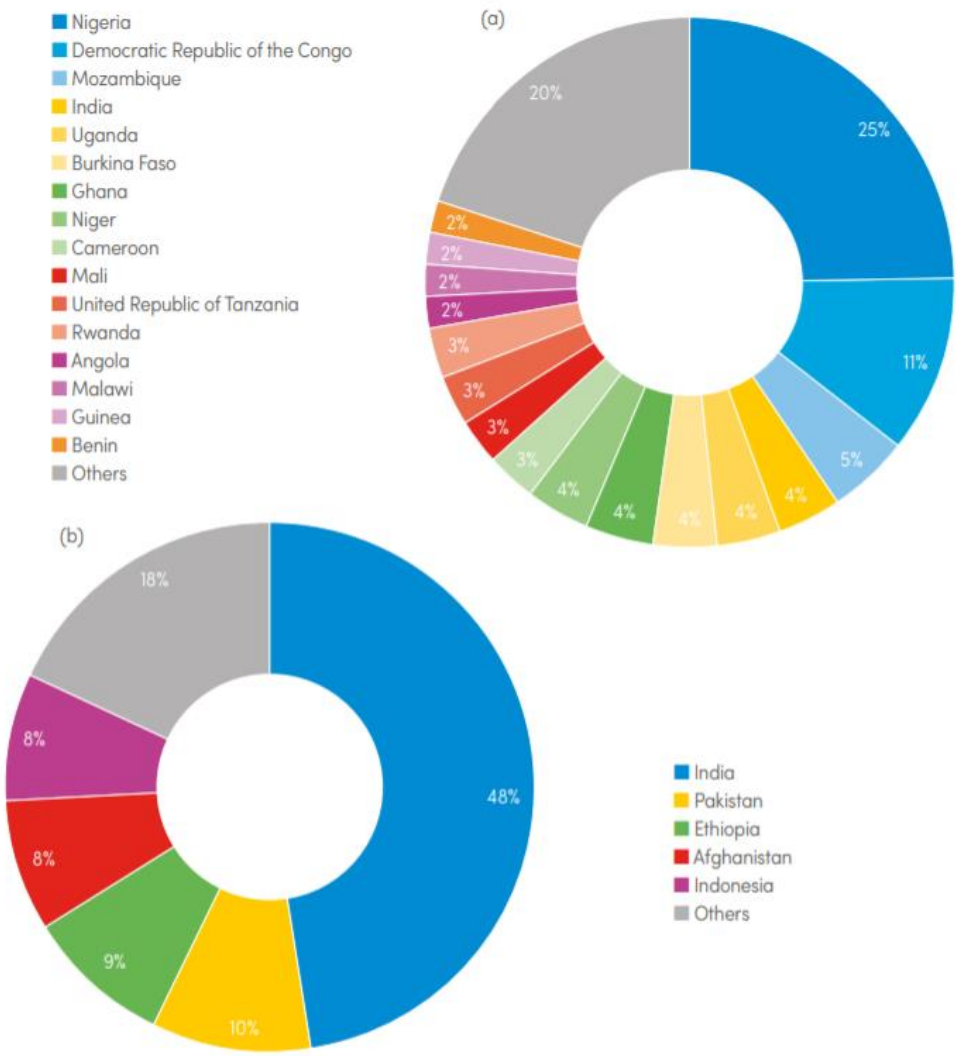


Figure 1.5. Distribution of estimated malaria cases per country reported by the WHO. *a) Distribution of total estimated malaria cases in 2017 including P. falciparum and P. vivax. From the estimated total 219 million malaria cases, 5 countries comprised nearly 50% of all the estimated cases: Nigeria (25%), Democratic Republic of Congo (11%), Mozambique (5%), India (4%) and Uganda (4%). In this case, most of the total malaria cases correspond to P. falciparum (approximately 211 million estimated cases). b) Distribution of estimated malaria cases in 2017 for P. vivax. From the 7.45 million estimated cases of P. vivax in the world, nearly 50% of the estimated cases come from India (48%), followed by Pakistan (10%), Ethiopia (9%), Afghanistan (8%) and Indonesia (8%).*

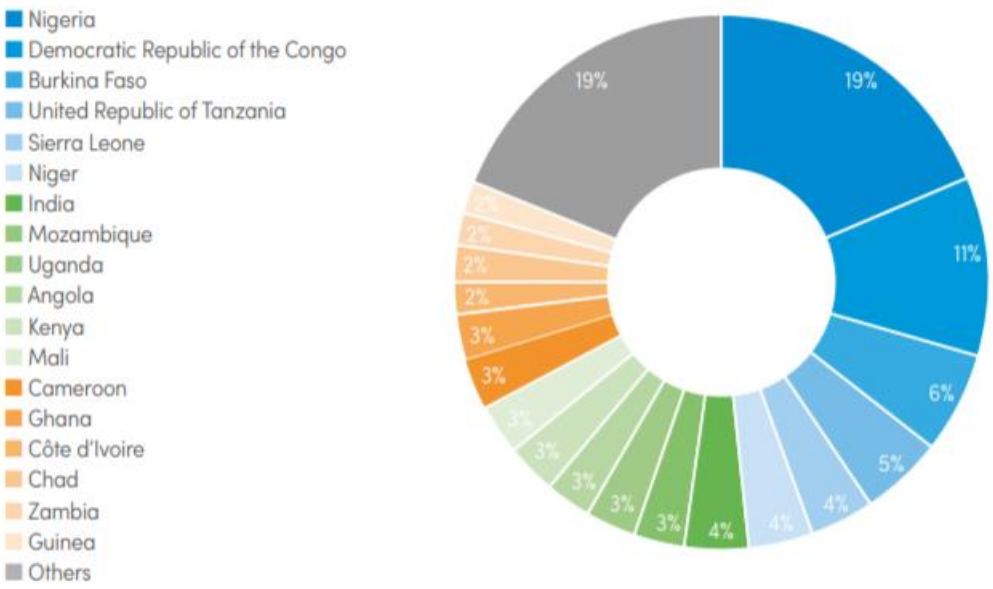


Figure 1.6. Distribution of estimated malaria deaths per country reported by the WHO. *From the 435,000 estimated malaria deaths in the world in 2017, 18 countries accounted for nearly 80% of the total deaths. These countries are in Africa, except for India. Nigeria is the country with the most estimated deaths (19%). Most of the estimated deaths cases are in children under 5 years old. Source: WHO Malaria report 2018.*

1.2.3 Mechanism of transmission and parasite life cycle

The mechanism of transmission of malaria is well characterised. In the case of *P. falciparum*, the parasite is transmitted to humans by the female mosquito *Anopheles* during blood-feeding. The mosquito seeks blood to nurture its eggs and it bites humans (also any warm-blooded animal). While doing this, it injects the sporozoites (the human-infecting stage of the parasite). The sporozoites move among the skin cells and then reach the circulatory system. The sporozoites then reach the liver where they transform and multiply into merozoites. In the case of *P. vixax*, the parasite can stay for a month or even years as hypnozoites inside hepatocytes, before transforming into merozoites

and initiate a blood-stage. This is the reason why *P. vivax*, different from P. falciparum, can cause relapses of the disease.

For initiating the blood-stage of the parasite, the merozoites are released from the hepatocytes in the form of hepatocyte-derived vesicles called merosomes (a special process that maintains the host cell membrane integrity) [80]. Merosomes, unrecognized by the host immune system, are transported to the blood circulation. Each merosome contains hundreds of merozoites and is disintegrated in pulmonary capillaries, further releasing the merozoites [81]. The merozoites then infect the circulating red blood cells where they further transform into trophozoites. Inside the red blood cells, trophozoites grow and divide into schizonts that contain approximately 30 daughter merozoites. Merozoites are then released for subsequent red blood cell invasion and replication [82]. This cycle lasts 48 hours in the case of *P. falciparum* and 72 hours in the case of *P. vivax* [83]. This is the so-called blood-stage and it is responsible for most of the malaria clinical symptoms.

The invasion of red blood cells by the merozoites is mediated by different ligands and host cell receptors. In the case of *P. vivax*, the parasite only infects Duffy blood group-positive erythrocytes. For this, the parasite uses the Duffy-binding protein and reticulocyte-binding homology proteins (mostly found in reticulocytes) [84], [85]. In the case of *P. falciparum*, different host cell receptors and invasion mechanisms come into play. In this case, varieties of Duffy-binding-like (DBL) homology proteins and reticulocyte-binding-like homologous proteins recognize different erythrocyte receptors that seem to be redundant in their invasion function [86], [87].

Invasion of red blood cells is also possible thanks to specialized apical secretory organelles called micronemes, rhoptries and dense granules. The merozoites use these organelles to push their way through the red blood cell membrane and finally to establish themselves in the cytoplasm [88].

When infected individuals are bitten again by *Anopheles* mosquitoes, they ingest female and male gametocytes that were formed during the blood-stage. These gametocytes are a product of a process called gametocytogenesis that occurs during the growth and division of some merozoites inside the red blood cells [89]. After ingestion by the female mosquito, the gametocytes mature into microgametes (male) and macrogametes (female), during a process called gametogenesis. This process takes 1-4 days for *P. vivax* and 8-10 days for *P. falciparum* [90]. The microgamete and the macrogamete then mate in the gut of the mosquito by fusion of nuclei and forming a zygote that further develops into an ookinete. The ookinete then penetrates the midgut wall of the mosquito, where it encysts into a formation called oocyst. Inside the oocyst, the ookinete nucleus divides to produce thousands of sporozoites in a process called sporogony. This process lasts 8-15 days [90]. The oocyst then ruptures and releases sporozoites inside the mosquito gut. After 10-18 days, only a few hundred sporozoites migrate to the salivary glands in order to be injected in humans and start the cycle again [91].

The life cycle of *Plasmodium* can also be divided into sexual and asexual stages. Humans can be considered to support the asexual stages of the parasite, while the mosquitos the sexual stages.

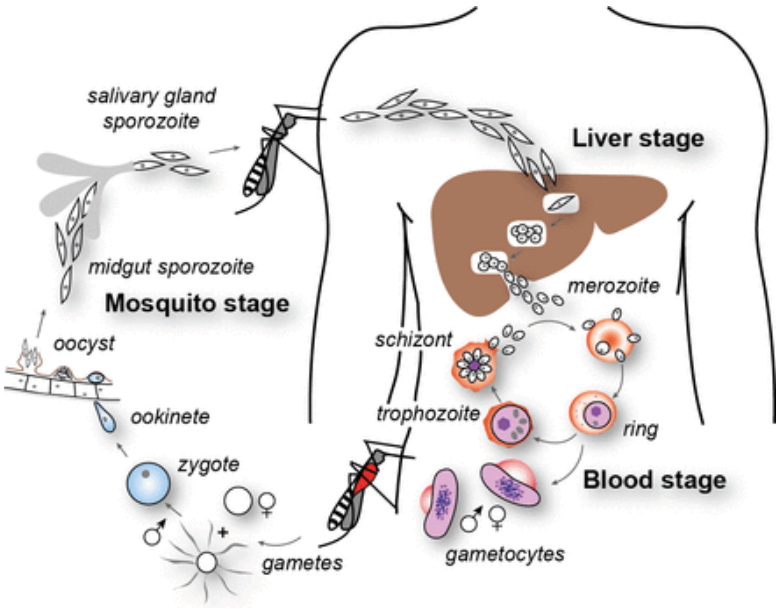


Figure 1.7. Life cycle of Plasmodium spp. *The life cycle of the Plasmodium parasite is well characterised. The female mosquito Anopheles injects the sporozoites, the human-infecting form of the parasite. The sporozoite moves among skin cells to reach the human blood circulation. Then it reaches the liver where it transforms into merozoites. In the case of P. vivax, the parasite can last months or even years in the form of hypnozoites. Merozoites are formed in the liver inside merosomes. After being released, the merozoites infect and multiply in the red blood cells (blood-stage of the parasite). Gametocytes formed during the blood-stage can be ingested by the Anopheles mosquito. Gametocytes then mature into female and male gametes. The female and male gametes then mate in the mosquito gut to form a zygote. The zygotes then transform into ookinetes and subsequently oocysts. This process culminates in the formation of new sporozoites (released from the oocysts) that move to the mosquito salivary glands after some days. When these mosquitos bite another human, the life cycle starts again with the sporozoites being injected. Source: http://jcb.rupress.org/content/198/6/961.abstract*

1.2.4 Naturally-acquired immunity to malaria and correlates of protection

Natural-acquired immunity to malaria is the capacity of individuals living in malaria-endemic areas to develop an adaptive immunity against the *Plasmodium* parasite, evading or ameliorating the clinical symptoms of malaria [92]. Several studies have been carried out describing this phenomenon. For example, natural-acquired immunity is thought to be dependent on the age of the individuals and the duration of exposure to the parasite [93]. This has been observed mostly in Sub-Saharan Africa, where the people are more strongly exposed to the parasite. Naturally-acquired immunity to malaria is characterised by a marked reduction of parasitaemia and the reduction of severe symptoms of the disease and death. This naturally-acquired immunity is more characteristic of adolescents and adults that have been exposed to the parasite in the endemic areas for a longer period.

The mechanism of acquired immunity to malaria is not known and it is thought to be complex and diverse. For example, it is known that immunity is partly strain-specific and it might involve the participation of multiple proteins being expressed at different stages of the life cycle of the parasite. In addition, the proteins being expressed at different stages of the parasite life cycle can also be polymorphic. This might also affect acquired immunity since a person could become immune not only to one specific strain of the parasite but also to only one repertoire of proteins [93]–[95].

Despite several studies about the induced adaptive immunity to the parasite, it has not been possible to establish with certainty which host immune responses

might provide immunity. In addition, the repertoire of parasite proteins that are the target of the host immune effectors is not fully understood. Nevertheless and due to the complexity of the parasite, it is believed that acquired immunity involves the participation of multiple cell immune effectors against the multiple proteins and life cycle stages of the parasite [96]. The different studies describing possible mechanisms of immunity and correlates of protection to malaria can be classified into antibody and cell-mediated immunity. In the following sections it is described each one focusing on *P. falciparum*, the most devastating strain causing the disease. These studies comprise pre-clinical and clinical studies and target mainly two of the stages of the parasite: pre-erythrocytic and blood stages.

1.2.4.1 Mechanism of antibody immunity

One of the first evidence of the importance of antibody responses for the induction of immunity came from passive immunisation experiments. Purified IgG antibodies from long time exposed adult individuals transferred to chronically infected children strongly ameliorated the clinical symptoms of the disease and level of parasitaemia [97]. Later studies further described the potential of purified IgG antibodies against the merozoite proteins to block the infection of red blood cells. Among these proteins, MSP1, AMA1, and EBA175 were identified [92]. Other functions mediated by antibodies and helping in the control of parasitaemia have also been studied. As an example, certain IgG antibodies against the merozoites have the capacity to inhibit the growth of the parasites through the induction of complement fixation and opsonic phagocytosis [98]–[101].

Most of the studies concerning antibody-mediated protection focus on the blood-stage of the parasite. This is due to the general understanding that antibodies are most likely to play a crucial function in this stage. Nevertheless, antibodies against sporozoites and early liver stages have also been investigated. These antibodies could potentially inhibit the liver infection by thwarting the motility of the sporozoite, its migration through the liver cells or hepatocyte invasion [102]–[104]. For example, IgG antibodies induced against irradiated sporozoites (that do not develop into erythrocytic stages) have the capacity to provide high levels of protection in passive immunisation studies in humans and mice [105], [106].

1.2.4.2 Mechanisms of cell immunity

There are relatively scarce studies about the cellular immune responses targeting sporozoites and liver stages in humans during naturally acquired immunity. From surveillance and challenge studies in endemic areas, it is known that $CD8^+$ T cell responses (such as IFN-γ and TNF-α) against expressed proteins in the pre-erythrocytic stage of the parasite might be low, transient and dependent on the frequency of parasite exposure [107], [108]. Nevertheless, it has also been found that these IFN-γ responses might be associated with protection [109]–[111]. The participation in the naturally-acquired immunity of $CD4^+$ T cell responses is not clearly defined, but some correlations with protection have been found with the expressed proteins of the parasite in the liver stages CSP and LSA-1 [97], [112], [113].

Mouse studies have also contributed greatly to clarify the participation of T cell responses in protection. Experiments using irradiated or genetically attenuated

sporozoites have demonstrated that sterile protection is possible and is T cell-dependent, mainly mediated by $CD8^+$ T cell responses and IFN-γ-mediated immune responses [114]. Nevertheless, it has been found that $CD4^+$ T cell responses might also confer protection and be crucial in the formation of memory $CD8^+$ T cell responses [115], [116]. It has also been found that the killing of intracellular parasites by $CD8^+$ T cells is performed by cytolytic means (through the production of perforin and granzyme) and by non-cytolytic means (nitric oxide induced by IFN-γ) [93].

Similar results have also been found in immunisation experiments with humans. After vaccinating with attenuated sporozoites, protection has been linked to polyfunctional memory $CD4^+$ T cells responding mainly to sporozoites [117], [118], $CD107a^+$ $CD4^+$T cells and granzyme B-secreting $CD8^+$ T cells [117].

Cell-mediated immune responses to the blood-stage of the parasite are better characterised. It is known that monocytes, neutrophils, and lymphocytes participate in the arrest of parasite growth through the release of soluble factors such as tumour necrotic factor (TNF) and the elimination of merozoites by antibody-dependent cellular inhibition [100], [119], [120]. In addition, macrophages are known to kill intracellular parasites through an antibody-independent mechanism and the participation of the type II interferon, IFN-γ [121]–[123].

TNFα and IFN-γ seem to be key in the induction of cell-mediated immunity to the blood-stage of malaria. As an example, IFN-γ has been shown to control parasitaemia in mice [124]. Production of IFN-γ by $CD4^+$ T cells have shown to increase the production of antibodies [123]. In humans, $IFN\gamma^+$ and $TNF\alpha^+$ T-cell

responses have been associated with protection [110], reduction of parasitaemia [125] and reduction of severe malaria symptoms [126].

As previously stated, immunity against malaria is more likely to be the product of multiple immune effectors, both antibody and cell-mediated, targeting multiple proteins at different stages of the parasite. In the case of the pre-erythrocytic stage of malaria, immunity is found to be strongly dependent on $CD8^+$ T cell responses and IFN-γ-mediated responses, both in humans and mice.

1.2.5 Vaccine development for malaria

Multiple efforts have been made in order to produce a cost-effective vaccine for malaria. Vaccines for malaria can be divided into pre-erythrocytic, blood-stage and transmission-blocking vaccines. Pre-erythrocytic stage vaccines aim to induce protective immune responses against the first stages of infection of the parasites in the human host, mainly sporozoites and liver-stage forms, before they develop into merozoites. In the case of blood-stage parasite, vaccines aim to induce immunity by targeting merozoites and infected red blood cells. In the case of transmission-blocking vaccines (TBVs), these vaccines aim to block the parasite transmission by targeting the sexual stages of the parasite that are responsible for the transmission to the *Anopheles* vector. In a sense, these vaccines aim to prevent another person from getting the disease and not the person getting vaccinated. It is considered that transmission vaccines would target a smaller number of parasites compared to pre-erythrocytic vaccines. It is calculated that 10^{10}-10^{11} sporozoites are injected by a mosquito in one single bite, while each mosquito would ingest 10^3 macrogametocytes [127]. As an example of a TBV, a protein-based vaccine using the ookinete surface protein

Pfs25 was studied. It was found that the antibodies against this protein, drawn in during a mosquito bite, could block parasite development in the mosquito midgut, preventing transmission to other individuals [128].

Pre-erythrocytic stage vaccines have the advantage of potentially obstructing the development of the parasite into the merozoite form, responsible for the infection and disruption of red blood cells and the clinical symptoms of malaria. Therefore, pre-erythrocytic stage vaccines represent an ideal vaccination strategy compared to other stages. The following text describes the current state in the development of pre-erythrocytic vaccines focused on *P. falciparum*.

1.2.5.1 Vaccines for pre-erythrocytic stage malaria caused by *P. falciparum*

Some of the most promising vaccines candidates for pre-erythrocytic malaria are irradiated and live-attenuated sporozoites [129], [130]. These vaccines are shown to provide sterile protection in humans. In the case of the PfSPZ vaccine (an irradiated whole sporozoite-based vaccine), protection has been linked to a breadth of T and B cell responses and the formation of tissue-resident $CD8^+$ T cells in the liver [105], [131]–[133]. The efficacy and protection of the vaccine are being currently tested in endemic areas of the disease and also using a controlled human malaria infection approach (CHMI) with variably but generally positive outcomes [105], [132], [134]–[136].

In the case of genetically-attenuated parasites, their induced immunogenicity has been tested in humans [129], [130]. By administering the sporozoites to humans by mosquito biting, individuals could develop neutralising antibodies and T cell responses and no parasitaemia was observed. Nevertheless, its

protective efficacy has only been proved in mice so far. It is believed that increased efficacy in humans will require the arrest of the parasite development in later stages of the liver infection and not early stages as it has been currently tested [137].

RTS,S is the most advanced malaria vaccine to date. This pre-erythrocytic malaria vaccine is a virus-like particle (VLP) based on the Hepatitis B small protein (HBsAg) fused to the C-terminus region of the CSP (circumsporozoite protein) of *P. falciparum* [138]. The vaccine formulation, developed by GSK, also contains the adjuvant AS01. This is a liposome-based adjuvant containing two immunostimulants: 3-O-desacyl-4'-monophosphoryl lipid A and the purified saponin, QS-21. The adjuvant is described to increase the $CD4^+$ T and B cell responses from RTS,S, enabling protection. The vaccine has completed phase III trials and is currently undergoing pilot implementation in sub-Saharan Africa. In clinical studies in children, the vaccine reduces clinical cases of malaria by approximately 36-45%. In these Phase III trials, clinical symptoms were followed up for up to four years. The vaccine required four immunisation to achieve this level of protection [139]. The partial protection conferred by the vaccine was later to be correlated to antibodies against the CSP of the sporozoite [140].

The efficacy of a new version of RTS, S called R21 and developed by The Jenner Institute of the University of Oxford is also being studied. R21 contains more CSP antigens per VLP subunit compared to RTS, S. This is thought to induce higher immunogenicity levels against the parasite-relevant sections of the vaccine, potentially making it more effective than RTS,S [141]. The vaccine was tested with adjuvants AS01 and Matrix-M in Phase I/II clinical trials by the same institute and Matrix-M is being taken forward to field efficacy trials [137].

Viral vectors have been explored due to their capacity to induce high levels of $CD8^+$ T cell responses, a desirable feature for pre-erythrocytic stage malaria. One of the most advanced vaccines against pre-erythrocytic-stage malaria comprises the use of a chimpanzee adenovirus (ChAd63) in a prime-boost vaccination regimen with Modified Vaccinia Ankara (MVA) virus. The most widely tested antigen is TRAP, thrombospondin-related adhesion protein, that the parasite uses to attach to hepatocytes during host invasion. Vaccination using the aforementioned immunisation regimen and TRAP has reported variable levels of protection depending on the experiment set up (CHMI versus natural exposure) and age (adults versus infants) [142]–[144]. The use of CSP as the pre-erythrocytic antigen expressed by the viral vectors has failed to increase protection levels achieved by TRAP using the same viral vector immunisation strategies [145].

A summary of the vaccine platforms being tested for pre-erythrocytic stage malaria is described in **Table 1.5**. The table includes the advantages and disadvantages of using each vaccine platform, their reported induced immunogenicity and their stage of development (pre-clinical or clinical stages).

Table 1.5. Vaccines under development for pre-erythrocytic malaria.

Vaccine	Target	Use	Advantages	Problems	Status
RTS,S/AS01 (virus-like particle) [138]–[140]	C-terminal region CSP of *P. falciparum* containing $CD4^+$ T and B cell epitopes	Active immunization	Protection (partial) in clinical trials	Partial and short-lived protection, secondary effects (febrile convulsions cases have been reported)	Phase III and pilot implementation
R21/AS01 and R21/Matrix-M (virus-like particle) [141], [146]	C-terminal region CSP of *P. falciparum* containing $CD4^+$ T and B cell epitopes	Active immunization	Higher levels of antibodies and $CD4^+$ T cell responses towards CSP, compared to RTS, S, making it more effective than RTS,S. In human trials, 2-10 µg doses of R21 induce comparable humoral responses than 50 µg of RTS.S	To be determined in clinical studies, most likely waning protection as in the case of RTS,S	Phase I, IIa
FMP013 (virus-like particle) [137], [147]	Full CSP of *P. falciparum*	Active immunization	Induction of a wider breadth of antibodies against the CSP (inclusion of the N terminus region of CSP), compared to R21 and RTS,S	To be determined in clinical studies, most likely waning protection as in RTS,S and R21	*Pre-clinical*
PfSPZ (irradiated sporozoites) [105], [131], [133]	Whole sporozoite and early liver stages	Active immunization	Highest level of protection (sterile protection), well-tolerated	Higher protection levels by intravenous vaccination might compromise deployment of the vaccine, also, difficulties in the scale-up process of production of the vaccine	Phase IIb
GAP (genetically-attenuated	Whole sporozoite and	Active	High level of protection, well-	To be determined in clinical studies, difficulties during the scale-up process of	Phase I

Vaccine	Target	Use	Advantages	Problems	Status
parasites) [129], [137]	early liver stages	immunization	tolerated	the production of the vaccine	
Viral vectors (ChAd63-MVA prime-boost immunisation) [142], [145]	CSP (circumsporozoite protein) and TRAP (thrombospondin-related adhesion protein) *P. falciparum*	Active immunization	High levels of protective $CD8^+$ T cell responses. Intravenous immunisation further increases responses	Long-term immunity is under investigation	Phase I/II

1.3 Viral vectors for global infectious diseases

This section aims to provide a general view of the use of different viral-vectored vaccines for infectious diseases of global health importance. The section provides advantages and disadvantages of the use of the different viral vectors and further challenges concerning their use. The section highlights the use of adenovirus-based vaccines as one of the most promising viral-vectored vaccines to eradicate global infectious diseases, including malaria and MERS coronavirus. This section does not cover the use of genome-integrating viral vectors as these convey enormous challenges for deployment due to their low safety.

Viral vectors represent a promising tool for the eradication of infectious diseases that require a broad range of immune responses, including $CD8^+$ T cell responses and antibody responses. This is the case of intracellular pathogens, including viruses and intracellular bacteria and parasites. Viral vectors have been mostly employed for diseases where a commercial vaccine has not been achieved or where further improvements in immunogenicity and protection are needed. Examples of this are HIV, malaria, Dengue virus, SARS-CoV and Ebola virus. Viral vectors have been extensively studied in mouse models with some entering clinical trials.

Among the advantages of using viral vectors for vaccination over other vaccine platforms are the high transduction capacity of the viruses used for vaccination, therefore impacting on the antigen expression and immunogenicity. In addition, there is the avoidance of adjuvants, the robust induction of both T cell and

antibody responses, and also their safety and relatively rapid manufacturing [148].

The induction of a broad range of immune responses, especially T cell and antibody responses is accomplished due to the high capacity of the recombinant and attenuated viruses to infect the host cells and expressed the pathogen-relevant antigen (or antigens). The $CD8^+$ T cell responses are induced by this means by MHC Class I antigen presentation. The induction of high antibody responses is accomplished by the transgene expression of proteins being exposed to naïve B cell as secreted soluble proteins or anchored proteins on the surface of the host cells.

Viral vectors do not generally require adjuvants. Their virus components induce diverse innate responses including interferons and pro-inflammatory cytokines that impact the induction of adaptive responses [149].

Nevertheless, the use of viral vectors also has some disadvantages that need to be addressed before widespread deployment. One of the main potential disadvantages is the finding of anti-vector immunity for some strains of the viral vectors in the general population that may thwart the transduction efficacy of the virus, and therefore their immunogenicity. This has led researchers to investigate other strains that are not likely to be circulating among humans, such as the use of chimpanzee adenovirus. The complete evaluation of genetic stability and replication-deficiency is necessary prior to clinical research with this type of vaccine platform. In addition, large-scale studies require a relatively demanding GMP manufacturing infrastructure.

The following sections aim to provide a brief description of each type of viral vector being used for vaccination.

1.3.1 Vaccinia virus

Vaccinia virus is an enveloped DNA virus of the poxvirus family. This virus was historically used for eradicating smallpox; therefore, its protection potential has been demonstrated. There are various vaccinia virus strains that are under investigation for vaccination, among them the strains NYVAC, TROVAC and MVA [148]. These highly attenuated vaccines are not replication-competent *in vivo*, but others under development do have this feature, such as the strain LC16m8.

The large genome of vaccinia viruses has the advantage of permitting the insertion of large antigens (approximately 25 kb inserts in a 190 kb genome).

The immunological potential and efficacy of vaccinia-based vaccines have been tested in different human trials for different infectious diseases such as HIV [150]–[153], hepatitis B [154], influenza [155], malaria [156], [157] and tuberculosis [158].

1.3.2 Adenovirus vectors

Approximately 50 human adenovirus serotypes have been identified. Human adenovirus type 5 (AdHu5) has been most extensively studied for vaccine development. This is due to the high manufacturing yields characteristic of this virus, in addition to their strong induction of $CD8^+$ T cell and antibody responses, and broad tropism.

Adenoviruses are non-enveloped DNA viruses. In the case of AdHu5, the virus contains a 36 kb linear DNA genome. Due to the wide cellular distribution of the adenovirus receptor CAR (Coxsackie and Adenovirus receptor), this viral vector has a wide range of cellular targets, potentially helping in the development of a wider immunological response.

For use as vaccines, adenoviruses are rendered replication-deficient by the genetic deletion of the E1 genome region. The immunomodulating E3 region is also frequently deleted. This also allows the insertion of a transgene in this locus (up to a maximum of ~8 kb).

One disadvantage of using adenovirus-based vectors is the potential of anti-vector pre-existing immunity in the general population. Studies have been made concerning the genetic modification of the vector capsid proteins. This has resulted in the diminishing of anti-vector immunity and increased vaccine efficacy in different studies [159]–[161]. In addition to this, researchers are testing other adenoviruses serotypes found in non-human species, which are subject to lower pre-existing anti-vector immunity in humans. Such vectors include chimpanzee adenovirus type 63 (ChAd63), which has been successful in clinical trials [162].

Recombinant Adenovirus vectors have been examined in diverse clinical trials against HIV [163], [164], influenza [165], malaria [142]–[144] and tuberculosis [166]. In the case of malaria, this vaccine platform is usually tested as a prime with MVA as a boost resulting in even higher levels of immunogenicity.

1.3.3 Cytomegalovirus

Human cytomegalovirus is a member of the *Herpesviridae* family also known as human herpesvirus type 5. The human cytomegalovirus causes a life-long, usually asymptomatic infection in healthy hosts. The enveloped and generally non-pathogenic virus contains a relatively large 235 kb double-stranded linear DNA genome. The transgene capacity of the virus is at least 6 kb [167].

The advantage of using human cytomegalovirus as a vaccination agent is due to its capacity of inducing a broad range of T cell responses, comprising approximately 10% of the entire $CD4^+$ and $CD8^+$ T cell memory compartment. This is accompanied by a usually long-lived quality of these responses, also in elderly individuals [168]. In mice, CMV-based vaccines have been shown to induce protective T cell responses against the Ebola virus, herpes simplex and tuberculosis [169]–[172]. In addition, rhesus cytomegalovirus (RhCMV) has been shown to provide considerable protection levels (approximately 50%) against a simian immunodeficiency virus (SIV) challenge in macaques. This protection was associated with memory T-cell responses [173].

1.3.4 Vesicular Stomatitis Virus

Vesicular Stomatitis Virus (VSV) is an enveloped bullet-shaped virus of the family *Rhabdovirus*. The virus has an 11 kb negative-sense RNA genome. Different advantages of this virus have attracted research attention. For example, the virus induces robust cellular and antibody responses and has a high production titre. The cargo transgene capacity of the virus is at least 4.5 kb.

The use of VSV for vaccination requires the attenuation by deletion of the G glycoprotein tail (a membrane fusion protein). This affects the capacity of the

virus to infect new cells. The attenuation of VSV is also accomplished by the downregulation of the nucleoprotein (N) expression. This negatively affects the capacity of the virus to form its nucleocapsid.

Since 2011, recombinant VSV has been studied as a vaccine for HIV-1 in clinical trials [174]–[177] and Ebola [178]–[180]. Results from these studies show robust induction of neutralising antibodies and modest $CD8^+$ T cell responses for Ebola. Nevertheless, for HIV, the results have been discouraging as the vaccine induced dramatically low levels of these responses [174]. But high efficacy was observed in Guinea for Ebola.

1.3.5 Measles virus

Measles virus is an enveloped spherical virus with a 16 kb negative-sense RNA genome. It is a member of the *Paramyxovirus* family. Despite its innate pathogenicity, it has been used as a vaccination platform since the 90s. Many of the attenuated measles viruses used today for vaccination are derived from the strain Edmonston. This strain was then adapted to different cell cultures, transforming the virus in a highly attenuated form [181]. Most of the knowledge about this type of vaccines comes from vaccination trials for the measles itself. Here, the vaccine has shown to induce durable protective antibody and T cell-mediated responses (mainly $CD4^+$ T cell responses) [182]–[184].

Among the advantages of using recombinant measles viruses are the low cost of large-scale manufacturing and the induction of robust responses of $CD4^+$ T cells for infectious diseases where this response is important (e.g. HIV). Nevertheless, one of the main disadvantages of using this vaccine platform is

the potential of encountering anti-vector immunity since measles vaccination is part of childhood immunisation programs in many countries.

Recombinant measles vaccines are currently under clinical assessment for HIV-1 [185] and Chikungunya [186]–[188]. In the studies for Chikungunya, they have shown to create robust antibody titres for 100% of the individuals tested (prime-boost vaccination regimen), with a good tolerability profile at lower doses of the vaccine [186].

1.3.6 Sendai virus

Sendai virus (SeV) is an enveloped and non-pathogenic, single-stranded RNA virus of the *Paramyxoviridae* family. This virus has been rendered replication-deficient by eliminating the F protein that mediates cell entry. The transgene capacity of the virus is low compared to other viral vectors (3.4 kb), limiting the use of SeV. Nevertheless, this virus has been tested in clinical trials as a vectored vaccine against the human parainfluenza virus type 1 [189], [190] and HIV [191], [192] showing robust and long term immunological responses (usually in combination with other vaccine platforms such as DNA and protein vaccines).

Table 1.6. Viral-vectored vaccines for global infectious diseases. *The table describes the main viral-vectored vaccine platforms being tested for global infectious diseases.*

Vaccine platform	Disease	Advantages	Problems	Status
Vaccinia virus [150]–[153] [154] [155] [156], [157] [158].	HIV, malaria, influenza, tuberculosis, hepatitis B	High and robust $CD8^+$ T cell responses, shown to provide protection in clinical trials, large capacity of transgene insertion	Variable protection levels in human trials, high demands for industrial manufacturing, side effects depending on dose, potential of anti-vector immunity	Phase I/II/III
Adenovirus [163], [164] [165] [142]–[144] [166]	HIV, malaria, Influenza, tuberculosis	High and robust $CD8^+$ T cell responses, shown to provide protection in clinical trials, wide tropism impacting on wider immunological responses	Possibility of anti-vector immunity impacting on the vaccine immunogenicity and efficacy, issues due to induced hepatoxicity, but that can be improved (high i.v. doses)	Phase I/II/III
Adeno-associated virus (AAV) [148]	HIV, Influenza, papillomavirus	Induce $CD8^+$ T cell responses	Lower immunogenicity than other viral vectors (example, adenovirus and MVA). Low production titres and small transgene insertion capacity. Safety concerns due to the still potential of genome integration	Pre-clinical
Cytomegalovirus (CMV) [173]	SIV	Broad range and high frequency of memory $CD8^+$ T cell responses (including non-canonical recognition of MHC II epitopes), protective in non-human primates	Safety concerns in immunocompromised individuals, still need further studies (clinical trials)	Phase I
Vesicular-stomatitis virus (VSV)	Ebola, HIV-1	Induces cellular and antibody responses against the transgene antigen, high production titres	Safety concerns depending on the ability to attenuate the virus	Phase I, II, III

Vaccine platform	Disease	Advantages	Problems	Status
Sendai virus [189]–[193]	Human parainfluenza virus type 1, HIV (replication-competent VSV)	Endurable T and B cell immunogenicity, highly transduction capacity, low genotoxicity due to the fast transgene expression	Low transgene insertion capacity, potential of anti-vector immunity in the general population, potential necessity to be used in combination with other vaccine platforms	Phase I
Measles virus	HIV-1, Chikungunya	Low-cost large-scale production, induction of both antibody and T cell responses	Implications of pre-existing immunity	Phase I, II

1.4 Recent improvements of adenovirus-based vaccines

From the viral vectors platforms previously described, adenoviruses represent one of the most powerful tools for the eradication of infectious diseases. This is due to their capacity to harbour large transgenes, the induction of high and broad levels of immunological responses (including $CD8^+$ T cells and antibodies), their safety and high production yield. Nevertheless, there is a space for improvements in adenovirus-based vaccines. These improvements are explained in this section.

In the case of adenovirus-based vectors, there are two main opportunities for immunogenicity enhancement. The first one is the avoidance of pre-existing anti-vector immunity. The second is the enhancement of transgene immunogenicity.

In the case of AdHu5, the negative effect of pre-existing immunity was made evident in clinical trials [194], [195]. Therefore, many groups have explored adenoviruses with lower seroprevalence in the human population, such as the case of low circulating human serotypes and simian ones. For example, human adenovirus type 26 and 35 have been tested clinically for HIV-1 (Phase I). Here, these adenoviruses showed induction of high antibody responses and modest T cell responses when performed in a prime-boost vaccination regimen with MVA [196]. Human adenovirus type 26 has also been tested in combination with MVA for Ebola in clinical trials, showing strong and durable antibody responses [197], [198]. In addition, the human adenovirus type 35 has been tested for HIV and has been shown to also be highly immunogenic and safe [199]–[202].

In addition to human serotypes, simian adenovirus-based vaccines have been tested in clinical trials. As an example, chimpanzee adenovirus type 3 (ChAd3) has been tested for Ebola Zaire in Phase I and II clinical trials [203]–[205]. This vector was shown to elicit strong antibody and T cell responses, increased by an MVA boost. In addition, the chimpanzee adenovirus type 63 (ChAd63) has been evaluated for malaria [144], [206], [207] and leishmaniasis [208] with competitive immunogenicity and safety.

The induced immunogenicity of the simian adenovirus was found to vary depending on the serotype, the route of administration and the host immune response. As an example, chimpanzee adenovirus type 7 (ChAd7) induces superior protective mucosal immunity in the respiratory tract, if administered by the mucosal route and as compared to a systemic administration. This superior immunogenicity has been linked to prolonged persistence in antigen-presenting cells that are enriched in the mucosal cavities [209].

In the case of the improvements towards the transgene immunogenicity, adenovirus-based vaccines have been recently engineered to further increase their levels of transgene expression. As an example, the immunogenicity of adenoviruses has been improved by allowing the virus to perform a round of replication (single-cycle adenoviruses) *in vivo*. These adenoviruses, therefore, can enter the host cells allowing a single round of replication and more transgene expression (approximately 30 to 100-fold copies of the transgene compared to completely replication-deficient adenoviruses). This has been possible through the deletion of the pIIIa structural gene [210]. The higher transgene immunogenicity of single-round replication adenoviruses compared to

completely replication-deficient adenoviruses have been shown in mice [211], [212].

Recent improvements in adenovirus transgene expression include the use of more potent promoters driving transgene expression and new leader sequences modulating the trafficking and presentation of antigens. As an example, the replacement of the innate leader sequence of a transgene antigen with a tissue Plasminogen Activator leader sequence (tPA-LS) has been described to increase the production and/or secretion of the antigen from AdHu5. This has been linked to higher transgene antibody responses in pre-clinical studies [213].

On the other hand, tPA-LS has shown to reduce the T cell immunogenicity of an HIV-1 chimeric antigen in a ChAd63-MVA prime-boost immunisation regimen in mice [214]. This tPA leader signal makes the expressed antigen to be directed to the endoplasmic reticulum and the cellular secretory pathway, therefore promoting the secretion of the antigen. Extracellular foreign proteins are then better targets for the induction of antibody responses and MHC Class II antigen-presenting cells. This then impacts on the levels of $CD4^+$ T cell responses [214], [215]. In the case of enhancing $CD8^+$ T cell responses, this can be achieved by dendritic cells that can present peptides by an MHC Class I complex after taking up the exogenous antigen (cross-presentation) [214].

The transgene immunogenicity can also be enhanced by the addition of different promoters controlling the transgene expression in adenovirus. As an example, the synthetic CAG promoter controlling the expression of the envelope glycoprotein of *Zaire ebolavirus* in AdHu5 induces higher transgene T and B cell immunogenicity than the promoter CMV (10-100 fold), providing higher

protection levels in mice [216]. As another example, the inclusion of the endogenous intron A in the CMV promoter sequence also induces higher transgene expression and immunogenicity levels in mice in AdHu5 [217].

The effect of other genetic designs for the enhancement of transgene expression and immunogenicity such as the inclusion of Kozak consensus have also been described for adenoviruses and generally accepted that have a positive effect on the transgene immunogenicity [218], [219].

Recent improvements in adenovirus vaccines also target their production process. Recently, chimeric chimpanzee adenoviruses have been investigated to achieve higher production titres. As an example, ChAdOx1, a chimeric chimpanzee adenovirus derived from the serotype Y25 has been recently created by inserting incorporating E4 gene products from AdHu5. This has positively impacted on the production yield of the virus compared to its parental virus [220]. ChAdOx1 has been already tested in clinical trials, inducing competitive T cell responses to other non-chimeric chimpanzee adenoviruses such as ChAd63 [221].

1.4.1 Adjuvants for adenovirus-based vaccines

Adjuvants are also recent improvements being studied for adenoviruses. This section aims to provide general information about their use for this type of viral vectors. Due to the extent of this topic, this has been divided into chemical traditional adjuvants and genetically-encoded adjuvants.

Traditionally, adjuvants are substances that in combination with an antigen induces a more robust immune response than the antigen alone [222]. The mechanism of the adjuvant might be related to their capacity to create an

antigen depot, induce cytokines and chemokines, recruit immune cells, enhance antigen uptake and presentation, and promoting antigen transport to the draining lymph nodes [222].

The effect of adjuvants has been for long studied in protein-based vaccines. Nevertheless, knowledge of their effect on viral vectors (including adenoviruses) is extremely scarce. This is more likely due to the understanding that viral vectors do not usually require the help of adjuvant to induce a robust immunological response.

Nevertheless, in the case of intracellular parasites such as *Plasmodium spp.*, there is a need for a further increment of transgene-specific $CD8^+$ T cell responses induced by viral vectors, including adenoviruses. This was exemplified by the finding that the most advanced viral-vectored vaccine for liver-stage malaria based on ChAd63 (and boosted with MVA) only induced 25 % sterile protection in naïve individuals [142]. In this case, adjuvants, either genetically-encoded or chemical, have been explored to try to achieve higher immunological responses from adenoviruses.

1.4.1.1 Chemical adjuvants

Chemical adjuvants have been shown to enhance responses to adenovirus-based vaccines. As examples, the chimpanzee adenovirus serotype 63 encoding the $CD8^+$ T cell-restricted epitope Pb9 from *Plasmodium berghei* was tested in a mixture with different commercial adjuvants. From 11 adjuvants tested, only 2 (CoVaccineHT™ and Abisco®-100) could enhance the protective levels of the vaccine which correlated with the CD107a expression of antigen-

specific $CD8^+$ T cells [223]. In the case of the adjuvant CoVaccineHT™, this adjuvant could even double the protection level of the adenovirus vaccine, an effect that was also dependent on the route of administration. The chemical adjuvants AddaVax and Matrix-M™ have also been tested with the chimpanzee adenovirus ChAdOx1 and AdHu5 for Rift Valley fever where they showed variable immunogenic profiles depending on the viral vector used, in mice [224]. As an example, AddaVax increased $CD8^+$ T cell responses of AdHu5, but not ChAdOx1; and Matrix-M did not have a significant effect on these cellular responses for either of the two viral vectors. In contrast, AddaVax induced higher transgene-specific antibody responses for ChAdOx1, but not for AdHu5. In a further experiment, Matrix-Q™ (an adjuvant similar to Matrix-M) did not have a positive effect on antibody responses with ChAdOx1 in cattle species [225]. This last finding highlights the need for further studies for the development of chemical adjuvants if they are to be tested in other species different from mice.

The adjuvants AddaVax, Abisco-100, and Carbopol have been also tested employing AdHu5 and MVA in a heterologous prime-boost immunisation regimen. Even though the adjuvant Carbopol showed a detrimental effect on the viral-vectored immunogenicity, the adjuvants AddaVax and Abisco-100 significantly enhanced the antigen-specific antibody responses in this vaccination regimen and for a malaria-related antigen in mice experiments [141].

Chemical adjuvants can also serve for better delivery of the viral vectors. DEG-PEI, a new polymeric delivery system, has shown to facilitate the mucosal administration of AdHu5 expressing the HIV Gag protein in mice, helping in the

enhancement of $CD8^+$ T cell and IgA antibody responses [226]. In addition, chitosan (a glycosylated natural polymer) has been also explored for delivering adenoviruses by the mucosal route. Chitosan has been tested with AdHu5 delivering the glycoprotein D of the bovine herpesvirus 1, this impacting on the protection levels in calves [227]. The immunological effects of chitosan are explained by the binding of the adjuvant to receptors including Dectin-1, macrophage mannose receptor and TLR 2 [228], and the slowdown of adenovirus infection, therefore prolonging the time of transgene expression.

In comparison with genetically-encoded adjuvants, chemical traditional adjuvants have some advantages and disadvantages. Among the first, there is the relative ease of adding low-cost substances to the vaccine formulation that do not require the generation and development of “adjuvant genes” and the subsequent molecular biology and virology steps for its insertion in adenovirus vectors. Nevertheless, chemical traditional adjuvants also have some constraints. As an example, chemical adjuvants might have more unpredictable effects on viral vectors. This has been the trend when testing chemical adjuvants, at least for adenovirus-based vaccines, resulting in the null or decreasing immunogenicity effect towards the transgene antigens. In addition, the co-delivery of chemical adjuvants with the transgene antigen cannot be assured. Therefore, the potential of targeting key immunological cells when using chemical adjuvants might be difficult.

1.4.1.2 Genetically-encoded adjuvants

Different genetically-encoded adjuvants have been tested with adenoviruses in order to enhance T cell and antibody-mediated immunogenicity. In this case, genetically-encoded adjuvants aim to express a component during T cell or B cell activation or proliferation, potentially impacting on the induction of effector and memory responses. As an example, the invariant chain (Ii) of the MHC Class II complex fused to the transgene antigens in adenovirus-based vectors has been successful in enhancing $CD8^+$ T cell responses in mice and non-human primates using the chimpanzee adenovirus type 63 and for malaria and Hepatitis C Virus antigens [229], [230]. The positive effect of the invariant chain has been attributed to their function in antigen presentation. In this case, the invariant chain acts as a scaffold for MHC Class II assembly in the endoplasmic reticulum [231], [232]. It also prevents binding to the MHC Class II complex of endogenous peptides [233] and it directs MHC Class II (and MHC Class I in cross-presentation) to endolysosomal compartments [234], [235].

Other genetically-encoded adjuvants have also been tested in adenoviruses. The tumour necrosis factor receptor superfamily (TNFRS) ligand, 4-1BBL, has been tested in AdHu5 to enhance $CD8^+$ T cell responses for malaria. In this research, a bicistronic viral vector was constructed encoding the *Plasmodium berghei* antigen Pb9 (a $CD8^+$ T cell epitope) and 4-1BBL. This approach allowed the enhancement of $CD8^+$ T cell responses against the peptide Pb9 in mice [236]. In this case, the enhanced effect was attributed to the known directional positive signalling induced by 4-1BBL during T cell priming. This results in a higher secretion of cytokines from T cell and dendritic cells and the subsequent higher antigen-specific T cell proliferation [237]–[241]. Several other TNFRS

ligands have been studied after being expressed by AdHu5. In this regard, BAFF, but not GITRL and CD27L, induced protection against a vaccinia virus expressing the HIV antigen Gag in a mouse model [242].

Ligands of Toll-like receptors (TLR) have been also explored to enhance adenovirus transgene immunogenicity as genetically-encoded adjuvants. This is due to the capacity of these ligands to enhance the uptake and presentation of vaccine antigens by antigen-presenting cells (APC), induction of APC maturation and migration of these APCs to the draining lymph nodes [243]. These processes then impact on the induction of $CD4^+$ and $CD8^+$ T cell responses. The inclusion of TLR 3 and 4 agonists as genetically-encoded adjuvants have been tested in AdHu5 for HIV [244], [245], Influenza A [246] and Leishmaniasis [247]. These studies have shown induction of higher $CD8^+$ T cell responses against the encoded antigen (compared to the no inclusion of the genetically-encoded adjuvant) in clinical trials.

STING agonists have also been proposed and tested as genetically-encoded adjuvants for adenoviruses. It has been described that the AdHu5 expression of a bacterial enzyme synthesising the STING agonist ci-diGMP enhances the immunogenicity against the capsid proteins of AdHu5 [248]. Nevertheless, the expression of the enzyme and the concomitant synthesis of ci-diGMP had a negative effect on the immunogenicity of another AdHu5 mixed with it and expressing Gag and toxin B antigens in mice. The enhancement in immunogenicity by the use of STING agonists has been linked to the induction of type I IFNs that in turn activate dendritic cells [249]–[251] and enhances T cell activation [252]. Type I IFNs have also been linked to a B cell activation and Ig class-switching [253]–[255].

Encoded heat-labile enterotoxins of *Escherichia coli* have also been tested in their ability to enhance neutralising antibodies from AdHu5. In this case, AdHu5 was engineered to express the enterotoxins LTB or LTR72 and these viral vectors were mixed with an AdHu5 expressing the A24 antigen from the human foot and mouth disease (HFMD). This enhanced the protection levels in mouse experiments [256].

The immunological effects of genetically-encoded adjuvants in adenoviruses are varied and most likely depend on the antigen tested, the animal model and the route of vaccination. One of the main advantages of genetically-encoded adjuvants over chemical adjuvants is the capacity of these adjuvants to make the host cells to synthesise the adjuvant, therefore impacting on the cost-effectivity of the vaccines. In addition, by these means, the adjuvant is co-delivered with the antigen, therefore, having the possibility to further refine immunological effects (co-deliver of both elements in the same cell). Among the disadvantages of genetically-encoded adjuvants is the necessity to incorporate non-human adjuvants to avoid the potential of triggering self-protein immunogenicity. This makes it more difficult to predict the desired effects of these types of adjuvants when moving from mice to human experimentation.

The following tables summarise main development efforts in the use of chemical (**Table 1.7**) and genetically-encoded adjuvants (**Table 1.8**) for adenovirus-bases vaccines. The tables include the reported effects in other vaccine platforms and in adenoviruses and the mechanism of action of each adjuvant.

Table 1.7. Chemical adjuvants successfully tested with adenovirus-based vaccines.

Adjuvant	Composition	Mechanism of action	Reported effect in other vaccine platforms	Immunological effect in adenovirus
ISCOMATRIX™ (Novavax)	Nanoparticles made of saponin mixed with synthetic cholesterol and a phospholipid [257]	Stimulation of infiltration of antigen-presenting cells in the site of injection and enhancement of antigen presentation (MHC Class I and II) in draining lymph nodes [257]	Reported increase of cellular (Th1/Th2, $CD8^+$ T) and antibody responses, prolonged immune responses and low allergic reactions [257]	Increased levels of protection correlated with serum IL-5, IL-6, and IFN-γ and $CD8^+$ $CD107a^+$ T cells, but not to antigen-specific $CD8^+$ T cell and antibody responses from spleens at the peak of immune response using ChAd63 for a malaria antigen (Abisco®-100) [223] Increased antibody responses for a ChAd63-MVA vaccination regimen for a malaria-related antigen (Abisco®-100) [141] Increased levels of protection correlated with neutralising antibodies for an envelope protein of Rift Valley fever using AdHu5 and ChAdOx1 in mice but not in cattle (Matrix-M/Q™) [224], [225]
AddaVax™ (InvivoGen)	Squalene-based oil-in-water nano-emulsion [258]	Working as a depot effect, the adjuvant helps in the recruitment and activation of APC and stimulation of cytokines and chemokines production by macrophages and granulocytes [258]	Reported increase in antibody titres and cellular responses through a more balanced Th1/Th2 response than those obtained with alum [258]	Increased levels of protection correlated with neutralising antibodies for an envelope protein of Rift Valley fever using AdHu5 and ChAdOx1 in mice. Furthermore, increased levels of $CD8^+$ T cell responses for AdHu5 [224] Increased antibody response for a ChAdOx1-based vaccine for rabies (G glycoprotein) [259]

Adjuvant	Composition	Mechanism of action	Reported effect in other vaccine platforms	Immunological effect in adenovirus
				Increased antibody responses with ChAd63 for a malaria-related antigen, also in combination with MVA. Also, a trend in induction of $CD8^+$ T cell responses [141]
CoVaccineHT™ (British Technology Group)	Oil in water emulsion. The oil phase consisting of synthetic sucrose fatty acid sulphate esters immobilised inside the oily droplets of squalene [260]	Maturation of dendritic cells depending on TLR4 [260]	Antibody and cell-mediated responses for a diverse range of vaccine platforms. Induction of memory responses and low reactogenicity than other oil in water emulsions, Increasing of protection levels.[261]	Increased levels of protection correlated with serum IL-5, IL-6, and IFN-γ and $CD8^+$ $CD107a^+$ T cells, but not to antigen-specific $CD8^+$ T cell and antibody responses from spleens at the peak of immune response using ChAd63 for a malaria antigen [223]
Montanide™ ISA (SEPPIC)	Water in oil emulsion formed by mannide monooleate surfactant and oil [262]	Activation of dendritic cells, increment of antigen uptake by DC [263]	Increased $CD4^+$ and $CD8^+$ T cell responses [262]	Increased $CD4^+$ T cell responses for AdHu5 expressing an antigen from foot and mouth disease in cattle [263] and *Mycobacterium* antigen Ag85A [264]
DEG-PEI	Biodegradable polymer-based on di-ethylene and polyethylene glycol	Retention of antigen in the mucosal cavities and better immune responses due to the formation of particles [265]	$CD8^+$ T cell responses and IgA antibody responses [266]	$CD8^+$ T cell responses and IgA antibody responses for an HIV-1 vaccine based on AdHu5 by intranasal vaccination [266]
Chitooligosaccharides and mannosylated polyethyleneimine-	Oligosaccharides and olisaccharised polymers	Retention of the antigen in the mucosal cavities and activation of dendritic cells by targeting of	Th1/Th2 responses impacting on the $CD4^+$ and $CD8^+$ T cell responses, and the enhancement of IgA	Th1/Th2 responses impacting on the $CD4^+$ and $CD8^+$ T cell responses, and the enhancement of IgA antibodies for AdHu5-Gag (HIV-1 vaccine)

Adjuvant	Composition	Mechanism of action	Reported effect in other vaccine platforms	Immunological effect in adenovirus
triethyleneglycol (mPEI)		these cells [265]	antibodies [267]	[267]
Chitosan	Biodegradable polysaccharide	Retention of antigen in the site of injection by the mucosal-adhesive properties of the adjuvant [265]	Increased levels of neutralising antibodies and protection [265]	Increase levels of protection for a bovine herpesvirus 1 using AdHu5 [227]

Table 1.8. Genetically-encoded adjuvants successfully tested for adenovirus-based vaccines

Adjuvant	Description	Mechanism of action	Reported effect in other vaccine platforms	Immunological effect in adenovirus
Tumour necrotic factor superfamily ligands (TNFSL)	Proteins belonging to the tumour necrosis factor superfamily in the host cells participating as co-stimulatory molecules during adaptive immune responses [268]	Induces positive feedback secretion of cytokines leading to T and B cell priming and proliferation [236]	Increased $CD4^+$ and $CD8^+$ T cell responses, increased protection levels [242]	Increased $CD8^+$ T cell responses from AdHu5 for malaria [236] and HIV-related antigens [242], increased protection levels in mice (4-1BBL, BAFF) Induction of $CD8^+$ T cell responses, but not protection levels for an HIV-1 antigen (IL-12p70) [242]
Escherichia coli heat-labile enterotoxins LTB or LTR72	Six-component protein deactivated by heat and secreted by *E. coli* during cell invasion [269]	Induces secretion of inflammatory cytokines, dendritic cell (DC) recruitment and activation, and initiation of antigen presentation [270]	Induced T cell and antibody responses (including IgA) in the mucous compartments, depending on the antigen and route of administration [270]	Increased neutralising antibodies and protection levels for foot and mouth disease in mice using AdHu5 [256]

Adjuvant	Description	Mechanism of action	Reported effect in other vaccine platforms	Immunological effect in adenovirus
Invariant chain (CD74, Ii)	Cluster of differentiation protein, part of the MHC Class II complex	Part of the MHC Class I/II antigen presentation, helping in the APC:T cell priming [271]	Increased $CD4^+$ and $CD8^+$ T cell responses and levels of protection [229]	Increased $CD8^+$ T cell responses, increased protection levels for LCMV [272], *Listeria monocytogenes* [273] and malaria [229], [271] using AdHu5 and ChAd63
Vibrio cholerae diguanylate cyclase (synthesises ci-di-GMP)	Bacterial enzyme expressing the second messenger cyclic di-GMP (CDG) [248]	Enhances antigen uptake and activation of dendritic cells [274]	Increased antibody and T cell responses ($CD4^+$ and $CD8^+$) [275]	T cell responses against the capsid proteins of AdHu5 and extracellular antigens mixed with the viral vector [248]
α-chain of C4b	α-helix domain of the inhibitor of the classical and lectin pathways of complement activation C4b [276]	Induces the oligomerization of immune complexes, most likely through Fcγ receptors binding or other unknown mechanisms [276]	Increased antibody responses [276]	Increased antibody and T cell responses from malaria-related transgenes in AdHu5, including protection levels in mice [276]
TLR 3/4 agonists	Structurally conserved microbial products (PAMPs; dsRNA and lipopolysaccharides) being sensed by Toll-like receptors expressed on sentinel cells such as dendritic cells and macrophages [277]	Uptake and presentation by APCs, induce maturation, and migration of APCs to draining lymph nodes [278]	Induction of pro-inflammatory cytokines and increase of Th1-related cytokines, increased protection levels [244]	Expression of TLR agonist from *Eimeria tenella* encoded by AdHu5 increased $CD8^+$ T cell responses against encoded HIV-1 Gag [244] TL3 agonists enhanced T cell transgene immunogenicity of an AdHu5 for Influenza A in humans [246] Increased $CD4^+$ and $CD8^+$ T cell responses and protection levels of an AdHu5 expressing TLR 4 agonist (GLA-SE) for a *Leishmania donovani* antigen [247]

1.5 Thesis aims and outline

Adenovirus-based vaccines represent a highly promising tool for the prevention of global infectious diseases. Nevertheless, their performance has not always been satisfactory when translated to human vaccination. Recent improvements in adenovirus-based vaccines try to tackle this issue by increasing the immunogenicity, safety profile and production yield of these viral vectors.

This thesis aims to explore recent improvements in adenovirus-based vaccination, focusing on two global infectious diseases: MERS coronavirus and malaria.

In the case of MERS coronavirus, the objective was to create and optimise a highly immunogenic vaccination regimen based on adenoviruses prime and poxvirus boost. The optimisation focused on the study of the immunological properties of different genetic designs modulating transgene expression.

In the case of malaria, the aim was to investigate the immunological effects of different adjuvants for a model adenovirus-based vaccine. These adjuvants, denominated spermidine and 2'3'-cGAMP, were further optimised to enhance the immunogenicity of the adenovirus-based vaccines.

1.5.1 Aims

1) To develop a highly immunogenic vaccination regimen for MERS coronavirus.
2) To assess the immunological effect of the adjuvant spermidine on adenovirus and poxvirus vectors.

3) To assess the immunological effect of the adjuvant 2’3’-cGAMP on adenovirus and poxvirus vectors.
4) To optimise the use of spermidine and 2’3’-cGAMP for their application with adenoviral and poxvirus vectors.

1.5.2 Outline

Chapter 2 describes the materials and methods used in this study.

Chapter 3 describes the creation of a highly immunogenic vaccination regimen against MERS coronavirus.

Chapter 4 explores the immunological effects of spermidine on vaccines.

Chapter 5 explores the immunological effects of 2’3’-cGAMP on vaccines.

Chapter 6 investigates the mechanisms of action of 2’3’-cGAMP and optimises the use of the adjuvant for vaccines.

Chapter 7 describes and summarises the results of these studies and discusses future directions.

Chapter 2 Materials and Methods

2.1 Ethics statement

Mice were used in accordance with the UK Animals (Scientific Procedures) Act under project license number P9804B4F1 granted by the UK Home Office. Animals were group-housed in individual ventilated cages under specific-pathogen-free conditions, with constant temperature, humidity with lighting on a 12:12 light-dark cycle (8 am to 8 pm).

To minimize distress and suffering during vaccination, animals were anaesthetized using vaporised isoflurane. Mice were monitored during the experiments by highly trained technicians and veterinaries for detecting changes in behaviour, the appearance of redness or swelling in the site of the injection. Blood draws were limited to a maximum volume of 15% of the total mouse blood volume in intervals of 28 days, in accordance with the terms of the project licence.

All animals were humanely sacrificed at the end of each experiment by an approved Schedule 1 method prior to collection of spleens and blood samples.

Shuttle vector cloning

For the construction of the Modified Vaccinia Ankara (MVA) viruses, the MERS S glycoprotein DNA sequence of the camel isolate Camel/Qatar_2_2014 was used (Genbank accession number: KJ650098.1). The DNA sequence was synthesised by GeneArt Gene Synthesis (Thermo Fisher Scientific). The DNA

sequence was cloned into a shuttle vector by In-Fusion PCR cloning (Clontech). The S glycoprotein gene was inserted between the upstream and downstream sequences of the F11 ORF of MVA.

A second version of this shuttle vector was created. For this, the mH5 (modified H5) promoter sequence was inserted directly upstream the S glycoprotein gene by In-Fusion cloning (also ending up being flanked by the F11 ORF upstream and downstream regions of MVA). The addition of the F11 ORF upstream and downstream regions flanking the S glycoprotein genes allowed their insertion in the MVA parental genome during the construction of the recombinant MVA viruses.

The expression of the MERS S glycoproteins from the shuttle vectors was evaluated by indirect fluorescence immunostaining (explained in detail in the result section). This expression was also evaluated by an in-house ELISA coating the plates with transfected HEK 293 cells and measuring the OD405 signal from bound antibodies (also explained in detail in the Results section).

For the construction of the replication-deficient (E1/E3-deleted) bicistronic human adenovirus type 5 vectors (AdHu5-mcGAS-TIPeGFP and AdHu5-**mut**mcGAS-TIPeGFP), the mouse cGAS gene (Genbank accession number: KC294567.1) was purchased from InvivoGen (France). The cGAS gene was then cloned into a shuttle vector by In-Fusion cloning (Clontech). The cGAS gene was inserted immediately downstream the human cytomegalovirus immediate-early (CMV-IE) promoter in the shuttle vector. This vector already contained a TIPeGFP synthetic gene under the control of the human Elongation Factor promoter EF1-α.

The TIPeGFP is a synthetic antigen comprising the peptides Pb9, P15 and AL11. These peptides are fused to an enhanced Green Fluorescence Protein (eGFP). The *Plasmodium berghei* $CD8^+$ T cell epitope Pb9 is an H-$2K^d$-restricted immunodominant protective epitope for pre-erythrocytic murine malaria [279]. The peptide P15 is a $CD4^+$ T cell peptide from *Mycobacterium tuberculosis* [279], [280]. The AL11 peptide is an H-$2D^b$-restricted $CD8^+$ T cell peptide coming from the gag protein of the Simian Immunodeficiency Virus (SIV) [281]. The inclusion of the eGFP enables the study of antibody responses against an encoded antigen and the study of an additional $CD8^+$ T cell peptide named $eGFP_{200\text{-}208}$ [282].

The catalytically inactive version of the cGAS gene (E211A and D213A, described in [283]) was created by site-directed mutagenesis of the aforementioned shuttle vector already containing the cGAS and TIPeGFP genes. These two created shuttle vectors contained AdHu5-specific E1 recombination sites flanking the two expression cassettes (cGAS and TIPeGFP) for posterior homologous recombination with a BAC parental genome of AdHu5.

2.2 Construction of viral vectors

Recombinant MVA viruses were created as previously described [284]. For this, chicken embryo fibroblast cells (CEFs) were infected with the MVA parental virus encoding a dsRed marker. The cells were also transfected with MVA shuttle vectors encoding the MERS spike glycoprotein gene and a GFP marker. This allowed the recombination of the shuttle vector with the MVA parental genome. Co-transfected cells were isolated by fluorescent selection using a

CyCLONE robotic sorting module of a MoFlo Flow cytometer (Dako Cytomation, Denmark).

Recombinant viruses were grown and further isolated by the plaque-picking method. MVA-MERS-S-F11 and MVA-MERS-S-mH5 viruses were confirmed to lack the dsRed marker and encode the MERS S glycoprotein gene by PCR and gel electrophoresis. The integrity of the DNA transgene sequence was further confirmed by PCR-Sanger sequencing. The recombinant MVAs were bulked up in 1500 cm^2 monolayers of CEFs cells, partially purified over sucrose cushions and titrated in CEFs cells according to standard practice. The purity and identity of the bulked-up recombinant MVAs were again verified by PCR-gel electrophoresis by the Jenner Institute Viral Vector Core Facility and Dr Naif Alharbi. MVA-MERS-S-F11, from vector cloning to virus isolation, was also performed by these parties.

The bicistronic human adenovirus type 5 vectors (AdHu5-TIPeGFP-mcGAS and AdHu5-TIPeGFP-**mut**cGAS) were prepared by Gateway® homologous recombination between the AdHu5 BAC parental genome and the shuttle vectors containing the mouse cGAS and TIPeGFP antigen. This homologous recombination technique is described in [236]. During the homologous recombination, the bicistronic cassette was inserted in the E1 locus of the AdHu5 parental genome. Resulting AdHu5 genomes were then derived in HEK293A cell lines (Invitrogen, Cat. R705-07). The resultant viruses were purified by CsCl gradient ultracentrifugation by the VVCF, as previously described [285]. The virus titres were determined on HEK293A cells using anti-hexon immunostaining assay using the QuickTiter™ Adenovirus Titer Immunoassay kit (Cell Biolabs Inc). The evaluation of expression of the

TIPeGFP gene from the bicistronic viral vectors was performed by fluorescence microscopy of the eGFP. The mean fluorescence intensity (MFI) was determined at different time points after infection by using a fluorescence microscope (Leica DMI 3000 B) and the software Q-Capture Pro 7 (Teledyne Qimaging). The images obtained were analysed using ImageJ software (NIH). The MFI was normalised against the maximum MFI recorded during the experiment. This maximum MFI corresponded to the eGFP signal measured from AdHu5-TIPeGFP. This viral vector was used at the same MOI of 1000 as the bicistronic viral vectors. All MFIs were measured from a single plate.

The evaluation of the expression of the cGAS gene from these viral vectors was not directly assessed. Nevertheless, the activity of the expressed cGAS was indirectly evaluated by detecting IFN-β using the mouse cell line Hepa 1-6 (ATCC CRL-1830). For this, bicistronic viral vectors were added to 96-well plates already containing the mouse cell line, at an MOI of 1000. The plate was incubated at 37 °C and supernatants were harvested at different time points after infection. The detection of mouse IFN-β from culture supernatants was performed using a mouse IFN-β ELISA kit (R&D Systems, Cat. 42400-1). The procedure was performed according to the recommended manufacturer's instructions.

2.3 Other viral vectors and antigens used in the study

Replication-deficient AdHu5 and MVA only expressing the TIPeGFP antigen were also employed in this study (referred to as AdHu5-TIPeGFP and MVA-TIPeGFP, respectively). AdHu5-TIPeGFP and MVA-TIPeGFP are referenced in [236] and [220], respectively. In the case of AdHu5, the antigen expression was

controlled by the EF-1α promoter and the expression cassette had been inserted in the E1 locus of the AdHu5 genome. These viral vectors were kindly provided by Adrian V.S. Hill.

For MERS coronavirus, two chimpanzee adenoviruses (based on ChAdOx1, described in [220]) expressing the MERS S glycoprotein gene were employed. Both ChAdOx1-based vaccines expressed the MERS S glycoprotein DNA sequence of the camel isolate Camel/Qatar_2_2014 (Genbank accession number: KJ650098.1). One of the viral vectors contained the human tissue Plasminogen Activator leader sequence (tPA-LS) fused to the N-terminus of the antigen. In both cases, the antigen expression was controlled by the human cytomegalovirus major immediate-early promoter (CMV-IE). These viral vectors were provided by Adrian V.S. Hill.

2.3.1 Virus-like particle R21

The virus-like particle R21 was provided by A. Hill, having been expressed in yeast using the small Hepatitis B surface antigen (HBsAg) fused to the C-terminus region of a part of the Circumsporozoite protein (CSP) of *Plasmodium falciparum* [141].

2.4 Adjuvant administration

2.4.1 Spermidine

Spermidine was administered at a 5 mM concentration in the drinking water, as previously described [286]. This drinking water was changed every 2-3 days for the whole duration of the experiments. For the positive control group, mice were

subjected to the spermidine treatment from 3 weeks before vaccination until culling. Negative control mice received normal drinking water.

Different durations of spermidine administration were also tested. This was performed by administering spermidine in the 5 mM concentration in the drinking water for 7 and 14 days after vaccination.

In the case of the intramuscular administration of spermidine, the different adjuvant doses were obtained from a filter-sterilised stock concentration of spermidine (diluted in PBS). The adjuvant doses were then injected in a maximum volume of 100 µL per mouse.

When indicated, the pH of the spermidine solutions to inject was changed by the addition of HCl. Final solutions were sterilised using a 0.22 µm membrane filter.

2.4.2 2'3'-cGAMP

2'3'-cGAMP Vaccigrade™ (InvivoGen) and 2'5'-GpAp (InvivoGen) were reconstituted in sterile PBS to obtain a master solution. This master solution was aliquoted and stored at -20 °C until final use. The different doses of the adjuvants (1-30 µg per mouse) were prepared by further diluting these master solutions in sterile PBS. These adjuvant doses were then added to vials carrying single doses of viral vectors previously obtained from a single vial. For comparison with 2'5'-GpAp, the adjuvants were added in an equimolar dose (corresponding to approximately 10 µg per mouse). Final injecting volumes were of 50 µL.

For the R21 vaccinations, a master solution of the R21 was diluted in sterile PBS to obtain a dose of 0.5 µg of R21 per mouse. Matrix-M1 (Novavax) and 2'3'-cGAMP were then added to these vials in an appropriate concentration to

obtain a dose of 6 μg and 10 μg per mouse, respectively. Final volumes to inject were of 50 μL per mouse.

2.5 Mouse immunogenicity

For MERS coronavirus-related studies, female BALB/c mice (Envigo, U.K.), aged 6–8 weeks, were immunised intramuscularly (upper leg thigh) with 10^8 IU of ChAdOx1 or 10^6 PFU of MVA. In prime only vaccination regimens, mice were vaccinated with ChAdOx1. For the analysis of immunogenicity, blood samples were taken 28 days after viral vector administration (peak of response). In heterologous prime-boost regimens, mice were vaccinated with ChAdOx1 (ChAdOx1-MERS-S-tPA-LS) and then boosted with MVA 28 days later. Mice were bled for analysis 14 days after boosting vaccination. In homologous prime-boost vaccination regimens, mice were vaccinated with MVA and boosted 21 days later. In this case, for immunogenicity analysis, mice were bled 21 days after boosting vaccination.

For malaria-related studies, female BALB/c, C57BL/6 (Envigo, U.K.) and C57BL/6 STING-knockout [287] (The Jackson Laboratory, U.S.A.), aged 7-8 weeks old, were injected intramuscularly (thigh muscle) with 10^7 IU of AdHu5-TIPeGFP or 10^6 PFU of MVA-TIPeGFP per mouse. Ex-breeder female BALB/c mice (Envigo, U. K.), aged at least 5-6 months old, were also vaccinated with these doses when specified. In the case of spermidine, boosting vaccination was performed by injecting i.m. 10^6 PFU of MVA-TIPeGFP per mouse. This boosting vaccination was performed 7-8 weeks after prime vaccination. For the analysis of the effect of spermidine on the peripheral blood mononuclear cells (PBMCs) immunogenicity or the serum antibody responses, blood samples were

obtained from the mouse tail by a puncture at different time points during the heterologous prime-boost vaccination regimen. For the analysis of the effect of spermidine on the spleen immunogenicity, these organs were taken at day 14 or week 7-8 after AdHu5 prime vaccination. Spleen harvesting from a mouse group was performed 1 week after MVA boosting vaccination.

For 2'3'-cGAMP experiments, spleens and blood were obtained from mice 14 days after vaccination with AdHu5-TIPeGFP for the analysis of cellular and humoral immunogenicity, respectively. In the case of MVA-TIPeGFP, spleens and blood were collected 7 days after vaccination. Intradermal vaccinations were performed from the ears of the mice and the immunological responses were analysed at the same time points as the intramuscular vaccination.

Bicistronic viral vectors (AdHu5-TIPeGFP-mcGAS and AdHu5-TIPeGFP-mutcGAS) were injected at a dose of 10^7 IU per mouse. For dose-response experiments, viral vectors were serially diluted from a master concentration to achieve the doses required (10^6-10^8 IU per mouse). For experiments mixing the bicistronic viral vectors with R21, 10^7 IU per mouse of the viral vectors were mixed with 0.5 µg of R21 (no chemical adjuvants added). In this case, all immunological responses were measured at day 14 after vaccination (humoral and cellular).

For experiments concerning the virus-like particle R21, this vaccine was injected in a 0.5 µg dose to female BALB/c mice (7-8 weeks old). Mice were boosted with the same vaccine formulation 3 weeks after priming vaccination. For the analysis of PBMCs and serum antibody responses, mice were bled by a tail puncture at different time points after prime and boosting vaccination.

For all experiments, spleens and PBMCs were analysed immediately after mouse extraction. For all serum antibody analysis, the blood was incubated at 4 °C overnight before centrifuging at 13,000 rpm for 4 minutes to obtain the serum. These sera were stored at -20 °C immediately until further analysis. All cellular responses from spleens and PBMCs were analysed using an *ex-vivo* IFN-γ ELISpot. For humoral responses from serum, these were analysed using an endpoint IgG ELISA.

2.5.1 ELISA

For the analysis of serum IgG antibody responses against viral vectors and virus-like particles, 96-well Nunc-Immuno Maxisorp ELISA plates were coated with 50 µL of the appropriate antigen diluted in PBS. Antigens, concentrations and sources are described in the following table.

Table 2.1. ELISA coating antigens

Antigen	Concentration	Source
MERS S1 recombinant protein	2 µg/mL	MyBioSource, USA.
Enhanced Green Fluorescence Protein (eGFP)	1 µg/mL	Millipore
$NANP_6$ polypeptide	2 µg/mL	ProImmune, U.K.
AdHu5 hexon protein	1 µg/mL	BioRad

A standard endpoint ELISA protocol was followed as previously described [288]. Serum was diluted 10 or 100-fold in PBS-Tween (PBS/T). This initial dilution was further diluted across the 96-well ELISA plate in 50 µL volumes. For each mouse sample, the ELISA test was performed in duplicates. The plate was

incubated for 1-2 hours before a washing step. Subsequently, goat anti-mouse total IgG conjugated to alkaline phosphatase (Sigma) and pNPP (p-nitrophenylphosphate) tablet substrate (SIGMA) were used in the assay. OD405 signal from each well was read using an ELISA microplate reader.

For all the endpoint IgG ELISAs performed, the endpoint titres were obtained after normalisation of the ELISA OD405 values with positive controls and expressed in ng/mL. Serum from a naïve BALB/c mouse was used as a negative control.

2.5.2 ELISpot

Splenocytes and PBMCs were harvested for analysis with *ex-vivo* IFN-γ ELISpot, described in [217], [289]. In brief, splenocytes and tail blood were treated with ammonium chloride-potassium (ACK) lysis buffer and plated on IPVH-membrane plates (Millipore) coated with 5 µg/ml anti-mouse IFN-γ (AN18) (Mabtech). Cells were re-stimulated with the peptides described in the following table.

Table 2.2. Peptides used to re-stimulate cells in ELISpot

Peptide	Concentration	Source
MERS S glycoprotein-specific peptide S291 (VYDTIKYYSIIPHSI [52])	2 µg/mL	ProImmune, U.K.
Pb9	1 µg/mL	ProImmune, U.K.
$eGFP_{200-208}$	1 µg/mL	ProImmune, U.K.
P15	2 µg/mL	ProImmune, U.K.
AL11	1 µg/mL	ProImmune, U.K.
AdHu5 hexon T cell peptide pool (PepTivator)	1 µg/mL	Miltenyi Biotech
T cell peptide pool spanning the C-terminus region of the circumsporozoite protein of *P. falciparum*	1 µg/mL	ProImmune, U.K.
$CD4^+$ T cell peptide from the C-terminus region of the *P. falciparum* CSP ($CSP_{326-345}$) [290]	1 µg/mL	Mimotopes Pty Ltd.

After re-stimulation, plates were incubated for 18-20 hours. IFN-γ spot forming cells (SFC) were detected after staining with 1 mg/mL of anti-mouse IFN-γ biotin (Cat No. R46A2, Mabtech), followed by 1 mg/mL of streptavidin-Alkaline Phosphatase (Mabtech). Development was performed using an alkaline phosphatase (AP) conjugate substrate kit (BioRad, U.K.).

IFN-γ spots were counted using an ELISPOT Reader System ELR04 (AID GmbH). Samples were analysed in technical duplicates. The final data is presented as spot-forming cells (SFC) per million splenocytes/PBMCs, after subtracting the background (number of IFN-γ SFC in wells containing cells and media only).

2.5.3 MERS pseudotyped neutralisation assay

MERS pseudotyped HIV-1 lentiviruses were produced as previously described [291]. In brief, HEK 293 cells were transfected with three plasmids using PEI solution. One of the plasmids encoded the MERS S glycoprotein gene sequence, mammalian codon optimised (var. 2c_EMC/2012, Genbank accession number: JX869059.2). The second plasmid was a lentiviral vector plasmid encoding a firefly luciferase denominated pCSFLW. The third plasmid was a second-generation lentiviral packaging construct plasmid expressing HIV-gag and denominated p8.91. Supernatants were harvested and filter-clarified 44-52 hours after transfection. Finally, supernatants containing the pseudotypes were stored at -80°C until further use. For the selection of a cell line reporting transgene expression, Huh7.5, HEK 293 and Vero E6 cell lines were incubated with serial dilutions of the MERS pseudotyped virus expressing firefly luciferase. The incubation was performed for 48 hours at 37 °C using NUNC™ F96 White Polystyrene plates. Firefly luciferase expression at was measured using Bright-Glo Luciferase Assay System (Promega). Thereafter, the plates were read using Thermo Scientific Varioskan® Flash and the software SkanIT™ (ThermoFisher Scientific).

For the MERS pseudotype neutralisation assay, camel and mouse serum samples were serially diluted in 96-well NUNC™ F96 white polystyrene plates. A determined concentration of the MERS pseudotypes was added to these wells. The plates were incubated for 1 h at 37 °C. After this incubation, Huh7.5 cells (10,000 cells per well) were added to the plates. Following a second 48 hours incubation at 37 °C, cells were lysed, and luciferase activity was measured using the Bright-Glo Luciferase System. IC90 neutralisation titres were calculated for each serum sample in technical duplicates. The IC90 neutralisation titre was considered as the serum dilution that provided a 90% neutralisation of the maximum luciferase signal.

2.6 Adjuvant *in-vitro* inhibition assays

The effect of 2'3'-cGAMP and mouse/human IFN-β on the AdHu5 transgene expression was evaluated by immunofluorescence microscopy and luciferase activity reading. For this, a replication-deficient AdHu5 expressing the firefly luciferase gene under a human CMV promoter (virus described in [292]) was employed. Negative controls used were mouse/human IFN-α and 2'5'-GpAp.

For the fluorescence microscopy, Hepa 1-6 cells were plated in a 96-well black plate (clear bottom) and incubated overnight. Replication-deficient AdHu5 expressing eGFP (AdHu5-TIPeGFP) was used to infect the cells at an MOI of 1000. 2'3'-cGAMP at a concentration of 83.3 mM was added to each well, and the plate was incubated at 37 °C for 1-2 days. The mean fluorescence intensity (MFI) from each well was determined at different time points after infection. The MFI was determined by using a fluorescence microscope (Leica DMI 3000 B) and the software Q-Capture Pro 7 (Teledyne Qimaging). The images obtained

were analysed using ImageJ software (NIH). The MFI was normalised against the maximum MFI recorded during the experiment.

For the luciferase activity reading, Huh7.5 (ATCC CRL-2117) and Hepa 1-6 cells (ATCC CRL-1830) were plated in a white 96-well plate and incubated overnight. Different concentrations of human IFN-β and IFN-α (1-10 ng/mL; R&D Systems) were added across the plate and then a single concentration of AdHu5 expressing the firefly luciferase gene was added (MOI 12). For analysing the effect of mouse IFN-α and β, a single concentration of 5 ng/mL was used. Luciferase activity was read 48 hours after infection using Bright-Glo Luciferase Assay System (Promega). The plates were read using Thermo Scientific Varioskan® Flash and the software SkanIT™ (ThermoFisher Scientific).

For confirming the effect of 2'3'-cGAMP on the AdHu5 transgene expression, the adjuvant was added at serial concentrations to Hepa 1-6 cells, 24 hours after infection. This was performed by removing the supernatant from Hepa 1-6 cells 24 hours after AdHu5 infection and immediately adding serial concentrations of the adjuvant dissolved in DMEM10 medium (41.65-1.30 mM). Luciferase was read 48 hours after infection using the Bright-Glo Luciferase Assay System.

2.7 Statistical analysis

The Mann-Whitney test was used for comparison of immunological responses between two vaccination treatments (including at specific time points). Kruskal-Wallis test with Dunn's post hoc was used for comparison of more than two vaccination treatments (including at specific time points).

Two-way ANOVA main effect analysis was used for analysing the immunological effect of the chemical adjuvants considering different routes of administrations and independent repetition of experiments. For R21, this statistical method was used for analysing the interactive effects of multiple chemical adjuvants.

Spearman's rank correlation was used for the pairwise comparison of ELISA titres and pseudotype neutralisation assay titres. A parametric t-test was used for the pairwise comparison of all the *in-vitro* experiment. GraphPad Prism (GraphPad software) was used for all the statistical analyses and to plot the data.

Chapter 3 Pre-clinical assessment of genetic designs modulating transgene expression (MERS coronavirus)

3.1 Introduction

The Middle East Respiratory Syndrome coronavirus (MERS-CoV) is a novel coronavirus of the genus Betacoronavirus first identified in 2012 from Saudi Arabia [1]. The infectious disease is characterised by fever, cough and shortness of breath. Pneumonia is typical, but not always present in the patients. Gastrointestinal symptoms such as diarrhoea have also been reported [5]. Nevertheless, other cases seem to be asymptomatic [293]. Furthermore, severe cases are characterised by respiratory failure (requiring mechanical ventilation and intensive care), especially in older people, people with a compromised immune system and people with a chronic disease such as renal disease, cancer, chronic lung disease and diabetes [5].

The numbers of reported cases for MERS-CoV have increased steadily since its identification in 2012. In its latest report, the World Health Organization (WHO) notifies the existence of 2,274 laboratory-confirmed cases for the disease with 806 associated deaths. This translates into a mortality rate of 35% for the disease [5]. The cases have been associated with a direct contact with camels (the camels being a major reservoir) and human to human transmission has been observed in hospital settings [5], [30], [294].

This section of the thesis contains material previously published with the permission of the co-authors.

It is worth mentioning that the mechanism of transmission for the disease is not fully understood. There is serological evidence that points out that the MERS coronavirus was circulating among camels before jumping in to human and that it is not possible for sheep, goats, cows, chickens and horses to contract the virus [24], [295]–[297]. Furthermore, camel to human transmission has not been directly observed (but only “associated”) and human to human transmission seems to be unsustained [5], [30].

Despite its global health impact, no licensed vaccine is available for the MERS-CoV. Several research organisations are working towards creating a cost-effective vaccine using different vaccine platforms and testing these in animal models [298]. Correlates of protection have been associated with a high concentration of neutralising antibodies, but also cellular responses seem to play an important part in protection. In this regard, replication-deficient viral-vectored vaccines (e.g. adenovirus, poxvirus) represent a promising tool in that they induce not only high levels of antibodies but also high levels of T cell responses, as has been tested for different pathogens including malaria, HIV, influenza and tuberculosis [299].

Here, we developed and tested the immunogenicity of new viral-vectored vaccines based on the highly immunogenic chimpanzee adenovirus Oxford University 1 (ChAdOx1) [220] and the Modified Vaccinia Ankara virus (MVA) [300] using the full-length spike (S) glycoprotein of the MERS-CoV [301].

The effect of two different genetic designs modulating transgene expression in these viral-vectored vaccines was tested with the aim to achieve higher immunogenicity. In the case of the ChAdOx1, the immunological effects of

fusing the tissue Plasminogen Activator leader sequence (tPA-LS) to the N-terminus of the S glycoprotein were tested.

Leader or signal sequences are N-terminal portions of a secretory or membrane protein that assists the protein across the membrane of the endoplasmic reticulum, where it is synthesized [302]. Leader sequences are cleaved from the protein, even when the protein has not been fully translated [303]. They are usually 16-30 amino acids long and they are characteristic of proteins that are meant to be secreted, translocated in cellular membranes or directed to organelles (Golgi, endoplasmic reticulum, endosomes) [302], [304].

Previous studies have shown that tPA-LS can have different immunomodulatory properties depending on the antigen being used for DNA and MVA vaccine platforms [305]–[309]. In the case of MVA, the effect on immunogenicity of a strong early promoter driving antigen expression (F11) was compared to the benchmark early/late promoter (mH5). Previous reports have established a positive effect on the cellular immunogenicity when using the F11 promoter [310], [311].

Furthermore, a neutralisation assay based on the replication-deficient HIV-1 lentivirus pseudotyped with the MERS S glycoprotein was developed in order to test the effect of the aforementioned genetic designs on the induction of neutralising antibodies.

This work was performed under the direction of Dr. Naif Alharbi. All viral-vectored vaccines except MVA-MERS-S-mH5 were manufactured by the Viral Vector Core Facility (VVCF) of The Jenner Institute of the University of Oxford.

This section of the thesis contains material previously published with the permission of the co-authors.

3.2 Construction of MVA-MERS-S-mH5, a benchmark MVA vaccine for comparison of immunogenicity

3.2.1 Vaccine design and production

In order to produce an MVA vaccine for the MERS coronavirus that could serve as a benchmark for comparison of immunogenicity of different genetic designs modulating transgene expression, an MVA vaccine expressing the MERS S glycoprotein (Genbank accession number: KJ650098.1) under the control of the poxvirus promoter mH5 was constructed [310], [312]. The complete cassette (mH5 promoter and antigen-coding sequence) was inserted in the F11L locus of the MVA genome as superior immunogenicity has been reported for this strategy when compared to inserting the cassette in the Thymidine Kinase (TK) locus of the MVA [310]. To accomplish this, a cloning and virus production strategy for having both the MERS antigen and the mH5 promoter in the F11L locus was designed. To ensure rapid clinical translation and manufacturing, the MVA was produced without a reporter gene.

In more detail, for the construction of the benchmark MVA vaccine, here referred to as MVA-MERS-S-mH5, the mH5 promoter sequence was amplified by PCR and inserted by In-Fusion cloning into a shuttle plasmid already containing the MERS S glycoprotein sequence (**Figure 3.1**). The mH5 sequence was inserted immediately 5' of the MERS S glycoprotein coding sequence, leaving the fused mH5 and MERS S glycoprotein sequences flanked by the F11R and F11L sites for the subsequent homologous recombination with the parental MVA.

This section of the thesis contains material previously published with the permission of the co-authors.

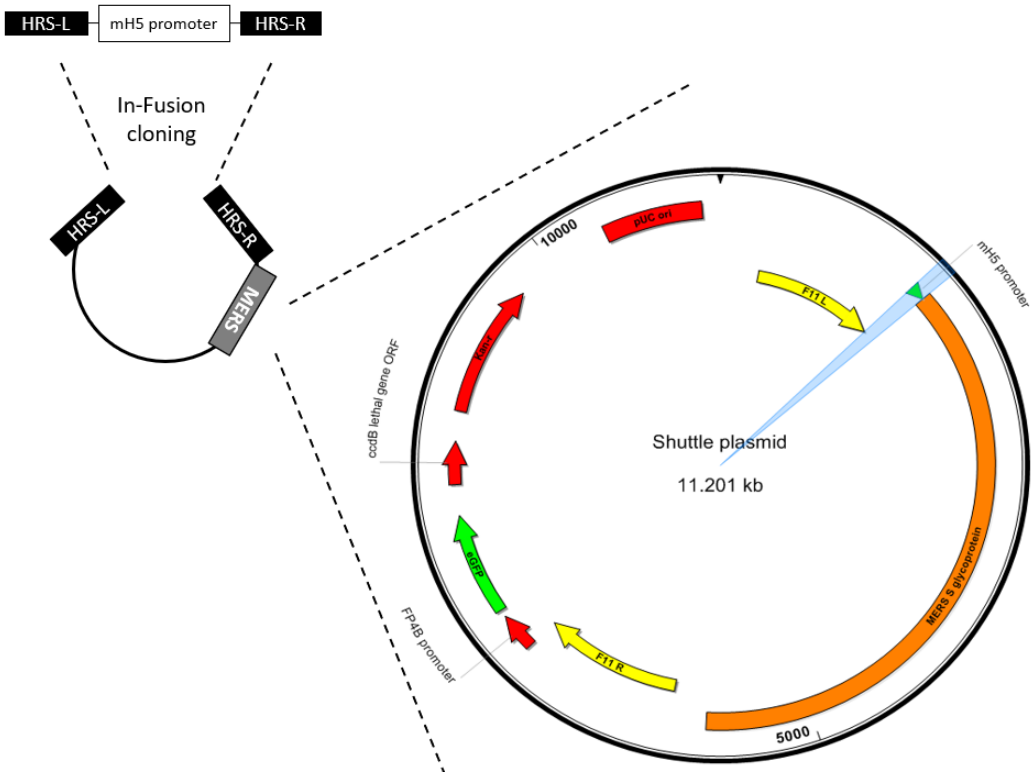


Figure 3.1. Cloning strategy for the construction of the shuttle vector. *The shuttle vector was made by In-Fusion cloning (Takara) of the mH5 promoter sequence into a pre-existing shuttle vector containing the MERS S glycoprotein. The mH5 promoter was fused immediately upstream the MERS S glycoprotein sequence. This strategy assured that both the mH5 promoter sequence and the MERS S glycoprotein sequence were flanked by F11 sites for posterior homologous recombination with the parental MVA. A GFP marker was included outside the F11 recombination sites.*

The F11 flanking sites from the shuttle vector (F11R and F11L) were used to insert, by homologous recombination, the mH5-MERS S glycoprotein cassette into the F11 locus of a parental MVA (**Figure 3.2**). For this, chicken embryo fibroblasts (CEF) were infected with the parental MVA and later transduced with the shuttle vector.

This section of the thesis contains material previously published with the permission of the co-authors.

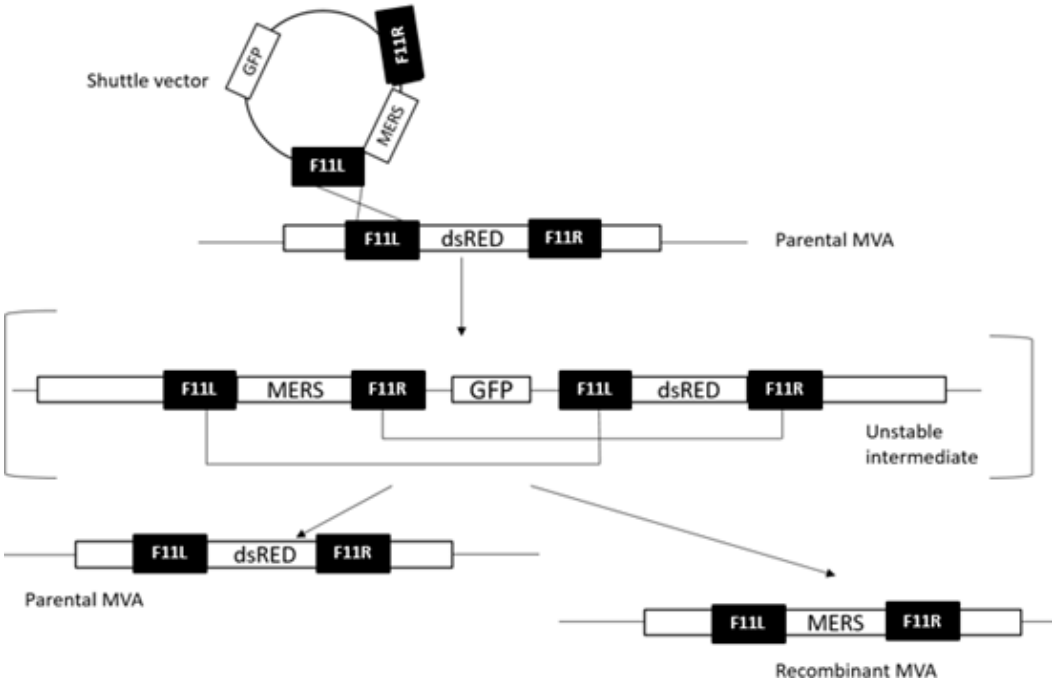


Figure 3.2. Transient-dominant selection (TDS) strategy for the construction of the recombinant MVA. *A fluorescence-based transient-dominant selection (TDS)* [313] *strategy was used for the construction of the recombinant MVA (MVA-MERS-S-mH5). The homologous recombination between the shuttle vector and the parental MVA occurred in two steps. The first step was a single crossover event that resulted in the integration of the full-length shuttle plasmid into the genome of the parental MVA. Due to the presence of direct repeats, a second crossover event occurred with the spontaneous formation of the parental MVA or the MVA-MERS-S-mH5. Initially, GFP and dsRED positive cells were sorted to isolate cells where the recombination had occurred. Subsequently, non-fluorescent virus preps which lacked either GFP or dsRED activity were selected: the lack of fluorescence indicating the presence of the MERS transgene cassette.*

The transduced CEF cells were isolated by flow cytometry after the detection of the dsRED and GFP markers coming from the parental MVA and the shuttle plasmid, respectively. These cells were later propagated *in vitro* and the GFP

and dsRED activities from these isolates were tracked by fluorescence microscopy until vanished (transient-dominant selection). Viruses which lacked GFP and dsRED activity after several plaque-picking rounds were finally selected (**Figure 3.3**).

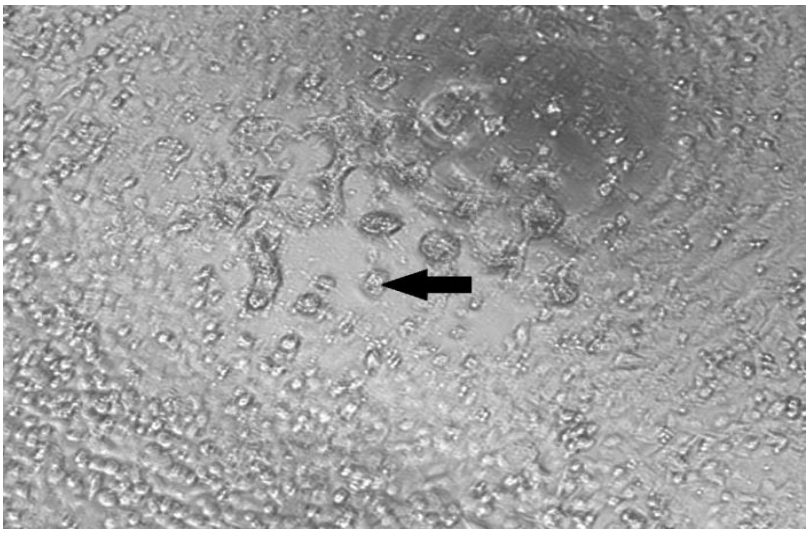


Figure 3.3. Microscopy of plaques used to isolate the recombinant MVA. *The plaques used to isolate the MVA-MERS-S-mH5 were characterised by the formation of syncytium (indicated here with a black arrow, ←). This feature along with the scarcity of DF-1 cells from the plaque area was used to identify and isolate the plaques by the plaque-picking method. GFP and dsRED activity was not detected from these plaques (not shown)*

The markerless plaques corresponding to the recombinant virus were further propagated in DF-1 cells. The recombinant viruses were finally extracted from the DF-1 cells by the freeze-thawing method and purified by CsCl ultracentrifugation (this final step was performed by the by the VVCF and Dr. Naif Alharbi).

This section of the thesis contains material previously published with the permission of the co-authors.

3.2.2 Virus titration and quality control

The identity of the purified recombinant MVA was confirmed by PCR using primers targeting the MERS S transgene sequence, followed by gel electrophoresis (**Figure 3.4**). The purity of the recombinant virus was confirmed by the same technique using primers targeting the dsRED marker sequence (only present in the parental MVA and not in the recombinant MVA).

This section of the thesis contains material previously published with the permission of the co-authors.

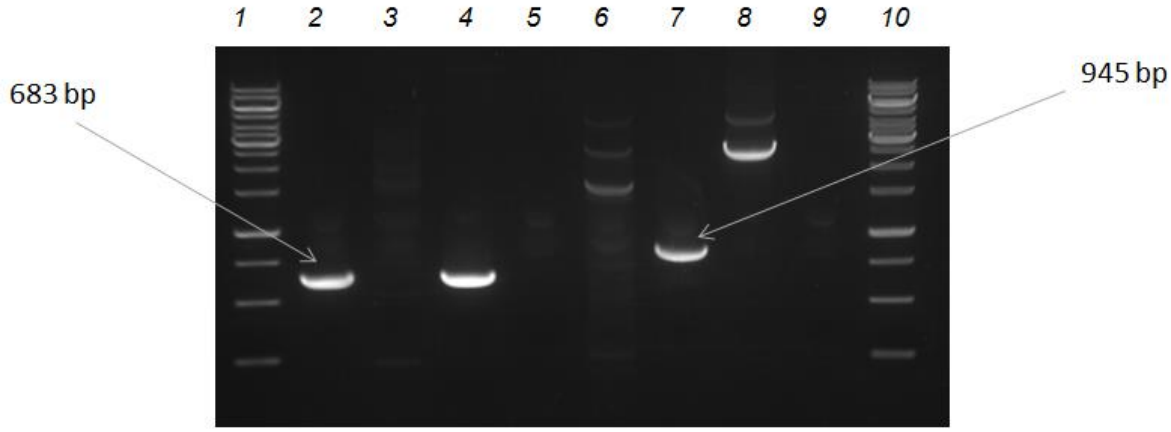


Figure 3.4. Confirmation of the identity and purity of the MVA-MERS-S-mH5. *The positive control for the identity of the MVA-MERS-S-mH5 is shown in lane 4 (band 683 bp) and corresponds to the shuttle vector previously used to insert the MERS cassette into the parental MVA. The positive control for testing the purity of the recombinant MVA corresponds to the parental MVA (lane 7, band 945 bp). Non-specific amplification bands were observed in lanes 6 and 8, most likely due to lack of optimisation of the conditions of the PCR.*

Lane	*Lane*
1. Smart ladder *2. MVA-MERS-S-mH5-specific PCR: MVA-MERS-S-mH5* *3. MVA-MERS-S-mH5-specific PCR: parental MVA* *4. MVA-MERS-S-mH5-specific PCR: shuttle vector (positive control)* *5. MVA-MERS-S-mH5-specific PCR: sterile distilled water (negative control)*	*6. Parental MVA-specific PCR: MVA-MERS-S-mH5* *7. Parental MVA-specific PCR: parental MVA (positive control)* *8. Parental MVA-specific PCR: shuttle vector* *9. Parental MVA-specific PCR: sterile water (negative control)* *10. Smart ladder*

This section of the thesis contains material previously published with the permission of the co-authors.

3.2.3 Evaluation of integrity of the mH5 promoter and the MERS S glycoprotein DNA sequences

The integrity of the promoter mH5 and the MERS S glycoprotein DNA sequences in the purified recombinant MVA was evaluated by the PCR-Sanger sequencing method. For this, 7 primers were designed to cover the whole mH5 promoter and MERS S glycoprotein sequence. After PCR amplification, these 7 fragments were sent to Sanger for sequencing.

The 7 reads were aligned and compared to a reference DNA sequence (the shuttle vector). A point mutation was found in one of these PCR fragments; nevertheless, this mutation corresponded to a synonymous mutation and therefore it did not compromise the final protein sequence (**Figure 3.5**).

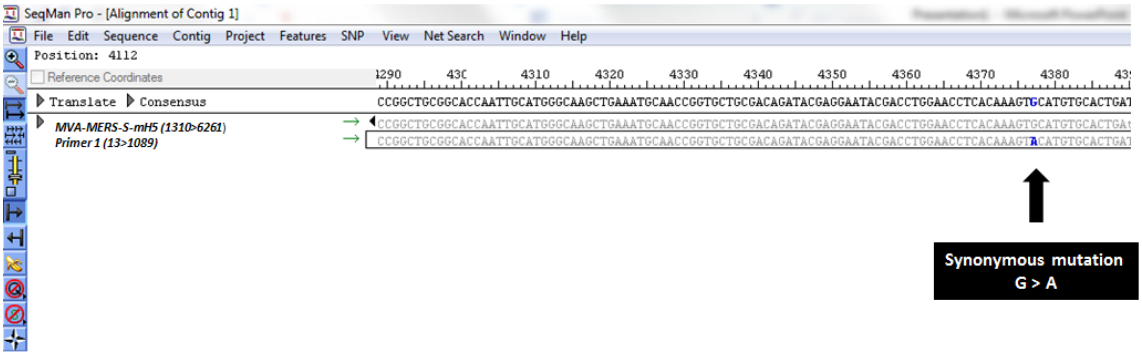


Figure 3.5. Sanger sequencing read showing a point mutation. *A point mutation in the MERS S glycoprotein region was found. This mutation was determined to be a synonymous mutation, meaning that the new codon produced by this mutation did not have an impact on the encoded amino acid (GTG>GTA, Valine).*

This section of the thesis contains material previously published with the permission of the co-authors.

3.2.4 Evaluation of expression of the MERS S glycoprotein

The expression of the MERS S glycoprotein was evaluated in HEK 293 cells by fluorescence immunostaining after transfection of these cells with the shuttle plasmid encoding the MERS S glycoprotein (**Figure 3.6**).

A B

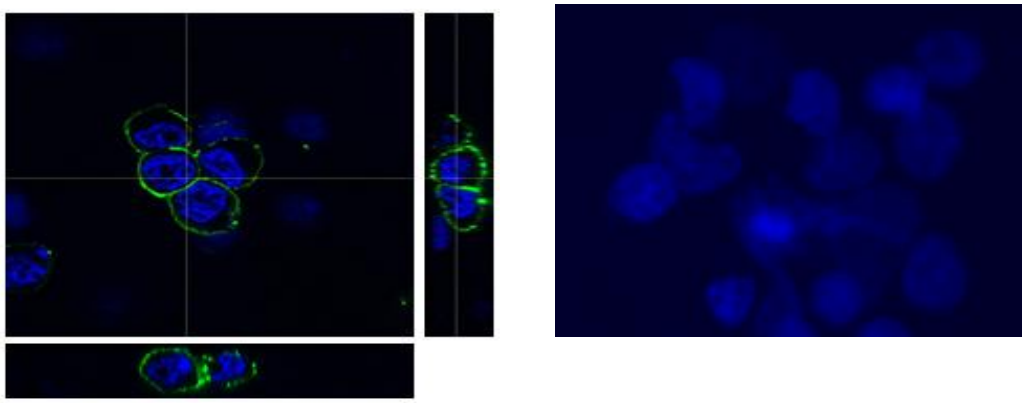


Figure 3.6. Confirmation of the expression of the MERS S glycoprotein by fluorescence immunostaining. *A. Indirect fluorescence immunostaining was used to confirm the expression of the MERS S glycoprotein on the cell membrane of HEK 293 cells (no permeabilization step was performed; green staining). The blue staining corresponds to DAPI. B. Negative control: HEK 293 cells transfected with shuttle plasmid with no MERS antigen incorporated.*

The expression of the MERS S glycoprotein was also confirmed by an in-house whole-cell ELISA targeting the expression of the MERS S-glycoprotein on the cell surface of the cells (**Figure 3.7**).

This section of the thesis contains material previously published with the permission of the co-authors.

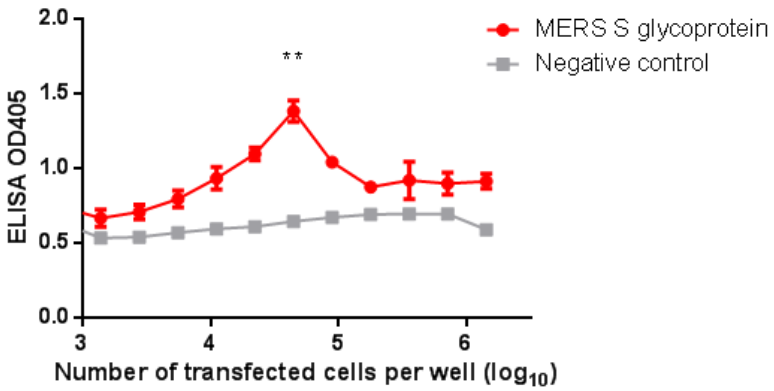


Figure 3.7. Confirmation of the expression of the MERS S glycoprotein by whole-cell ELISA. *HEK 293 cells transfected with a plasmid expressing the MERS S glycoprotein were serially diluted across a 96-well plate and fixed after 24 hours. A standard concentration of a mouse anti-MERS S glycoprotein serum was used as the primary antibody, followed by a rabbit anti-mouse HRP-conjugated secondary antibody. Mean ELISA OD405 values (+/- SEM) are shown in the panel for each HEK 293 cell dilution. Negative control: HEK 293 cells transfected with an empty plasmid (no MERS antigen). Statistical analysis: Student's t-test, **p<0.01, n=3.*

This section of the thesis contains material previously published with the permission of the co-authors.

3.3 Development of a pseudotype-based neutralisation assay

3.3.1 Pseudotyped virus construction

A pseudotype neutralisation assay was designed, optimised and employed for the titration of neutralising antibodies binding the MERS S glycoprotein. For this, replication-incompetent second-generation viruses based on the HIV-1 lentivirus were constructed, displaying the MERS S glycoprotein on the virus envelope and carrying the firefly luciferase gene to enable detection of virus infection (**Figure 3.8**).

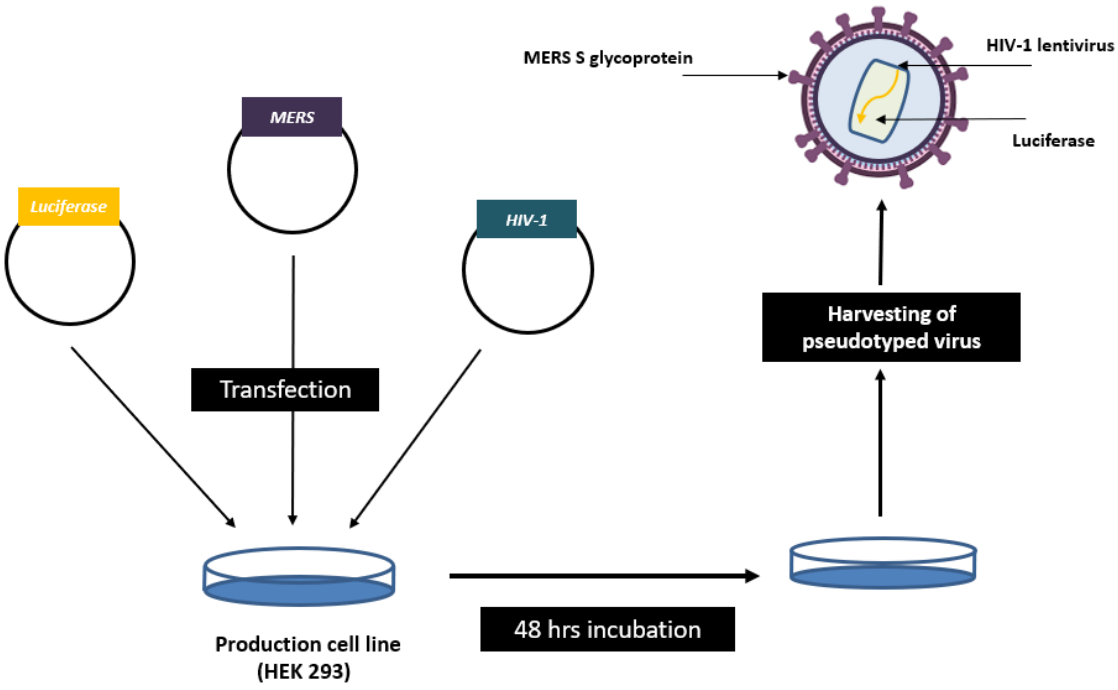


Figure 3.8. construction of the MERS S glycoprotein-pseudotyped viruses. *HEK 293 cells were seeded and transfected with three plasmids encoding the MERS S glycoprotein, a reporter gene for virus infection (firefly luciferase gene with an incorporated Ψ packaging signal) and the*

HIV-1 structural proteins. Pseudotyped viruses were harvested from the cell culture supernatants 48 hours after transfection and these were clarified and stored at -20 °C until further use.

An indirect ELISA was used to confirm the formation of the MERS S glycoprotein pseudotypes (**Figure 3.9**).

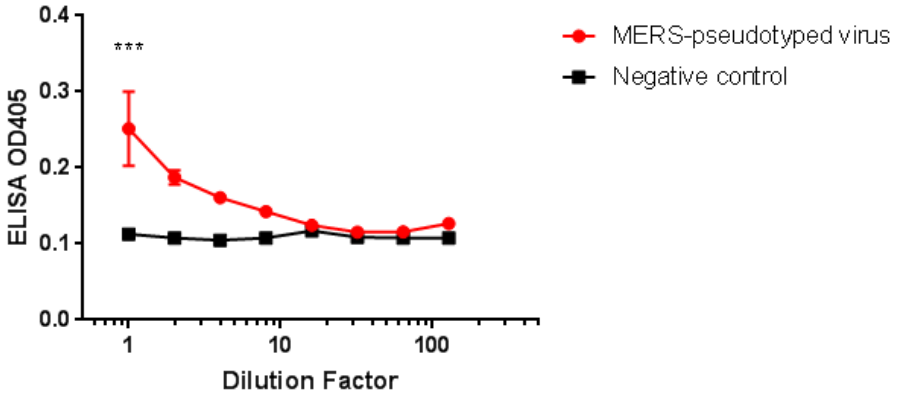


Figure 3.9. Confirmation of the formation of the MERS S glycoprotein pseudotypes by indirect ELISA. *MERS S glycoprotein pseudotypes previously harvested from the cell culture supernatants and purified by ultracentrifugation were serially diluted in a 96-well plate and used as an ELISA coating agent. Presence of the pseudotypes at each dilution was detected using a standard concentration of mouse anti-MERS S glycoprotein serum as the primary antibody, followed by rabbit anti-mouse alkaline phosphatase-conjugated secondary antibody. Mean ELISA OD405 values +/- SEM are shown for each virus dilution coating the ELISA plate. Negative control: empty virus (no MERS S glycoprotein). Statistical analysis: Student's t-test, ***p<0.001, n=3.*

This section of the thesis contains material previously published with the permission of the co-authors.

3.3.2 Selection of a reporter cell line

The pseudotype neutralisation assay would require the use of a cell line that can be infected by the MERS pseudotypes. For this, serial dilutions of the cell culture supernatant containing the MERS pseudotypes were incubated with various cell lines at a fixed cell density. The luciferase activity coming from the pseudotypes was measured at each serial dilution after addition of the substrate luciferin (**Figure 3.10**). The human hepatocyte cell line Huh-7 showed the highest luciferase activity dependent on MERS pseudotype concentration.

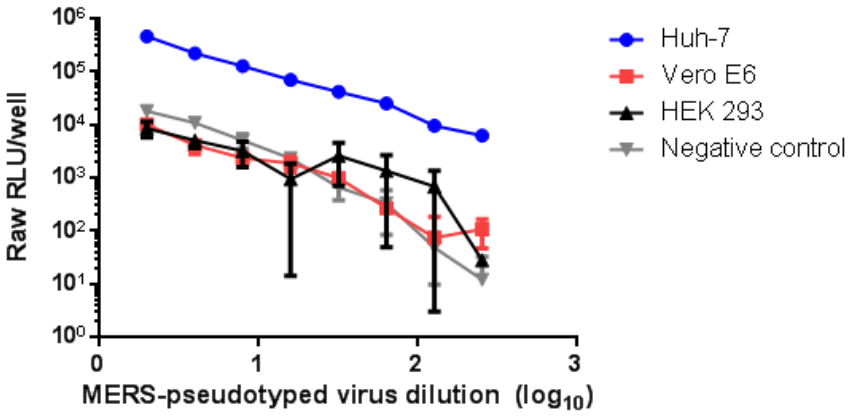


Figure 3.10. Luciferase activity dependent on MERS pseudotype concentration in different cell lines. *The luciferase activity coming from the MERS pseudotypes was measured at different virus dilutions for three different cell lines: Huh7, HEK 293 and Vero cells. Mean relative luminescence unit (RLU) values and range are shown at each virus dilution. Negative control: empty vector (no integrated MERS glycoprotein). The assay was performed in duplicates and repeated once showing similar results.*

This section of the thesis contains material previously published with the permission of the co-authors.

3.3.3 Development of the pseudotype-based neutralisation assay

In order to develop the pseudotype neutralisation assay, an in-house positive (MERS-CoV spike hyper-immune) and negative mouse control sera were serially diluted in a 96-well plate and incubated with a standard concentration of the MERS-pseudotyped virus. After this, a standard concentration of Huh-7 cells was added to the previous mixtures in the 96-well plate and further incubated for 48 hours. Luciferase activity was recorded at each serum dilution. A blocking effect of luciferase activity dependent on the positive serum concentration was observed (**Figure 3.12**).

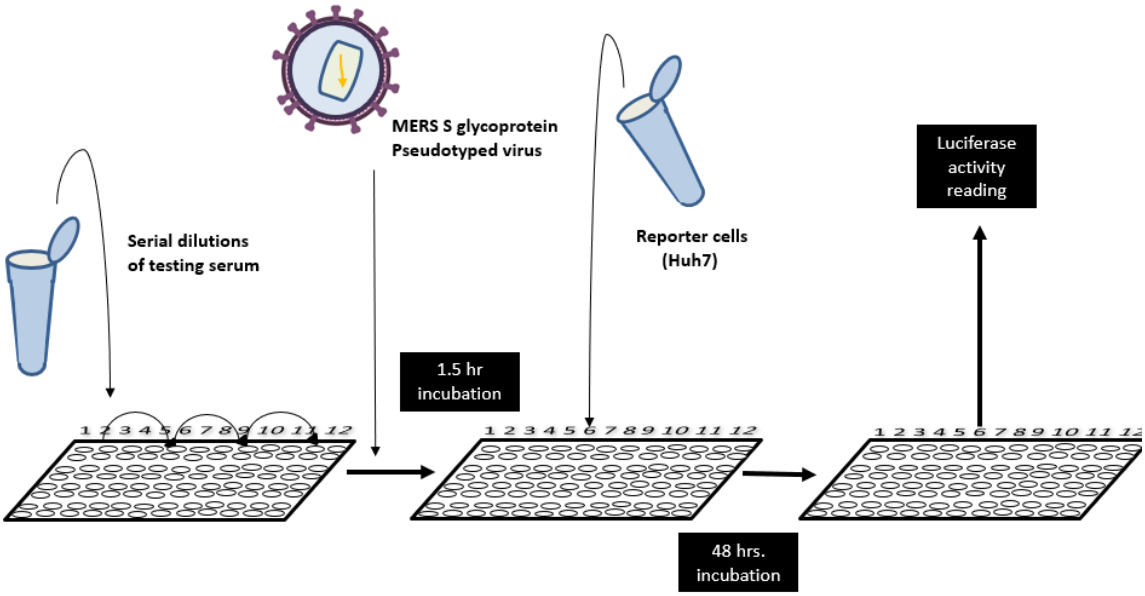
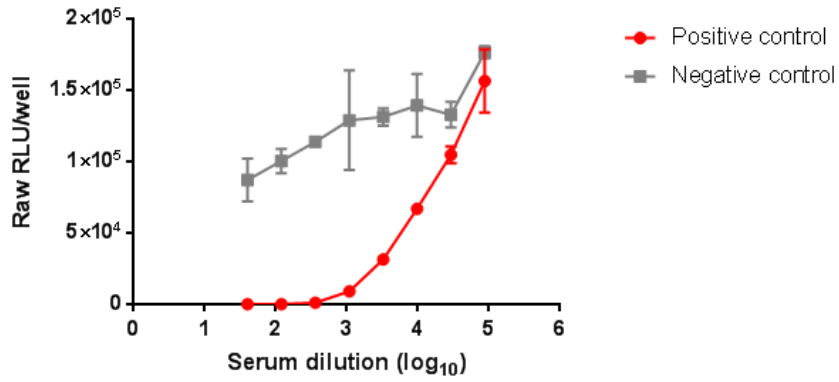


Figure 3.11. Development of the pseudotype neutralisation assay. *A standard concentration of cell culture supernatant containing the MERS pseudotyped virus was incubated with the positive and negative serum for 1.5 hrs at 37 °C. After this, the Huh-7 cells were added to each well and plates were incubated for 48 hours at 37 °C. Cells were lysed and a luciferin substrate was added to each well of the plate. Luciferase activity was recorded as at each serum dilution.*

This section of the thesis contains material previously published with the permission of the co-authors.

A



B

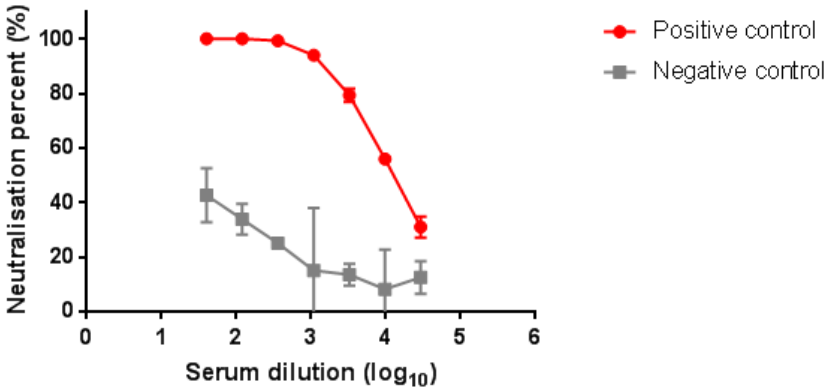


Figure 3.12. Luciferase activity dependent on neutralising serum concentration. *A. Luminescence raw values and range at each positive and negative serum dilution. Negative control corresponded to naïve serum. The assay was performed in duplicates and repeated once with similar results. B. Neutralisation percentage at each serum dilution of the positive and negative control. Raw luciferase activity at each serum dilution was normalised against the RLUs of a positive and negative virus control; this value was expressed as neutralising percentages (%). Mean neutralisation percentages (%) and range are shown at each serum dilution.*

This section of the thesis contains material previously published with the permission of the co-authors.

An IC90 value was calculated for the positive and negative serum after a non-linear regression fit using the Hill equation. This value was considered as the $\log_{10}$ of the serum dilution that produces a 90% neutralisation of the luciferase activity. The IC90 of the positive control was found to be at least 1,000 times higher than that of the negative control, meaning that the assay can actually be used to titrate neutralising antibodies that fall in this range.

3.3.4 Correlation with other serological assays

A strong correlation was observed between the pseudotype neutralisation assay and a microneutralisation assay performed by an external laboratory using BALB/c mice previously vaccinated with the MERS S glycoprotein (**Figure 3.13**). The external laboratory corresponded to the one of Stephan Becker (Institute of Virology, Philipps University of Marburg, Marburg, Germany). The purpose of the comparison between the two assays was to validate the pseudotype neutralisation assay and to know if the pseudotype neutralisation assay could work as a surrogate of the microneutralisation assay (this one has a higher biosafety level requirement, BSL-3). Microneutralisation assays use instead live MERS coronavirus to titrate serum neutralising antibodies. The assay is highly specific and sensitive for this purpose [314].

This section of the thesis contains material previously published with the permission of the co-authors.

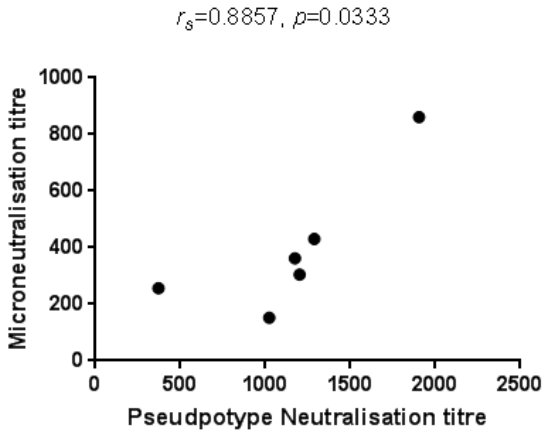


Figure 3.13. correlation between the pseudotype neutralisation assay and a microneutralisation assay. *6 BALB/c mice were vaccinated with 10^8 IU per mouse of a replication-deficient adenovirus type 5 vaccine encoding the MERS S glycoprotein. Serum was taken from the mice at day 28 and the neutralising antibodies were titrated using the pseudotype neutralisation assay (IC90 values) and a Microneutralisation assay (IC50 values) performed by an external laboratory. The pseudotype neutralisation assay was performed in technical duplicates. In the case of the Microneutralisation, the assay was performed in technical quadruplicates. Statistical analysis: Spearman correlation.*

The pseudotype neutralisation assay also had a strong correlation with an endpoint dilution ELISA performed by Naif Alharbi using samples from BALB/c mice immunised with different MERS viral-vectored vaccines and vaccination regimens (**Figure 3.14**).

This section of the thesis contains material previously published with the permission of the co-authors.

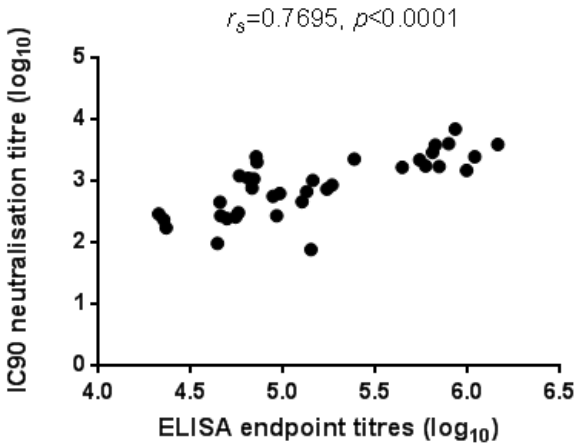


Figure 3.14. correlation between the pseudotype neutralisation assay and an endpoint dilution ELISA. *BALB/c mice were vaccinated with different regimens of replication-deficient adenovirus and MVA vaccines encoding the MERS S glycoprotein (32 mice in total). Serum was taken at different times for each vaccine regimen and tested by the pseudotype neutralisation assay and an endpoint titre ELISA based on the MERS S glycoprotein. Assays were performed in duplicates for each serum sample. Statistical analysis: Spearman correlation.*

The pseudotype neutralisation assay also had a strong correlation with an endpoint dilution ELISA for camel samples previously exposed to the MERS coronavirus and performed by an external laboratory (**Figure 3.15**).

This section of the thesis contains material previously published with the permission of the co-authors.

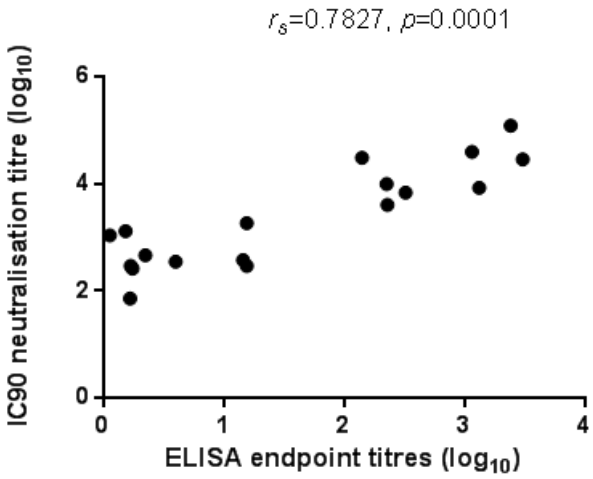


Figure 3.15. correlation between the pseudotype neutralisation assay and an endpoint dilution ELISA for camel samples. *Camel samples previously exposed to the MERS-CoV were tested with the pseudotype neutralisation assay and an endpoint dilution ELISA performed by an external laboratory. Samples were tested in triplicates and both assays were repeated once. Statistical analysis: Spearman correlation, n=18.*

This section of the thesis contains material previously published with the permission of the co-authors.

3.4 Effect on immunogenicity of the tissue plasminogen activator leader sequence (tPA-LS) in the chimpanzee adenovirus ChAdOx1

3.4.1 Experiment design

The pseudotype neutralisation assay was used to test the effect on humoral immunogenicity of the insertion of the human tissue plasminogen activator leader sequence (tPA-LS) at the N-terminus of the MERS S glycoprotein encoded by a replication-deficient chimpanzee adenovirus vaccine (ChAdOx1), as compared to the same virus with no tPA-LS included (**Figure 3.16**).

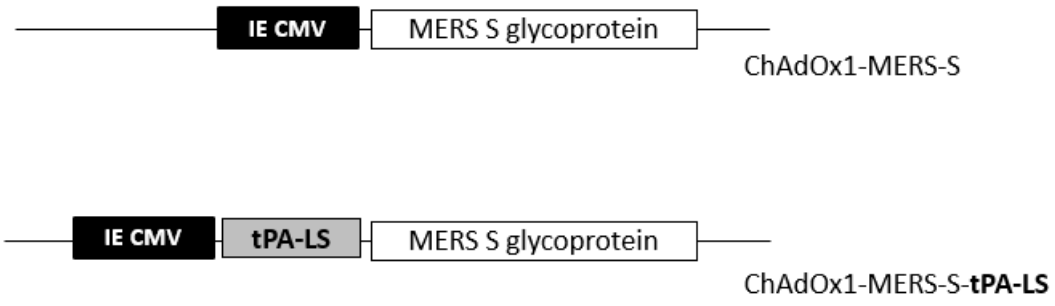


Figure 3.16. Schematic representation of the ChAdOx1 MERS vaccines. *Two ChAdOx1-based vaccines encoding the MERS S glycoprotein under the control of the immediate-early human CMV promoter were designed and constructed by Dr. Naif Alharbi and produced by the VVCF. In one of the vaccines, the human tissue Plasminogen activator leader sequence (tPA-LS) was fused at the N-terminus of the MERS S glycoprotein. Both antigens were inserted in the E1 locus of the chimpanzee adenovirus genome.*

This section of the thesis contains material previously published with the permission of the co-authors.

3.4.2 Humoral immunogenicity

The use of the tPA-LS significantly enhanced the neutralising antibody titres against the MERS S glycoprotein, as compared to the viral vector with no tPA-LS (**Figure 3.17**).

A



B

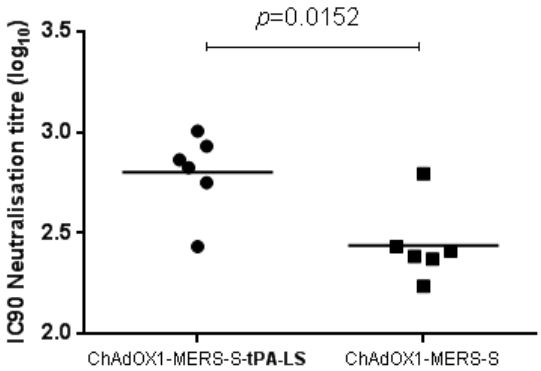


Figure 3.17. Effect on neutralising antibodies of the use of the tPA-LS in the ChAdOx1 vaccine. *A. BALB/c mice were vaccinated intramuscularly with 10^8 IU of ChAdOx1 vaccines per mouse. Serum was collected 28 days after vaccination and analysed with the pseudotype neutralisation assay. B. Comparison of neutralising antibody titres against the MERS S glycoprotein. Data are representative of two independent mouse experiments. The group means are indicated with a horizontal line. Statistical analysis: Mann-Whitney test, n=6.*

This section of the thesis contains material previously published with the permission of the co-authors.

3.5 Effect on antigen immunogenicity of an early/late promoter modulating transgene expression in MVA

3.5.1 Experiment design

The MVA-MERS-S-mH5 previously constructed was used as a benchmark MERS vaccine for testing the effect of an alternative strong early promoter (F11) on the vaccine's humoral and cellular antigen immunogenicity (**Figure 3.18**). This effect was studied in the context of heterologous and homologous prime-boost vaccination regimen with the chimpanzee adenovirus vector ChAdOx1 due to the higher immunogenicity achieved by this method of vaccination [315].

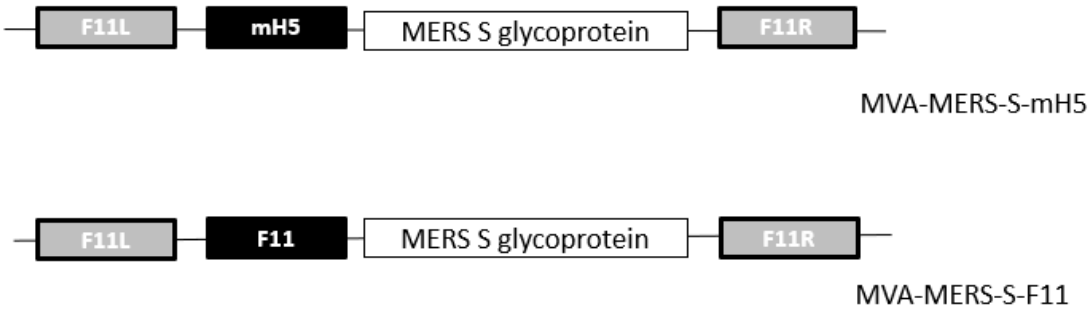


Figure 3.18. Schematic representation of the MVA vaccines used to test the effect of a strong early promoter (F11). *An MVA expressing the MERS S glycoprotein under the control of the strong early promoter F11 (here designated as MVA-MERS-S-F11) was constructed by Dr. Naif Alharbi and the VVCF. A benchmark MVA vector was constructed (MVA-MERS-mH5), based on the early/late promoter mH5. Both antigen cassettes were inserted in the F11 locus of the MVA genome by homologous recombination with the flanking F11L and F11R regions.*

This section of the thesis contains material previously published with the permission of the co-authors.

3.5.2 Homologous prime-boost vaccination regimens

In the context of a homologous prime-boost vaccination regimen, the strong early promoter F11 did not impact on the humoral immunity against the MERS S glycoprotein. Nevertheless, it had a positive effect on the T cell responses against this antigen as compared to the benchmark promoter mH5 (**Figure 3.19**).

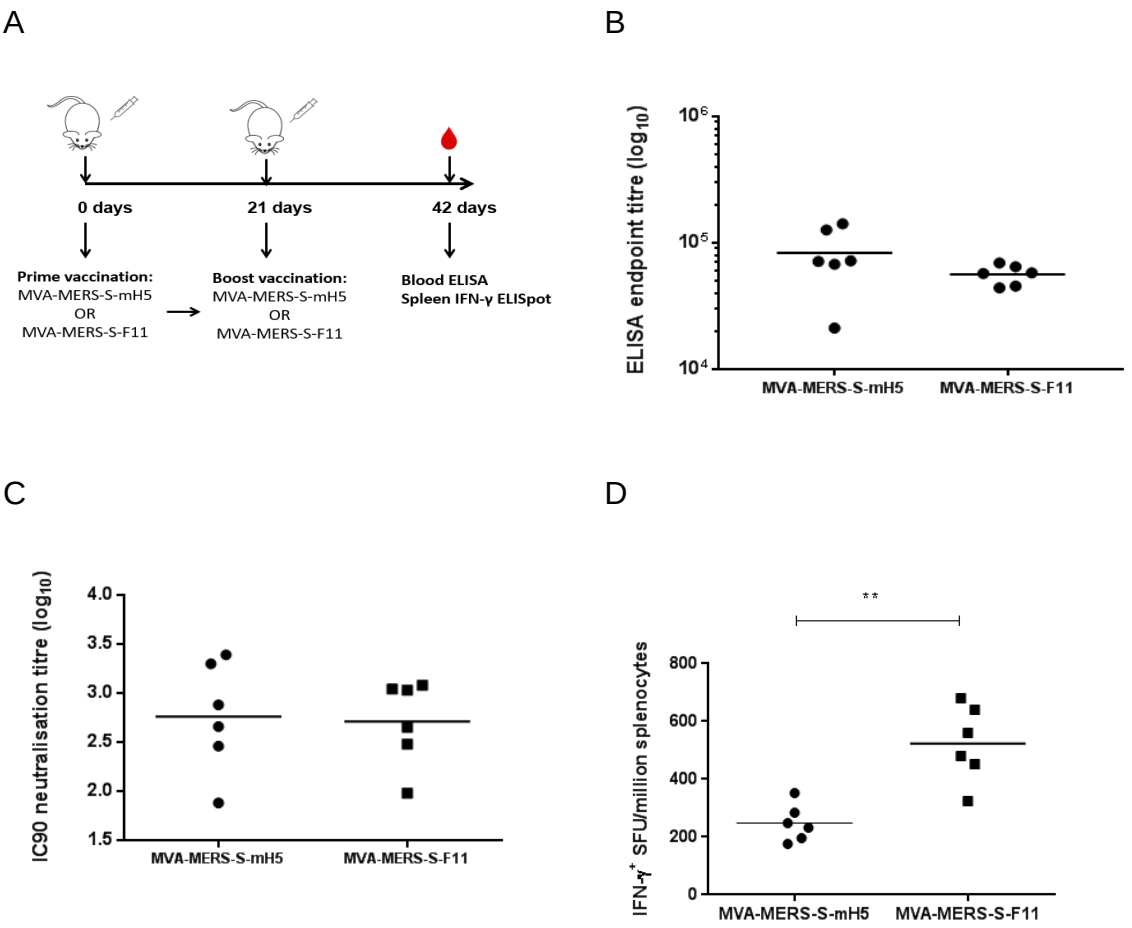


Figure 3.19. Effect on immunogenicity of the use of the strong early poxvirus promoter F11 compared to the benchmark promoter mH5 in a homologous prime-boost vaccination regimen. *A. BALB/c mice were primed with 106 PFU per mouse of MVA-MERS-S-mH5 or MVA-MERS-S-F11 and boosted with 106 PFU per mouse again 21 days after priming. Serum and spleens were collected 42 days after the first vaccination. B. Antibody titres against the MERS S glycoprotein measured by an endpoint ELISA. C. Neutralising antibody titres measured by the pseudotype neutralisation assay. D. T cell responses against a MERS S glycoprotein peptide This section of the thesis contains material previously published with the permission of the co-authors.*

*(S291) measured by IFN-γ ELISpot. Data are representative of two independent mouse experiments. C. Statistical analysis: Mann-Whitney test. **p<0.01.*

3.5.3 Heterologous prime-boost vaccination regimens

In the context of a heterologous prime-boost vaccination regimen with ChAdOx1, the F11 promoter did not have a significant positive impact on either the humoral or cellular immunogenicity against the MERS S glycoprotein; this is as compared to the mH5 benchmark promoter (**Figure 3.20**). There was a statistically non-significant trend in the same direction as the effect seen when the MVA was used alone, towards enhanced T cell responses with the F11 promoter.

This section of the thesis contains material previously published with the permission of the co-authors.

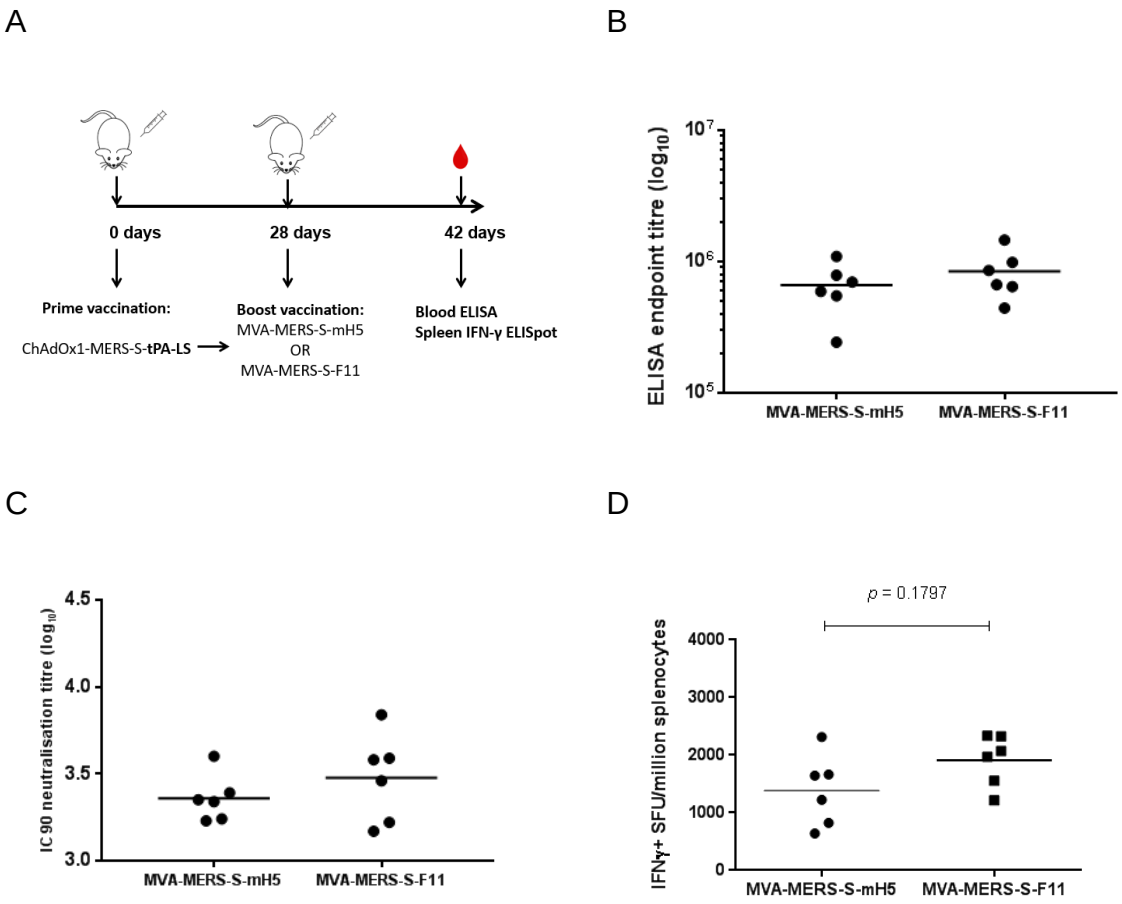


Figure 3.20. Effect on immunogenicity of the use of the strong early poxvirus promoter F11 compared to the benchmark promoter mH5 in a heterologous prime-boost vaccination regimen. *A. BALB/c mice were primed with 10^8 IU per mouse of ChAdOx1-MERS-S-tPA-LS and boosted with 10^6 PFU per mouse 28 days after priming with either MVA-MERS-S-mH5 or MVA-MERS-S-F11. Serum and spleens were collected 42 days after the first vaccination. B. Antibody titres against the MERS S glycoprotein as measured by an endpoint ELISA. C. Neutralising antibody titres as measured by the pseudotype neutralisation assay. D. T cell responses against a MERS S glycoprotein peptide (S291) as measured by IFN-γ ELISpot. Data are representative of two independent mouse experiments. Statistical analysis: Mann-Whitney test.*

This section of the thesis contains material previously published with the permission of the co-authors.

3.5.4 Vaccination immunogenicity comparison based on their induced neutralising antibodies

An overall comparison of the neutralising antibodies elicited by the vaccination regimens was made, bringing together the pseudotyped lentivirus neutralisation assay and the viral vectors described above. The heterologous prime-boost vaccination regimens resulted in higher neutralising antibody titres compared to the homologous prime-boost vaccination regimens. The heterologous vaccination regimens also outperformed single vaccinations with the ChAdOx1 vaccines (**Figure 3.21**).

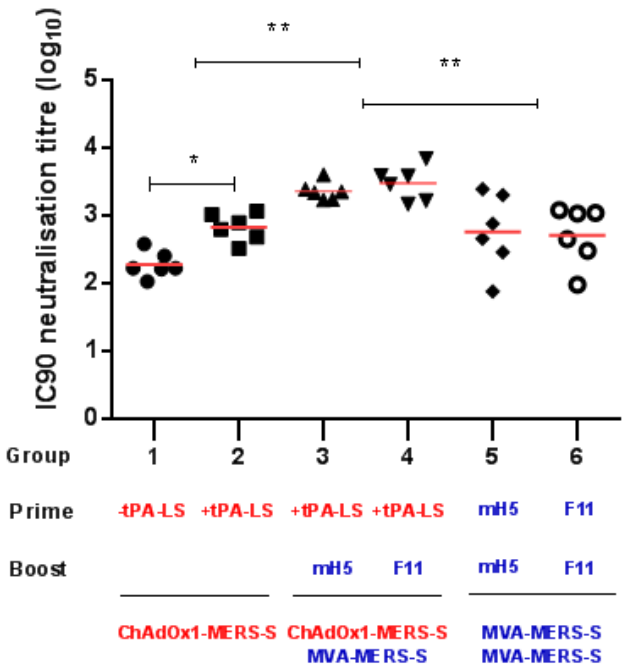


Figure 3.21. Comparison of the neutralising antibody titres from the vaccination regimens. *Neutralising antibody titres from each vaccination regimen were put together in one single panel. Means are shown for each vaccination regimen as a horizontal red line. Statistical analysis: Kruskal-Wallis test with Dunn's post hoc. *p<0.05, **p<0.01.*

This section of the thesis contains material previously published with the permission of the co-authors.

3.6 Discussion

The work described in this chapter has contributed to the Jenner Institute's development of candidate vaccines for MERS-CoV, as well as helping to guide strategies to optimise the immunogenicity of viral vector vaccines against other pathogens. The pseudotype neutralisation assay allowed a more informative assessment of the humoral responses induced by newly developed and highly immunogenic vaccines for the MERS coronavirus based on viral vectors. In the validation work undertaken here, the assay was able to discriminate among the different vaccination regimens tested (prime-only and heterologous/homologous prime-boost vaccination regimens) and the different genetic designs to try to boost the immunogenicity against the encoded MERS antigen (F11 versus mH5 and tPA-LS versus no tPA-LS). The development of this type of assays greatly contributes to the advancement of the study of the functionality of antibodies without the need of high biosecurity training and facilities, as it would be the case when employing the MERS-CoV.

In the field of vaccine development, the MERS pseudotype neutralisation assay has been used by other laboratories to evaluate the immunogenicity of different vaccination regimens based on proteins and DNA vaccines, as well as to evaluate the impact of different MERS S glycoprotein subunits on the induction of neutralising antibodies [57].

In the development of the pseudotype neutralisation assay described in this chapter, this showed a strong correlation with a microneutralisation assay and an endpoint titre ELISA using vaccinated mouse samples The neutralisation assay also showed a strong correlation with an endpoint titre ELISA using MERS-CoV-infected camel

sera, meaning that the assay can potentially be used to measure the immunogenicity of both vaccinated and naturally-exposed animal models.

In establishing the neutralisation assay, it was noted there was a higher susceptibility of the pseudotyped virus to infect Huh7 cells. The higher susceptibility of the Huh7 cell line to be infected with the MERS pseudotypes in our experiments might reflect the higher expression of the receptor dipeptidyl peptidase 4 (DPP4) from this specific cell line [316]. Nevertheless, more studies are needed to confirm this in our experiment set up.

Although most of the previous studies about prime-boost viral vector immunisations with adenovirus and MVA have focused on the optimisation of T cell responses (in fact the present study was originally conceived to optimise T cell responses), here we observed that a second vaccination with MVA has the capacity to further boost the neutralising antibody titres against transgene-encoded antigens. This leads to the possibility that if a ChAdOx1-only vaccination is not enough to protect against the MERS-CoV and this protection is correlated to neutralising antibody titres against the S glycoprotein, a prime-boost vaccination regimen with ChAdOx1-MVA would be ideal. Also, adding the MVA boost could well increase the durability of protection induced by ChAdOx1 alone.

In the case of the tissue Plasminogen activator leader sequence, its positive immunological effect against transgene-encoded antigens has been shown previously with other antigens, vaccine platforms (DNA and MVA vaccines) and infectious diseases [305]–[309]. Nevertheless, it has been shown that the inclusion of the tPA-LS might have a negative effect on cellular responses while favouring humoral responses [305]. It was interesting to find in our experiments that the tPA-

LS had a null effect on T cell responses while at the same time boosting antibody responses. This might reflect the fine balance between the two types of immune responses in vaccination and the intricate antigen processing mechanisms behind such immune responses.

In the case of humoral responses, it is known that the tPA-LS induces a higher expression/secretion of the antigens and that this might serve to a better B cell activation [309]. Nevertheless, this higher antigen expression and secretion might also contribute to cross-priming events impacting on $CD8^+$ T cell responses.

The experiments comparing the immunological effects of the early strong promoter F11 compared to the benchmark early/late promoter mH5 in MVA were mainly conceived to test if the early promoter activity of F11 could positively impact on the antigen-specific T cell responses, as a correlation between early antigen expression from poxvirus-based vaccines and T cell responses has been observed [284]. In agreement with these studies, the F11 promoter induced higher $IFN\gamma^+$ T cell responses against the MERS antigen compared to mH5. In addition, humoral responses were also evaluated with no statistically significant difference observed.

Based on the results presented in this chapter, the ChAdOx1-MERS-S-tPA-LS was selected to go to clinical manufacturing and it is now in clinical testing, with a phase I trial in Oxford nearing competition and a phase Ib trial in Saudi Arabia about to begin (ClinicalTrials.gov Identifier: NCT03399578) [317].

Chapter 4 Pre-clinical assessment of spermidine as an adjuvant for viral-vectored vaccines (malaria)

4.1 Introduction

Adjuvants represent a powerful tool to optimise the immunogenicity of viral-vectored vaccines. While viral-vectored vaccines have displayed outstanding immunogenicity and protection levels for different infectious diseases in animal models (e.g. malaria, Influenza, HIV), this effect has not always been translated to human trials [223], [318]. Therefore, the use of adjuvants (as in the case of other vaccine platforms) could represent a good option to try to enhance the immunogenicity of viral vectors.

To our knowledge, few studies have assessed the possibility to enhance the immunogenicity and protection levels of viral-vectored vaccines using chemical adjuvants. In one of these studies, an adenovirus-based vaccine encoding the $CD8^+$ T cell-restricted epitope Pb9 from *Plasmodium berghei* (a mouse malaria model in the laboratory) was tested in a mixture with different commercial adjuvants. From 11 adjuvants tested, only 2 could enhance the protective levels of the vaccine which correlated with the CD107a expression of antigen-specific $CD8^+$ T cells [223]. In the case of the adjuvant CoVaccineHT^{TM}, this adjuvant could even double the protection level of the adenovirus vaccine, an effect that was also dependent on the route of administration. The chemical adjuvant Matrix-M^{TM} has also been successfully used as an adjuvant for a recombinant Modified Vaccinia Ankara (MVA) virus for Influenza increasing both its humoral and cellular responses [319]. The same adjuvant has also been tested with a chimpanzee adenovirus (ChAdOx1) and the human

adenovirus type 5 (AdHu5) for Rift Valley fever where it showed different immunogenic profiles depending on the viral vector used [224].

Other adjuvants such as AddaVax, Abisco and Carbopol have been tested using AdHu5 and MVA in a heterologous prime-boost immunisation regimen. Even though the adjuvant Carbopol showed a detrimental effect on the viral-vectored immunogenicity, the adjuvants AddaVax and Abisco significantly enhanced the antigen-specific antibody responses [141].

The aforementioned studies highlight the promising implications of testing new adjuvants for viral vectored vaccines, but it also sheds light into the difficulty of this approach.

One of the desired effects of adjuvants for use in viral vectors is to increase the frequency/quality of memory $CD8^+$ T cells for infectious diseases where a good $CD8^+$ T cell response is necessary to combat the disease. This is the case of pre-erythrocytic malaria for which a correlation between $CD8^+$ T cell responses and protection has been found in animal models and humans [142], [320].

Spermidine is a recently discovered adjuvant that could potentially help in the frequency of memory $CD8^+$ T cells for pre-erythrocytic malaria. The compound (a naturally occurring polyamine) has the capacity to enhance $CD8^+$ T cell memory and recall responses when administered in the drinking water to old BALB/c mice (2 years old) [286]. In this research, it was established that memory $CD8^+$ T cell responses were significantly reduced in autophagy-deficient mice (Atg7 -/-) and that elderly mice also had reduced autophagy and $CD8^+$ T cell responses, nevertheless, treatment with spermidine could overcome these defects.

Spermidine is present in all living organisms and it is required for the maintenance of cell homeostasis. This polyamine influences a variety of biological process, including cell growth and proliferation, tissue regeneration, nucleic acid stabilization, enzymatic modulation, and regulation of translation [321]–[323]. In addition, it exhibits anti-inflammatory and antioxidant properties and enhances the metabolism of mitochondria [324]. External administration of spermidine has been linked to various beneficial effects on ageing and age-related diseases, including extension of lifespan [325]–[328], cardio- and neuroprotection [326], [329]–[331], and avoidance of immunosenescence of memory T cells (including $CD8^+$ T cells) [286]. Many of these features have been linked to the capacity of spermidine to ensure proteostasis through stimulation of cryoprotective autophagy [332]–[334]. Therefore, spermidine would deter the intracellular accumulation of toxic debris (a process characteristic of cell ageing), protecting the organisms from age-associated conditions such as cancer, neurodegeneration and cardiovascular diseases [335]–[337].

From a cell and molecular biology perspective, spermidine induces autophagy through the inhibition of several acetyltransferases [327], including EP300 [338]. These acetyltransferases negatively regulate autophagy [339]. Therefore, to counteract the effect of these enzymes will have a positive effect on autophagy levels, deterring age-related diseases (**Figure 4.2**). Nevertheless, more studies are needed to fully understand the mechanisms of action of spermidine in the upregulation of autophagy and its contribution to deter immunosenescence.

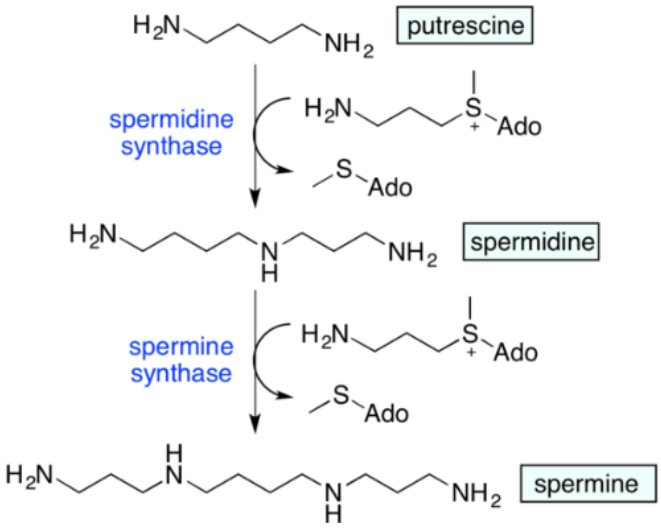


Figure 4.1. Biosynthesis of spermidine from putrescine. *Spermidine is a natural polyamine found in all living organisms. The compound is synthesised from putrescine by the enzyme spermidine synthase. This enzyme transfers an aminopropyl group to putrescine for the formation of spermidine. On the other hand, putrescine is synthesised from ornithine, an amino acid. Spermidine is synthesised in the cytoplasm. The net cytosolic content of spermidine is regulated by the catabolism of the compound by a process of acetylation and oxidation, extracellular intake and secretion* [324]. *Ado=5-adenosyl. Source: Andrew Murkin.*

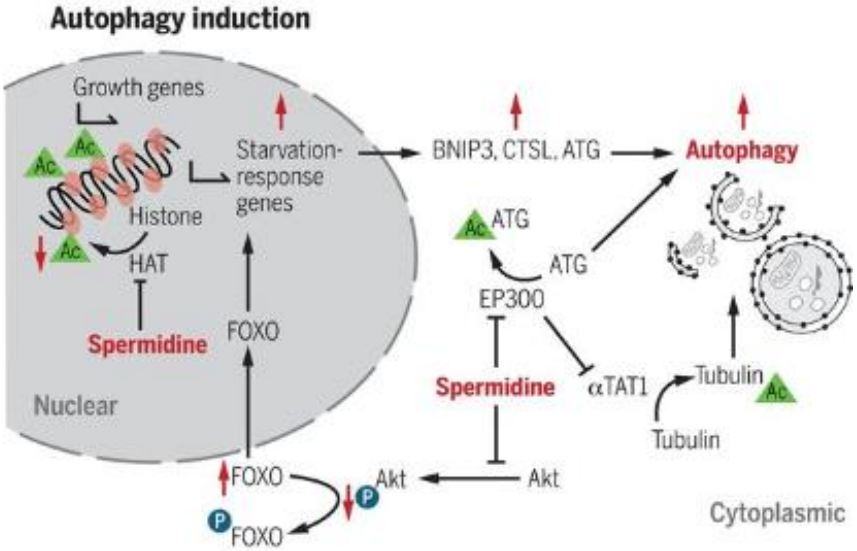


Figure 4.2. Autophagy induction by spermidine. *Autophagy induction can be accomplished by the administration of spermidine. This compound inhibits the acetyltransferase EP300, primarily resulting*

in autophagy-relevant cytosolic protein deacetylation. Sustained autophagy control by spermidine is mediated through induction of autophagy-relevant gene transcription. This process involves the regulation of FOXO transcription factor and the inhibition of histone acetyltransferases, all of this resulting in an epigenetic transcriptional reprogramming [340]*. Nevertheless, more studies are needed to fully understand the mechanisms of action of spermidine in the upregulation of autophagy. Source: https://science.sciencemag.org/ content/359/6374/eaan2788/tab-figures-data*

The series of experiments described in this chapter set about to determine whether spermidine could be used to enhance effector $CD8^+$ T cell responses and memory $CD8^+$ T cell responses induced by viral-vectored vaccination. While the previous study established the effect of spermidine in old mice that are autophagy-deficient, other studies have indicated that spermidine can boost autophagy levels in young mice [326], [341], [342], opening the potential for the use of spermidine to enhance the $CD8^+$ T cell responses in all age groups.

It is worth remembering that malaria is one of the most devastating infectious diseases in the world. According to the WHO, in 2017 there were 219 million malaria cases worldwide, including 435,000 confirmed deaths. Many of the cases were located in Sub-Saharan Africa (92%). In regard to the prevalence of different malaria species, most of the cases were due to the strain *Plasmodium falciparum,* except in the Americas where *Plasmodium vivax* was more prevalent [79]. Despite this global scale of disease burden, no effective vaccines are commercially available for malaria. Therefore, any strategy with the capacity to enhance immunity could have a significant impact.

4.2 Effect of spermidine on effector IFNγ$^+$ CD8$^+$ T cell responses from viral vectors

4.2.1 Experiment design

To evaluate a possible effect of spermidine on the effector CD8$^+$ T cell responses induced by viral vectors, replication-deficient adenovirus type 5 (AdHu5) was chosen as the test viral-vectored vaccine model for initial proof of concept studies. The deletion of the adenoviral E1 and E3 genes abrogates the replication of the virus in host cells and allows the insertion of larger antigens to enable the simultaneous assessment of CD4$^+$ T cells, CD8$^+$ T cells and antibodies [343].

An AdHu5 vector encoding a synthetic polypeptide string under the control of the human Elongation Factor 1-α (hEF1-α) promoter and comprising the peptides Pb9, P15 and AL11 fused to an enhanced Green Fluorescence Protein (eGFP) was utilised in this study (**Figure 4.3**). The *Plasmodium berghei* CD8$^+$ T cell epitope Pb9 is an H-2K^d-restricted immunodominant protective epitope for pre-erythrocytic murine malaria [279]. The peptide P15 is a CD4$^+$ T cell peptide from *Mycobacterium tuberculosis* [279], [280] and the AL11 peptide is an H-2D^b-restricted CD8$^+$ T cell peptide coming from the gag protein of the Simian Immunodeficiency Virus (SIV) [281]. The inclusion of the eGFP enables the study of antibody responses against an encoded antigen and the study of an additional CD8$^+$ T cell peptide named eGFP$_{200-208}$. This is an H-2K^d-restricted peptide [282]. The complete synthetic polypeptide has been denominated TIPeGFP (acronym of Tuberculosis, Influenza, *Plasmodium*, enhanced Green Fluorescence Protein). These peptides serve for a dual purpose, which is to test immunogenicity and efficacy for multiple infectious diseases [236],

[280]. The viral vector also induces a well-characterised effector and memory T cell profile, useful when assessing the effect of adjuvants [344]–[346].

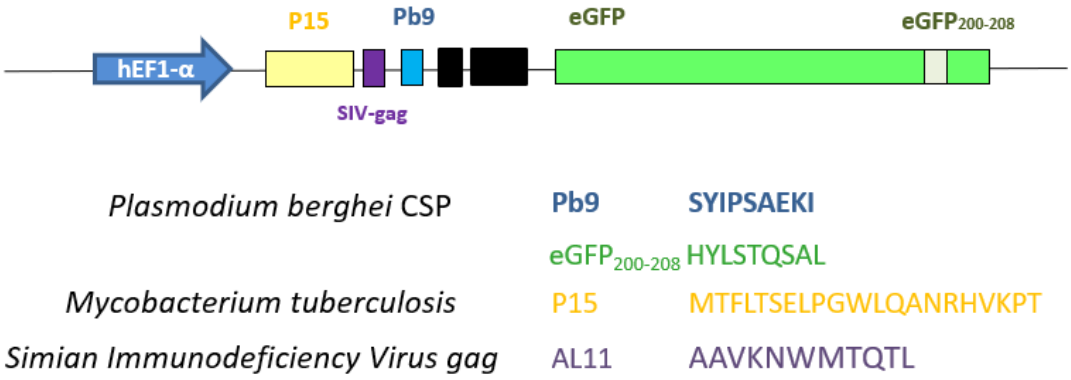


Figure 4.3. Scheme of the transgene peptides encoded by the viral vectors. *The synthetic polypeptide comprises the CD4⁺ T cell peptide P15 fused to the CD8⁺ T cell peptides SIV-gag (AL11) and Pb9. These peptides are fused to the N-terminus to the enhanced Green Fluorescence Protein (eGFP) that bears an additional CD8⁺ T cell peptide denominated $eGFP_{200-208}$. The complete synthetic polypeptide has been denominated TIPeGFP (acronym of Tuberculosis, Influenza, Plasmodium, enhanced Green Fluorescence Protein). Black boxes correspond to the peptides PyCD4 and Py3, not used in the present study.*

For the experiments, the viral vector was injected intramuscularly at a standard dose of 10^7 IU per BALB/c mouse and cellular responses were measured from spleens at the peak of response (day 14) [236], [347], [348]. To determine the effect of the adjuvant on the endurance of the transgene-specific T cell responses, the frequency of antigen-specific cells in peripheral blood mononuclear cells (PBMCs) was determined at different time points after vaccination.

Due to the well-known correlation between the frequency of IFNγ-secreting $CD8^+$ T cells induced by viral vectors and protection in the aforementioned pre-erythrocytic malaria model [346], the potential effect of spermidine on the frequency of these cells was of most interest. *Ex-vivo* IFN-γ ELISpot technique was therefore employed whereby cells are stimulated with the $CD8^+$ T cell peptide Pb9.

Typically spermidine was given in the drinking water *ad libitum* at a 5 mM concentration to the positive control group, this concentration having previously been described to have an effect on antigen-specific $CD8^+$ T cells [286]. Spermidine administration began 21 days before vaccination and the spermidine solution was changed every 2-3 days until the end of the experiment. A negative control group was included where the mice were vaccinated with the same viral vector dose and given normal water to drink.

4.2.2 Spermidine enhances the frequency of effector Pb9-specific $IFN\gamma^+$ $CD8^+$ T cells from spleens at day 14

To determine whether the addition of spermidine to the drinking water of mice could enhance the immune response, mice were given spermidine in the drinking water for 3 weeks prior to vaccination and until sacrifice (day 14 after vaccination). To test if the spermidine administered at the same concentration (5 mM) at different time frames after vaccination (without administration *prior to* vaccination) could also induce an immunological effect, spermidine was supplied for 7 and 14 days after vaccination. A negative control consisting of the AdHu5 without any spermidine in the drinking water was also employed.

Administration of spermidine significantly enhanced the frequency of IFNγ^{+} CD8^{+} T cells against the Pb9 peptide encoded by the AdHu5 at day 14. In pair-wise comparisons of individual groups, this only reached statistical significance when comparing the negative control (no spermidine) and positive control (spermidine administration from 21 days prior to vaccination and through the whole experiment) groups (**Figure 4.4** B). It was however apparent that there was a dose-response relationship between duration of administration and immunogenicity (by linear regression $^{**}p$=0.0030). A linear regression model was used rather than a curved dose-response due to the limited number of doses (durations) for which data was available, and the lack of evidence of curvature in the data. Therefore, it can be established that there is a positive significant effect of spermidine on this type of response depending on the spermidine intake.

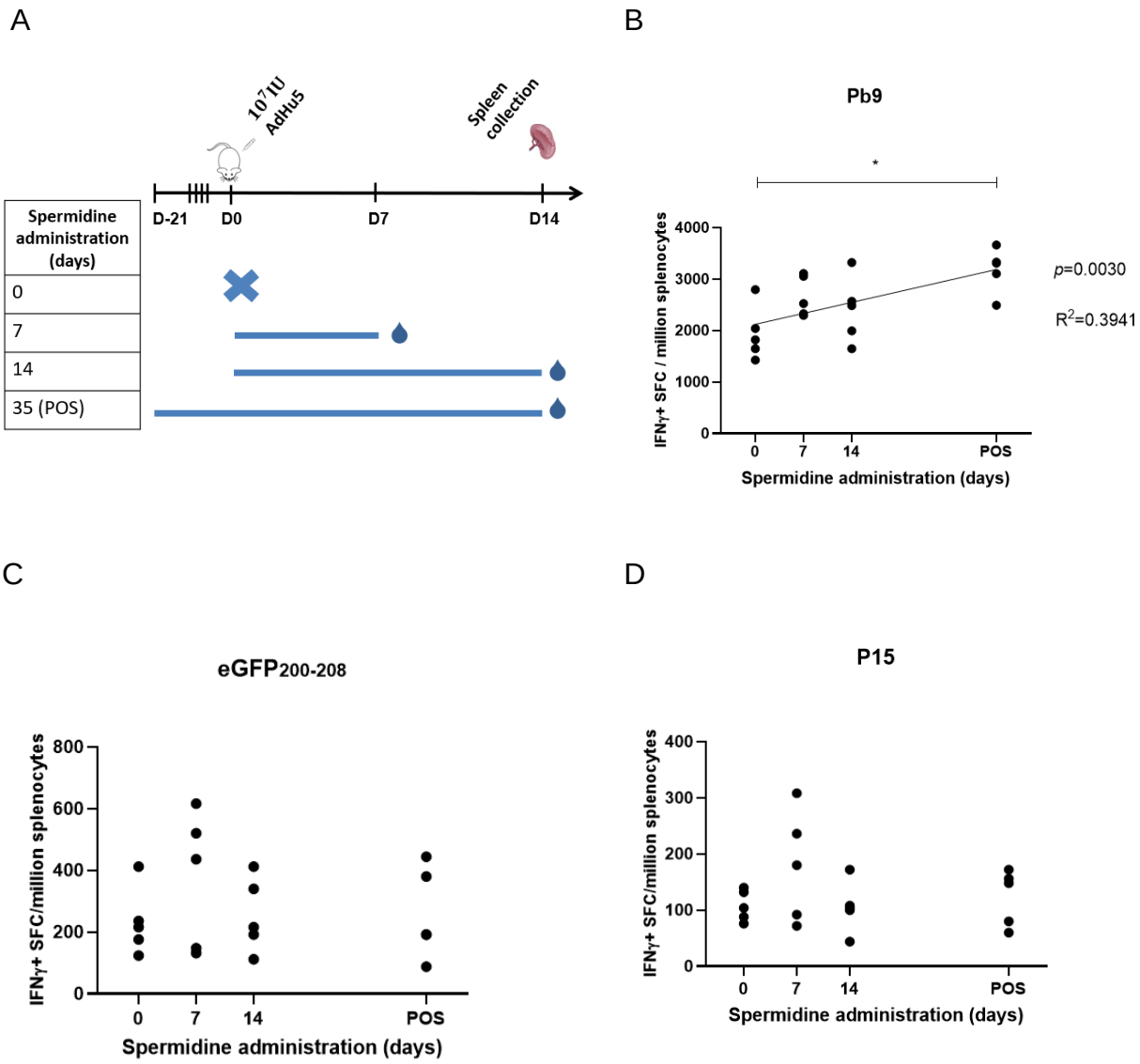


Figure 4.4. Effect of spermidine on the frequency of IFNγ⁺ T cells at day 14 from spleens. *A. 5 BALB/c mice per group were vaccinated intramuscularly with a standard dose of replication-deficient AdHu5-TIPeGFP (10^7* IU/mouse*). In the positive control group ("POS" in X-axis), spermidine was given in the drinking water at a 5 mM concentration from 21 days before vaccination and through the duration of the experiment (35 days in total). For the negative control group, mice were only given normal water to drink ad libitum ("0" in the X-axis). One testing group was given spermidine in the drinking water for the first 14 days after vaccination ("14" in the X-axis). The other testing group was given spermidine for the first 7 days after vaccination ("7" in the X-axis). For all mice groups, spleens were collected 14 days after vaccination and analysed by an ex-vivo IFN-γ ELISpot stimulating cells*

*with Pb9, $eGFP_{200-208}$ and P15 peptides. B. Frequencies of IFNγ$^+$ CD8$^+$ T cells against the peptide Pb9. C. Frequencies of IFNγ$^+$ CD8$^+$ T cells against the peptide $eGFP_{200-208}$. D. Frequencies of IFNγ$^+$ CD4$^+$ T cells against the peptide P15. For B, the statistical analysis was performed by the Mann-Whitney test, *p<0.05.R^2 and p-value shown correspond to a linear regression model. Statistical difference between the positive and the negative control groups for Pb9 was obtained in a further repetition of the experiment shown in* ***Figure 4.11*** *B.*

4.2.3 Spermidine does not influence the persistence of effector IFNγ$^+$ CD8$^+$ T cells from PBMCs

To evaluate whether spermidine could influence the persistence of effector IFNγ$^+$ CD8$^+$ T cell responses for Pb9, the immune response was monitored in the blood at multiple time points after vaccination. After evaluating the T cell responses using an ELISpot technique, no effect on the persistence of the cellular response against the peptide Pb9 was observed, comparing between the positive and the negative control groups (**Figure 4.5**).

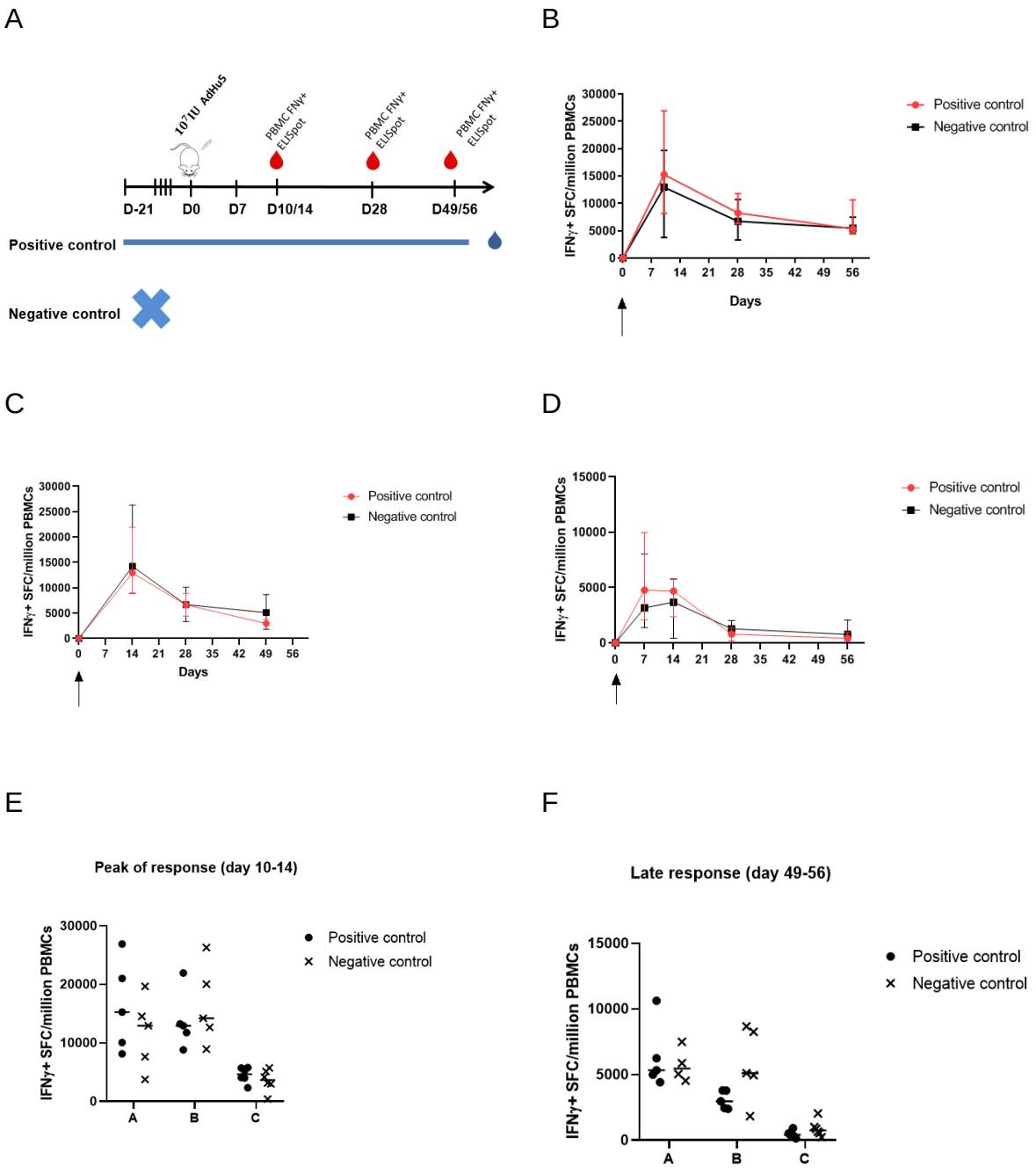


Figure 4.5. Effect of spermidine on the IFNγ⁺ CD8⁺ T cell responses against Pb9 from PBMCs. *A. Effect on the endurance of IFNγ⁺ CD8⁺ T cell responses under the administration of spermidine. Positive control group: spermidine (5 mM) given in the drinking water ab libitum 21 days before vaccination and through the whole duration of the experiment. Negative control group: normal water given ab libitum. Both groups were vaccinated with 10^7 IU AdHu5 at day 0 (black arrow below the panel). This experiment shown in panel B was repeated twice with similar results (biological replicates shown in panels C & D). E and F panels show the T cell responses at the peak of the immune response (day 10-14) and late response (day 49-56), respectively. These data were obtained from*

panels B, C & D. For panels B, C & D, the median + range is shown for each time point (n=5, 6). For E & F, horizontal lines represent the group medians. Statistical analysis: Mann-Whitney test for each time point and mouse groups, p>0.05.

4.3 Spermidine does not influence the re-activation of memory CD8$^+$ T cell responses

Given autophagy has been shown to play a role in memory formation and recall responses, it was important to determine whether administration of spermidine could also have an impact on memory T cells induced by viral vectors. For this, a heterologous vaccination regimen was selected for two reasons. The first was to circumvent the creation of anti-vector immunity, therefore potentially obscuring the results of the effect of spermidine on the memory T cells. The second reason was for translational purposes: adenovirus-MVA prime-boost vaccination regimens represent one of the most promising vaccination schemes to date to protect against the infectious diseases where a strong and long-lived CD8$^+$ T cell responses are required [142], [320], [348]; therefore, to try to increment the immunogenicity of this prime-boost vaccination regimen with adjuvants would be of extreme value.

A PBMC IFN-γ ELISpot technique was selected to allow the tracking and evaluation of the differences in the frequency of antigen-specific IFNγ$^+$ CD8$^+$ T cells right after the MVA boost for each mouse group. It could be inferred that any statistically significant difference in the frequency of antigen-specific IFNγ$^+$ CD8$^+$ T cells after the MVA boost would reflect the state of the memory CD8$^+$ T cells previously induced during the adenovirus priming. This approach for evaluating the effect of spermidine on the memory T cells by the measurement of the frequency of antigen-specific T cells after a boost vaccination or challenge has been previously employed [286].

Therefore, AdHu5 vaccinated BALB/c mice (described above) were boosted with a standard dose of MVA encoding TIP (Pb9) on day 49 or 56 after prime vaccination.

The frequency of IFNγ$^+$ CD8$^+$ T cells against the Pb9 peptide for the two groups (with and without spermidine) was measured 1 week after the MVA boosting (peak of response). No statistically significant effect of spermidine upon the frequencies of PBMCs and splenocyte IFNγ$^+$ CD8$^+$ T cells was seen at either of the two time points, immediately before the MVA boosting and 1 week after the MVA boosting (**Figure 4.6**), indicating that spermidine does not have an effect on the re-activation of Pb9-specific memory IFNγ$^+$ CD8$^+$ T cells.

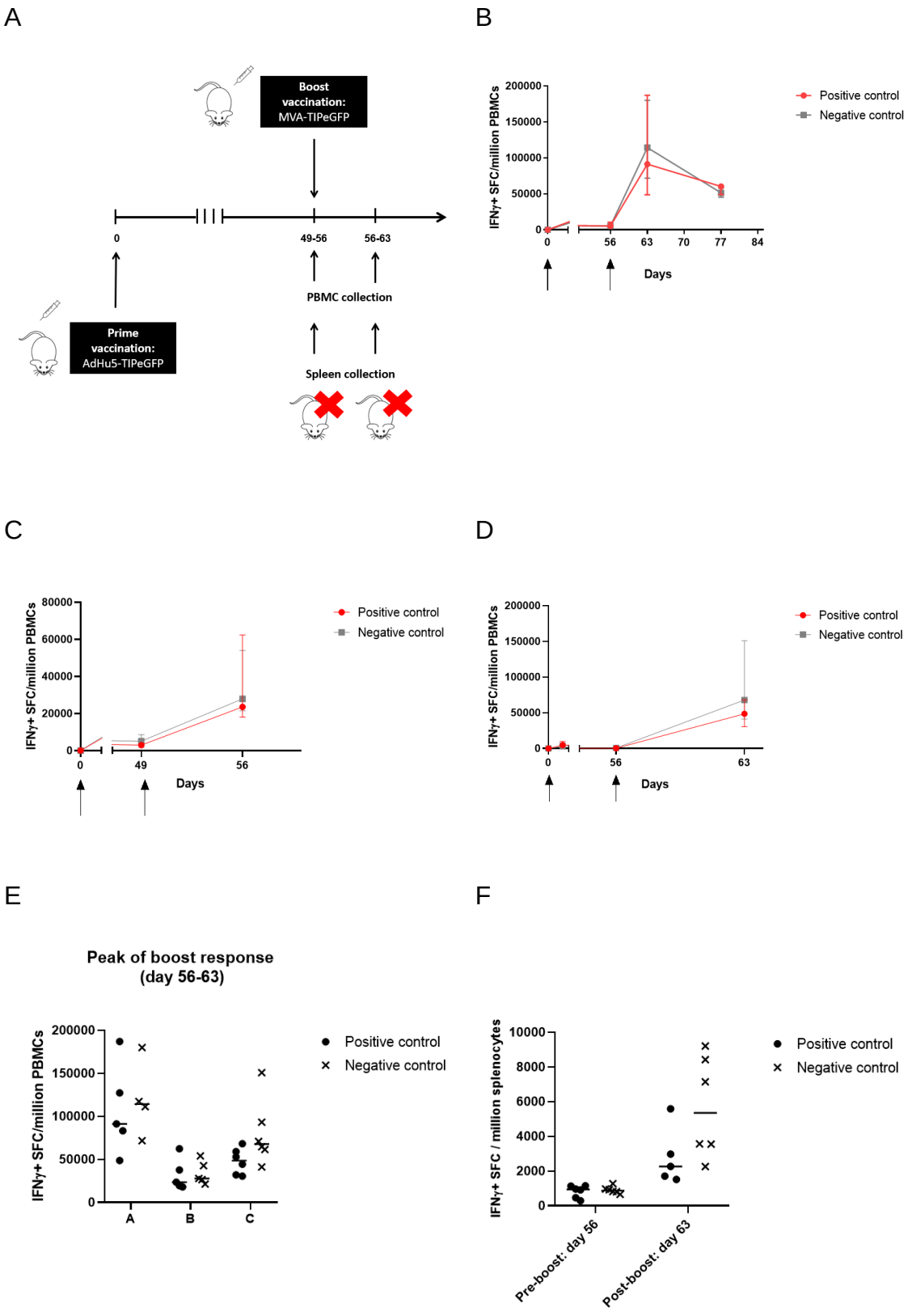


Figure 4.6. Effect of spermidine of the Pb9-specific memory CD8⁺ T cells. *A. Scheme of the vaccination regimen used in this study. All experiments involving PBMC (i.e. panels B-E) were performed using the same cohorts of mice for which data up to day 56 was shown in* ***Figure 4.5****.*

Positive control corresponds to the mouse group subjected to administration of spermidine in the drinking water (5 mM) from day 21 before vaccination until finalization of the experiment. Negative control: no spermidine administration, only vaccination. Two additional cohorts of mice were sacrificed at day 56 and 63 to provide pre-boost and post-boost splenocyte data shown in panel 4 F.

Panels B-D show kinetics of IFNγ$^+$ CD8$^+$ T cell responses against the peptide Pb9 from PBMCs during adenovirus-MVA prime-boost vaccination (black arrows below the panel represent the immunisation time points), with each panel depicting the results from a separate cohort of mice.

Panel E shows combined post-boost vaccination responses at the peak of the immune response (1 week after boosting) from all three of the replicate cohorts which are shown individually in panels B-D.

Panel F shows pre-boost (day 56 after prime vaccination) and post-boost (day 63, 1 week after boost) immune responses from spleens.

Statistical analysis for all panels: Mann-Whitney test for each time point and mouse group, p>0.05.

For panels B, C & D, data-points correspond to the medians + range (n=5, 6). For E & F, horizontal lines represent the medians for each mouse group.

4.4 Spermidine does not influence the antibody responses induced by viral vectors

Due to the capacity of the previously used viral vectors to express and induce IgG antibody responses against an encoded eGFP antigen, serum antibody responses against this antigen were measured 1 week after MVA boosting. The objective of this experiment was to determine if spermidine could influence the antibody titres at this time point, therefore reflecting a potential effect on the re-activation of memory antigen-specific B cells. The IgG ELISA antibody titres showed no significant effect of the adjuvant on the anti-eGFP antibody responses as compared to the negative control (no adjuvant in the drinking water) (**Figure 4.7**).

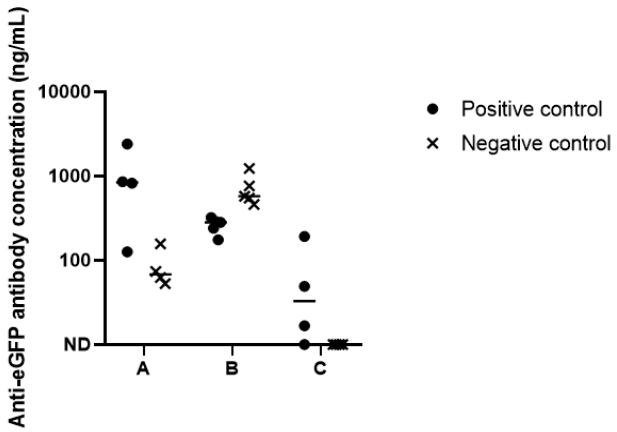


Figure 4.7. Effect of spermidine on the antibody responses induced by viral vectors. *IgG antibody responses from serum against the encoded antigen eGFP were measured 1 week after MVA boosting (day 56-63) employing samples from the mice previously used for measuring T cell responses (**Figure 4.5** and **Figure 4.6**). Positive control: spermidine administration in the drinking water (5 mM) from 21 days before vaccination until day 56-63. Negative control: no spermidine administration, only vaccination. The experiment was performed three times (biological replicates, shown as A, B & C). Medians are shown as black horizontal lines for each mouse group. Statistical analysis: Mann-Whitney test, p>0.05, n=4, 5.*

4.5 Spermidine does not influence the immunogenicity of the virus-like particle R21

In order to determine if spermidine could influence the immunogenicity of non-vectored antigens, spermidine was tested in combination with the highly immunogenic virus-like particle for malaria called R21. This vaccine comprises the C-terminal region of the circumsporozoite protein (CSP) of *Plasmodium falciparum* fused to the Hepatitis B Small protein (HBsAg) (**Figure 4.8**).

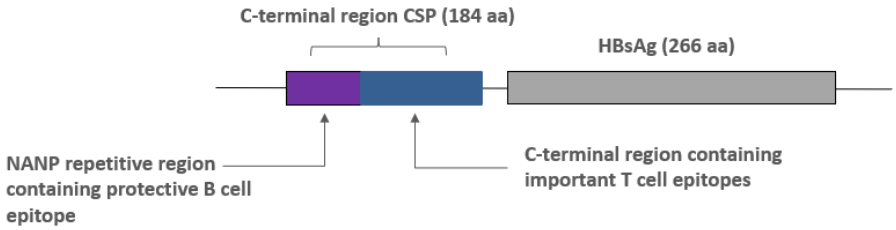
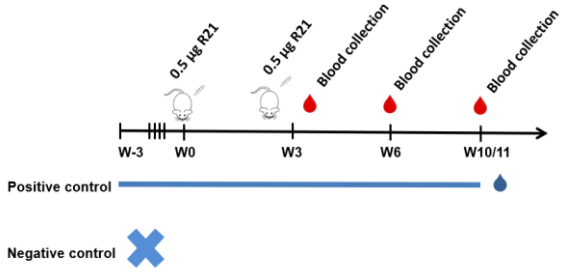


Figure 4.8. Schematic representation of the vaccine R21, a highly immunogenic virus-like particle for malaria. *The vaccine is composed by the C-terminal region of the circumsporozoite protein (CSP) of P. falciparum NF54 fused to the N-terminus of the Hepatitis B surface antigen protein (HBsAg). The CSP region also contains 19 NANP repeats from the central repeat region which contain protective B cell epitopes. In addition, the C-terminal region contains important T cell epitopes. The addition of the HBsAg allows the self-assembling of the virus-like particle produced in yeast exposing the CSP region on the surface of the VLP* [141].

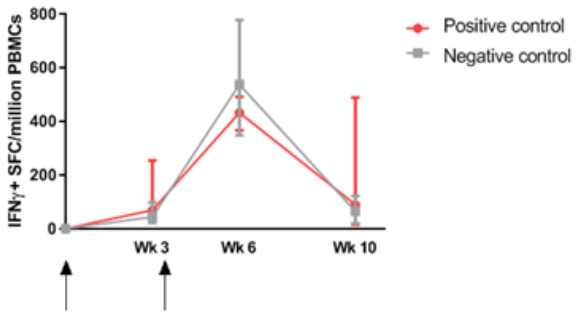
BALB/c mice were vaccinated with two i.m. doses of 0.5 μg of R21 given 3 weeks apart. PBMC responses to the C-terminal CSP peptides were measured at different time points by ELISpot. IFNγ$^+$ T cell responses as measured by ELISpot showed a non-significant effect of spermidine on this type of responses either after the priming or boosting vaccination (**Figure 4.9** B). In addition, the serum IgG antibody titres against the CSP region of the R21 did not show a significant effect either after priming or boosting vaccination (**Figure 4.9** C). Results at weeks 6 and 10, both with and without spermidine, showed a surprising pattern and were inconsistent with previous experience with this antigen. Ideally, this experiment would have been repeated, but this was not pursued due to the overall lack of an encouraging trend towards a beneficial effect of spermidine.

Therefore, this experiment suggested that, as had previously been found for viral-vectored vaccines, spermidine does not enhance B or T cell responses to the R21 non-vectored vaccine.

A



B



C

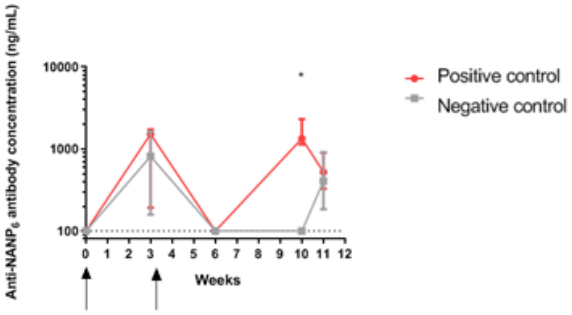


Figure 4.9. Effect of spermidine on the R21 immunogenicity. *A. Spermidine treated and control BALB/c mice were i.m. vaccinated on day 0 and day 21 with 0.5 µg R21 with serum blood sampled at different time points to measure T cell and antibody responses. Positive control: spermidine in the drinking water (5 mM) administered 3 weeks (21 days) before vaccination and throughout the whole duration of the experiment. Negative control: no spermidine, only vaccinations. B. PBMC IFNγ⁺ T cell responses against the circumsporozoite protein (CSP) region of R21 during a homologous prime-boost vaccination regimen (black arrows below the panel represent the time points of vaccination). C. Serum IgG anti-$NANP_6$ antibody responses obtained from the same mouse experiment as panel B. Medians + range are shown for each time point in both graphs. Statistical analysis: Mann-Whitney test for each time point, *p<0.05, n=4.*

4.6 Effect of the intramuscular administration of spermidine on the immunogenicity of viral-vectored vaccines

Prolonged oral administration of spermidine is not a practical means of adjuvanting vaccines for clinical use. Therefore. we wanted to establish whether spermidine could be administered along with the viral vectors by the intramuscular route. Since no previously published information regarding intramuscular injection of spermidine could be found, we initially prepared a range of concentrations of filter-sterilised spermidine solutions dissolved in PBS and administered them intramuscularly to BALB/c mice as a first approach. Unfortunately, the injected concentrations (0.1-1000 mM) in a 50 µL volume per mouse resulted in immediately visible mouse discomfort. After culling the mice and dissecting the site of injection (thigh muscle), tissue damage was observed (as reflected by the change of colour in the muscle fibres in the site of injection).

The physicochemical properties of spermidine were investigated to determine the possible causes of this tissue damage. An extreme alkaline pH was the most plausible cause for this: the pH of spermidine was measured at the concentrations previously indicated resulting in an extreme alkaline pH of at least 10.5 (**Figure 4.10Figure 4.10**).

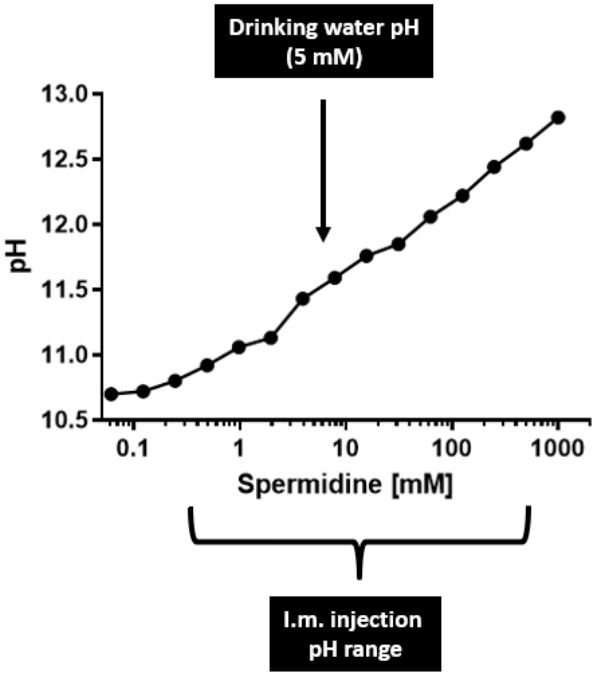


Figure 4.10. pH of spermidine at different concentrations in PBS as measured by a pH-meter. *Black boxes indicate the pH correspondent to the concentrations previously used when administering spermidine in the drinking water (5 mM) and by intramuscular (i.m.) vaccination (1-1000 mM) in a 50 µL volume per mouse.*

The pH of the spermidine solutions was therefore adjusted to a physiological pH of 7.4 by the addition of hydrochloric acid (HCl) and then filter-sterilised. 50 µL volumes of 0.14-14 mM solutions of spermidine at neutral pH were then administered intramuscularly, with close observation of mice during the recovery period. No evidence of discomfort was seen, and so it was concluded that pH had indeed been the cause of the adverse reaction to the unadjusted spermidine.

4.6.1 Spermidine injected intramuscularly does not influence the IFNγ$^+$ CD8$^+$ T cell responses induced by viral vectors

Different concentrations of spermidine at a pH of 7.4 were mixed with a standard dose of AdHu5 and injected intramuscularly, such that the mice received a dose of 10^7 IU of AdHu5 and 0.14-14 mM of spermidine in a 50 µL volume per mouse.

It is important to note that the maximum spermidine dose delivered intramuscularly would be several orders of magnitude less than that delivered orally. We had considered the feasibility of emulating the amount of spermidine hypothetically ingested by the positive control group (5.25 x 10^{-4} moles, based upon a daily water intake of 3 mL/day/mouse for 35 days). This dose was impossible to achieve in one single vaccination shot considering the maximum vaccination volume feasible to inject by the intramuscular route of administration is 50 uL and the maximum achievable spermidine concentration of 14 mM, and hence a maximum dose of 7 x 10^{-7} moles. Multiple shots would compromise the translational applicability of the experiment. Therefore. we tested a range of concentrations of spermidine, extending up to this practical limit (0.14-14 mM).

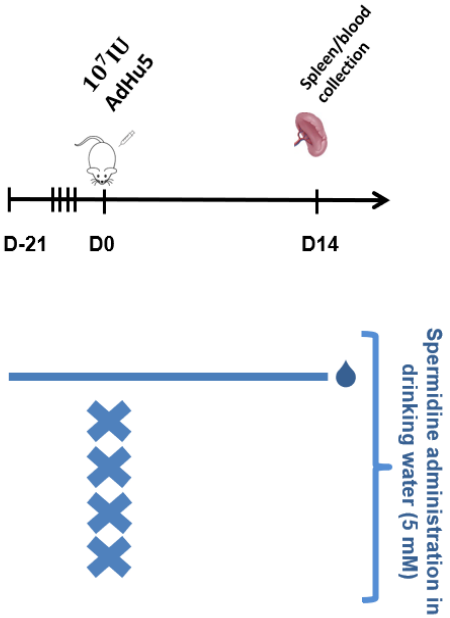
As previously indicated, positive and negative mouse controls used in this experiment corresponded to spermidine administered in the drinking water (5mM) from 21 days prior the vaccination and through the whole experiment; the negative control corresponded to the vaccination with the standard intramuscular administration of AdHu5 alone (no spermidine).

IFNγ$^+$ CD8$^+$ T cell responses against the encoded peptides were measured 14 days after vaccination (peak of response) and these showed that spermidine injected

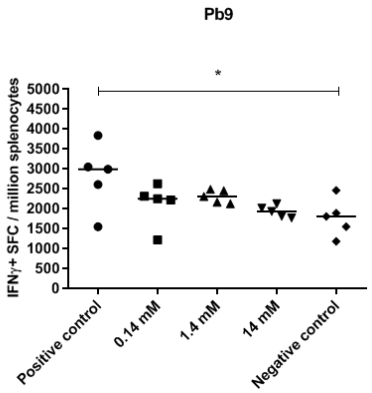
intramuscularly along with the adenovirus vector did not enhance the transgene-specific cellular responses at the spermidine concentrations tested (**Figure 4.11**).

A

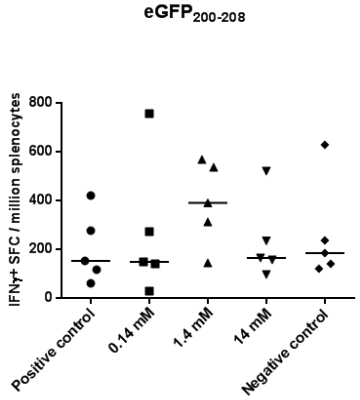
Group name	Administration spermidine in drinking water, 5 mM (days)	Administration of spermidine in a 50 µL shot along with AdHu5 (mM)
Positive control	35	0
0.14 mM	0	0.14
1.4 mM	0	1.4
14 mM	0	14
Negative control	0	0



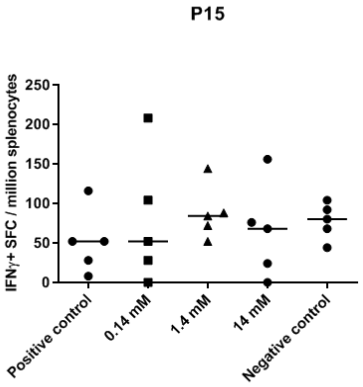
B



C



D



E

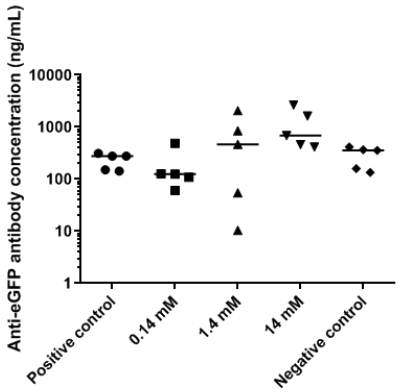


Figure 4.11. Effect of spermidine on the antigen-specific T cell and B cell responses induced by AdHu5 injected intramuscularly. *A. BALB/c mice were injected intramuscularly with increasing doses of spermidine (50 μL at 0.14-14 mM per mouse; pH 7.4) mixed with an equal dose of AdHu5-TIPeGFP (1x10^7 IU/mouse). B. Pb9-specific IFNγ$^+$ CD8$^+$ T cell responses as measured by an IFN-γ ELISpot at the peak of response (day 14). C. eGFP$_{200-208}$-specific IFNγ$^+$ CD8$^+$ T cell responses as measured by an IFN-γ ELISpot (day 14) D. P15-specific IFNγ$^+$ CD4$^+$ T cell responses as measured by an IFN-γ ELISpot (day 14). E. Anti-eGFP IgG antibody titres as measured by an endpoint IgG serum ELISA (day 14). Positive control: spermidine in the drinking water (5 mM) administered 21 days before vaccination and throughout the whole duration of the experiment. Negative control: no spermidine. Statistical analysis: Kruskal-Wallis test with Dunn's post hoc test, *p<0.05. Horizontal lines represent the medians for each mouse group.*

4.7 Effect of spermidine in older mice

The effect of spermidine on the viral-vectored immunogenicity was also evaluated in older BALB/c mice. Previously, it has been described that the T and B cell responses against vaccination in old mice (22-24 months old) can be significantly enhanced utilising the spermidine administration regimen previously described [286], [349]. Therefore, it was hypothesised that a similar significant effect could be observed

from the viral-vectored vaccination regimen if older BALB/c mice were to be employed. With this purpose, ex-breeder BALB/c mice (at least 5-6 months old, Envigo) were vaccinated with the heterologous prime-boost vaccination regimen. The Pb9-specific $CD8^+$ T cell responses and the IgG anti-eGFP antibody responses were measured at different time points after prime and boosting vaccination using a PBMC $IFN\gamma^+$ ELISpot and an endpoint IgG serum ELISA, respectively.

The Pb9-specific $CD8^+$ T cell responses and the anti-eGFP antibody titres at different time points indicated a non-significant effect of spermidine of these type of responses as compared to the negative control (no spermidine added to the drinking water) (**Figure 4.12**).

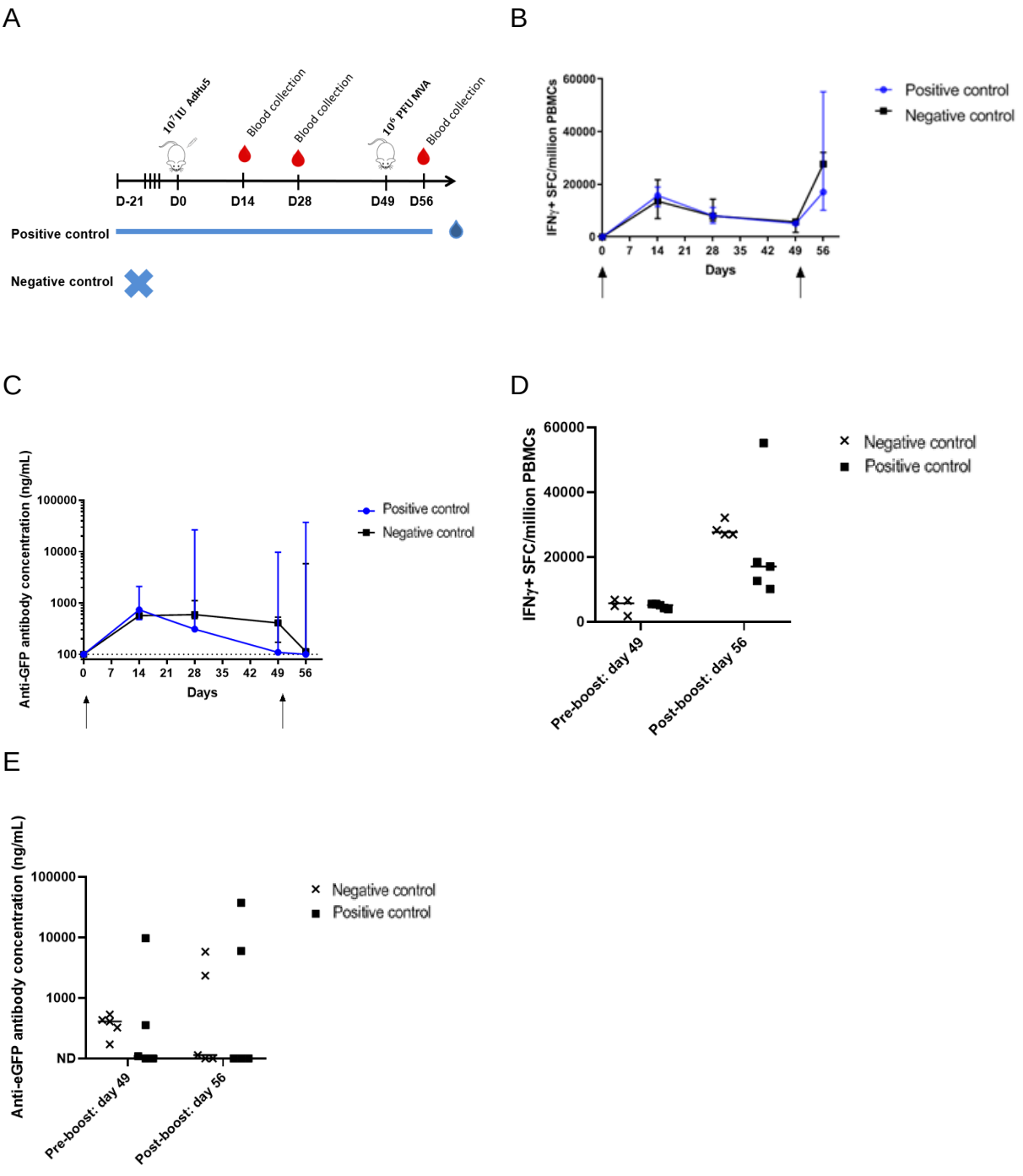


Figure 4.12. Effect of spermidine on the T and B cell responses against viral vectors using older BALB/c mice. *A. Female BALB/c mice of at least 5-6 months old were used in this study. Spermidine was administered in the drinking water (5 mM) 21 days before first vaccination and throughout the whole duration of the experiment (positive control). No spermidine in the drinking water was used as a negative control. B. Pb9-specific CD8⁺ T cell responses at different time points after vaccination as measured by a PBMC IFNγ⁺ ELISpot. C. Anti-eGFP antibody titres at different time points after vaccination as measured by an endpoint IgG serum ELISA. Medians + range at each time*

point are shown in both panels. Black arrows below the panel show the time points used for vaccination (AdHu5 followed by MVA). D. Pre-boost (day 49 after prime vaccination) and post-boost (day 56, 1 week after boost) immune responses from PBMCs obtained from panel B. E. Pre-boost (day 49 after prime vaccination) and post-boost (day 56, 1 week after boost) antibody responses obtained from panel C. For D & E, horizontal lines represent the group medians. Statistical analysis: Mann-Whitney test for each time point, p>0.05, n=5.

4.8 Discussion

This work demonstrated that spermidine, when administered in the drinking water to young BALB/c mice (6-11 weeks old), has the capacity to enhance the peak of effector IFNγ^{+} CD8^{+} T cell responses against the immunodominant protective pre-erythrocytic malaria peptide Pb9 from spleens. Sustained enhancement of the cellular immunogenicity against this peptide could not, however, be observed from PBMCs looking at later time points 49-56 days after vaccination, or after boosting. This was also the case for the R21 vaccination when the PBMC responses after priming and boosting were evaluated.

In the case of the spleen T cell responses, it can be hypothesised that spermidine has the capacity to increase the autophagy levels from these immunological cells, therefore impacting on the induction of effector CD8^{+} T cells induced by the viral vector. This immunological effect has been observed in old mice whose autophagy levels are naturally depleted [286].

The capacity of spermidine to increase autophagy levels in young mice has been observed in different tissues [326], [342], [350], although the capacity to boost the autophagy levels in immune cells in young mice has not, to our knowledge, previously been reported.

The finding that spermidine did not influence the re-activation of memory $CD8^+$ T cells was puzzling, especially after confirming the enhancement of the peak of response at day 14 after adenovirus priming. It is possible that the present experimental design lacked sensitivity for a potential enhancement on the re-activation of memory $CD8^+$ T cells (for example, only the IFN-γ phenotype from the $CD8^+$ T cells was analysed).

Having explored the effect of orally administered spermidine, it was assessed whether intramuscularly-administered spermidine might have a useful adjuvanting effect. A positive effect of spermidine on the immunogenicity of viral vectors when administering this adjuvant by the intramuscular route could not be observed. The dose which could be administered intramuscularly was substantially lower than that deliverable by oral administration. Tolerable injection of spermidine also required a previous change of pH: this pH change might also be deleterious. It is known, however, that spermidine's effect on autophagy is mediated through an effect upon hypusination (conversion of the lysyl residue to the uncommon amino acid hypusine) of eIF5A (Eukaryotic Translation Initiation Factor 5A) [349]. This will occur at physiological pH, regardless of the pH of the delivery vehicle. The lower dose of spermidine achieved by intramuscular injection, or a pharmacokinetic effect, seem the most likely explanations for the lack of adjuvantation by intramuscular spermidine.

To our knowledge, only one study has reported the modulation of autophagy through the administration of spermidine by injection. This report described the employment of injections of spermidine in young mice by the intraperitoneal (i.p.) route in order to modulate autophagy levels from muscle tissue [342]. They employed 10 i.p. injections of spermidine for 10 consecutive days at 5-50 mg/kg of mouse weight

(hypothetically calculated as 0.14-1.4 M in one single 50 µL vaccination volume for a ~20 mg mouse). It is hypothesised that spermidine administered by this route of vaccination would represent a better option than the intramuscular route. Nevertheless, this approach would likely require repetitive injections, negatively impacting upon the translational applicability of the adjuvant, and the intraperitoneal route itself has very limited translationally applicability in the vaccination field in humans.

To our knowledge, this is the first time spermidine has been proven to enhance the immune response to vaccination in young mice (6-11 weeks old), specifically in the enhancement of effector $CD8^+$ T cell responses. The work here extends previous findings regarding the potential use of spermidine to enhance $CD8^+$ T cell responses. Nonetheless, the findings are somewhat discouraging for the translational applicability of spermidine as a vaccine adjuvant, due to its problematic alkalinity and the inability to administer adequate doses by a practical route. Despite this, it seems that the modulation of autophagy could be used to enhance effector $CD8^+$ T cell responses. It may be that another autophagy-enhancing molecule, with more favourable physicochemical properties and dose-response relationships, could represent a more valuable adjuvant.

Chapter 5 Preclinical assessment of 2’3’-cGAMP as an adjuvant for viral-vectored vaccines (malaria)

5.1 Introduction

2’3’-cGAMP (cyclic guanosine monophosphate-adenosine monophosphate) is a naturally-occurring substance with potential as a vaccine adjuvant, including for viral-vectored vaccines. 2’3’-cGAMP is a natural second messenger involved in the sensing of cytoplasmic DNA from viruses and bacteria in mammalian host cells (**Figure 5.1**). The compound is produced by the cyclic GMP-AMP synthase (cGAS) in the cytoplasm after the interaction of the enzyme with cytoplasmic DNA. 2’3’-cGAMP later interacts with STING (Stimulator of Interferon Genes), a protein found in the endoplasmic reticulum. Subsequently, STING activates other proteins (IKK, TBK1) leading to activation of transcription of type I interferon IFN-β and other cytokines and chemokines [351]. Therefore, 2’3’-cGAMP is part of an intracellular molecular mechanism for the activation of innate immune responses and subsequent activation of adaptive responses against intracellular pathogens [352].

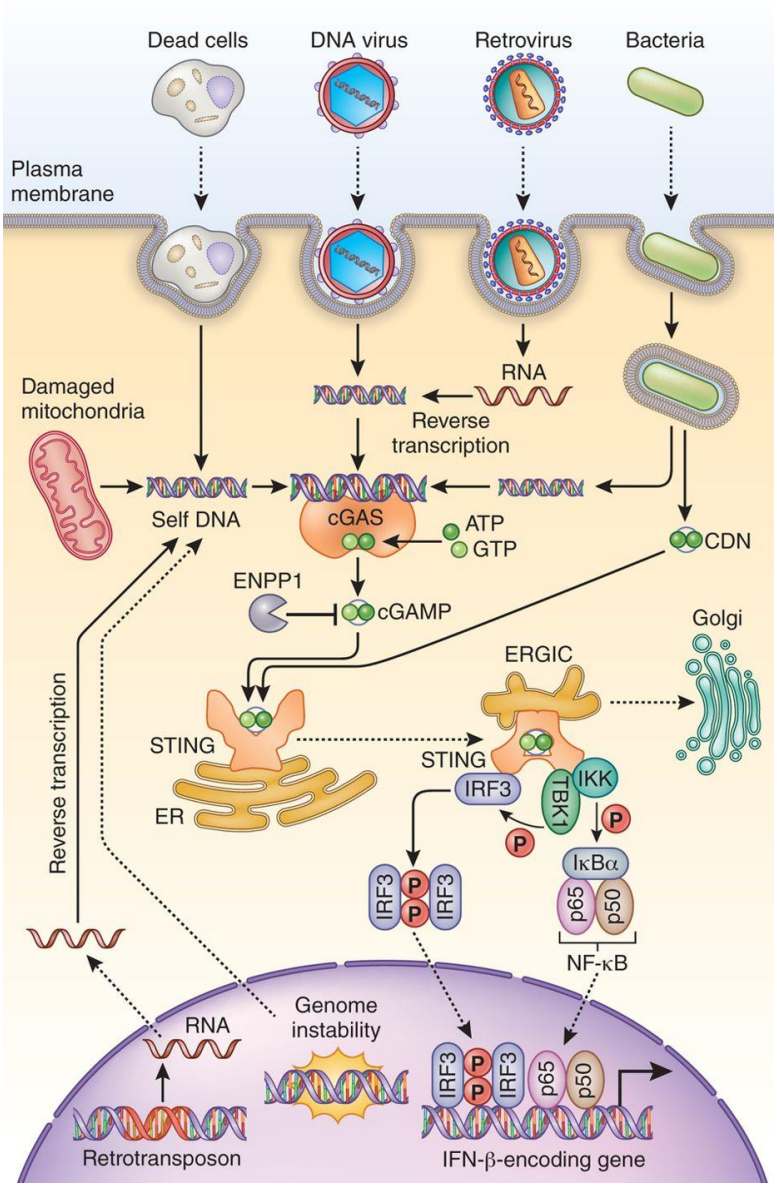


Figure 5.1. Scheme of the cGAS-STING pathway. *DNA found in the cytoplasm coming from a virus or bacteria (or even self-DNA under pathological conditions) interacts with and activates cGAS (cyclic GMP-AMP synthase). cGAS, in turn, catalyses the production of 2'3'-cGAMP from ATP and GTP. The produced 2'3'-cGAMP then interacts with STING (Stimulator of Interferon Genes) found in the endoplasmic reticulum. After trafficking to the ER-Golgi intermediate compartment (ERGIC) and the Golgi apparatus, STING activates IKK and TBK1. TBK1 then phosphorylates STING which recruits IRF3 for phosphorylation by TBK1. Phosphorylated IRF3 dimerizes and enters the nucleus, where it interacts with NF-kB to turn on the expression of type I interferons and other immunomodulatory proteins* [351]*. Image source: https://www.nature.com/articles/ ni.3558*

The capacity of 2’3’-cGAMP to participate in the induction of adaptive responses from DNA viruses, retroviruses and bacteria has led researchers to hypothesise that the compound could serve to enhance the immunological responses induced by vaccines. 2’3’-cGAMP has thus been tested as a chemical adjuvant with different vaccines for multiple infectious diseases such as Influenza and *Streptococcus pneumonia,* with different studies also performed using the model antigen ovalbumin (OVA) [274], [353]–[355].

The various studies of 2’3’-cGAMP suggest that its immunological effects might be dependent on the antigen tested, the vaccine platform and even the route of vaccination. As an example, 2’3’-cGAMP has been reported to function as an adjuvant increasing the cellular and humoral responses induced by the H5N1 and H1N1 Influenza vaccines only when injected by the intradermal route and not the intramuscular route [353]. This inconsistent behaviour suggests a need for further studies about the behaviour of the compound with different vaccine platforms and antigens.

Immunological effects of 2’3’-cGAMP may be principally mediated through activation of dendritic cells. It has been observed that 2’3’-cGAMP has the capacity to directly activate dendritic cells (as measured by the surface markers MHC Class II, CD40, CD86 and CD80) [355]–[357]. This, in turn, promotes the cross-priming of $CD8^+$ T cells [356], [357]. This mechanism has been recently shown to result in the enhancement of $CD8^+$ T cell responses against the model protein-based vaccine OVA [358]. The mechanism is also consistent with the observation that the greatest effect of 2’3’-cGAMP upon immunogenicity of influenza H5N1/HIN1 vaccines is achieved after intradermal (rather than intramuscular) administration [353]: the dermis is thought to offer greater scope for

2’3’-cGAMP action than the muscle, due to the larger number of dendritic cells present.

Due to the proven capacity of 2’3’-cGAMP to significantly enhance the immunogenicity of various vaccines, it is of interest to explore whether this adjuvant could serve to improve the T cell and B cell immunogenicity induced by viral-vectored vaccines. Therefore, the work reported in this chapter sought to assess the effect of 2’3’-cGAMP upon the immunogenicity of two model viral vectors: Adenovirus type 5 (AdHu5) and Modified Virus Ankara (MVA) virus, both encoding the model antigen ‘TIPeGFP’.

TIPeGFP is a synthetic antigen designed for the study of $CD8^+$ and $CD4^+$ T cell and antibody responses induced following vaccination responses in BALB/c and C57BL/6 mice [236], [280]. It is a fusion polypeptide comprising the peptides Pb9 (an H-$2K^d$-restricted $CD8^+$ T immunodominant protective malaria peptide from *Plasmodium berghei* [279]), P15 (a *Mycobacterium tuberculosis* I-A^d-restricted $CD4^+$ T cell peptide [10], [11]) and AL11 (an H-$2D^b$-restricted $CD8^+$ T cell immunodominant peptide coming from the gag protein of the Simian Immunodeficiency Virus [281]). This polypeptide is fused to the enhanced Green Fluorescence Protein (eGFP), which induces an antibody response in mice and also contains an H-$2K^d$-restricted $CD8^+$ T cell peptide known as $eGFP_{200\text{-}208}$ [282]. These model vectors thus allowed assessment of the effect of 2’3’-cGAMP upon $CD8^+$ and $CD4^+$ T cell responses, and upon humoral responses. They also potentially allow challenge studies to demonstrate an effect upon protection against the relevant diseases (albeit somewhat artificial due to the use of single epitopes).

Finally, this work aimed to analyse the effect of the adjuvant on the anti-vector immune response against the structural proteins of the Adenovirus type 5, specifically the hexon protein which is the major constituent of the capsid of the viral vector [361].

Together, therefore, these studies assessed the effect of 2'3'-cGAMP upon the immune responses to both the vector itself and the transgene-encoded antigens.

To my knowledge, this depth of assessment of the use of 2'3'-cGAMP as a chemical adjuvant for enhancing the immunogenicity of viral-vectored vaccines has not been previously performed. Therefore, this work may assist the development of safe and effective adjuvants for this type of vaccine platform and the refinement of other adjuvants with similar properties as 2'3'-cGAMP.

5.2 Effect of 2’3’-cGAMP on the immunogenicity of virus-encoded antigens

5.2.1 Experiment design

As explained above, AdHu5 and MVA viruses encoding the TIPeGFP antigen are two model viral-vectored vaccines which induce readily measurable B and T cell responses. In order to assess whether 2’3’-cGAMP could be used to enhance the immunogenicity against virus-encoded antigens, 2’3’-cGAMP was mixed in a previously described dose (10 µg per mouse [354]) with a standard dose of either AdHu5 or MVA virus (10^7 IU and 10^6 PFU per mouse, respectively [236], [347], [348]). The vaccines were injected intramuscularly or intradermally to wild type BALB/c mice and the frequency of IFNγ$^+$ T cells from spleens reacting against these encoded peptides were measured at the peak of the immune responses (day 14 for AdHu5 and day 7 for MVA after vaccination [236], [347]) by *ex-vivo* IFN-γ ELISpot. The effect of 2’3’-cGAMP on antibody responses against the enhanced Green Fluorescence Protein (eGFP) encoded by the viral vectors was also evaluated using an endpoint serum IgG ELISA at the same time points for each viral vector. Responses induced by the vectors with 2’3’-cGAMP were compared to those in control groups receiving the viral vectors alone injected by the same routes of administration (no adjuvant added).

5.2.2 2’3’-cGAMP diminishes the immunogenicity of AdHu5-encoded antigens when administered intramuscularly

Initially, the effect of 2’3’-cGAMP on the transgene-specific immunogenicity induced by intramuscularly-administered AdHu5 was assessed. As determined by *ex-vivo* IFN-γ ELISpot using splenocytes, a consistent detrimental effect on the T cell immunogenicity against all the AdHu5-encoded peptides Pb9, P15 and $eGFP_{200-208}$ was observed (**Figure 5.2** B-D). A similar detrimental effect was observed for anti-eGFP IgG antibody responses (**Figure 5.2** E).

These trends did not reach statistical significance effect when groups within individual experiments were compared pair-wise by Mann-Whitney test. When results from replicate experiments were considered together using a two-way ANOVA main effect analysis, however, it was found that the adjuvant caused a statistically significant detrimental effect on the T cell immunogenicity against Pb9 and $eGFP_{200-208}$ (**p*=0.0320 and **p*=0.0484, respectively) but not for the P15 or the anti-eGFP IgG antibody responses (*p*>0.05).

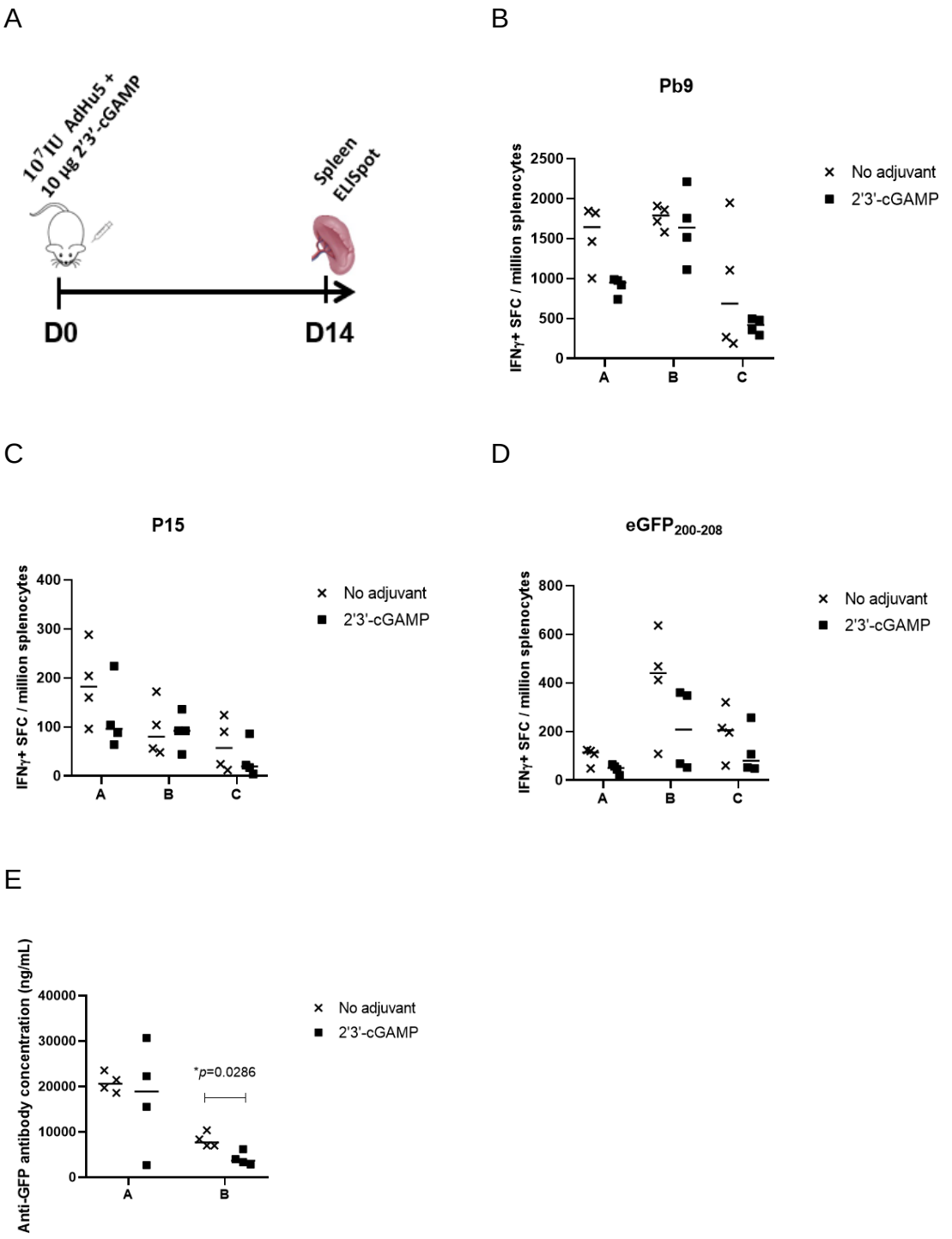
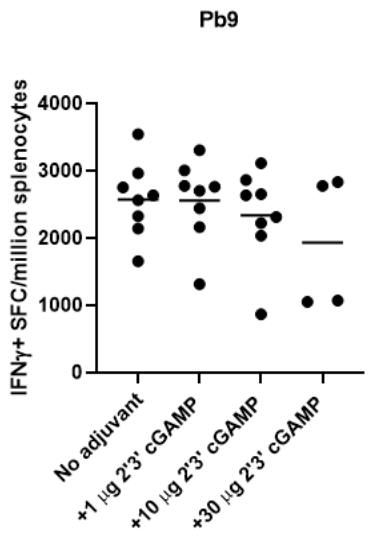


Figure 5.2. Effect of 2'3'-cGAMP on the cellular immunogenicity against the transgene antigens encoded by AdHu5 by the intramuscular route of vaccination*. A. BALB/c mice groups were injected intramuscularly with 10^7 IU of AdHu5 expressing TIPeGFP with or without 10 µg of 2'3'-cGAMP per mouse. Spleens were harvested at day 14 after vaccination. B. IFNγ⁺ CD8⁺ T cell responses against the peptide Pb9 (murine malaria protective peptide). C. IFNγ⁺ CD4⁺ T cell responses against the peptide P15 (Mycobacterium tuberculosis). D. IFNγ⁺ CD8⁺ T cell responses*

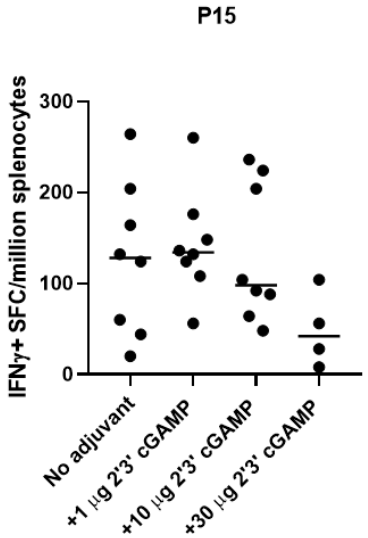
*against the peptide eGFP$_{200-208}$ (from enhanced Green Fluorescence Protein, eGFP). E. Anti-eGFP IgG antibody responses. For all panels, B, C & D represent independent repetition experiments. Statistical analysis: Mann-Whitney test between treatments within a single experiment (no adjuvant versus 2'3'-cGAMP) (*p<0.05); see text regarding two-way ANOVA analysis. Black horizontal lines for each mouse group represent the group medians.*

In order to determine whether 2'3'-cGAMP affected transgene immunogenicity in a dose-dependent manner, the standard dose of AdHu5 was mixed with increasing doses of 2'3'-cGAMP (1-30 µg per mouse) and injected intramuscularly. *Ex-vivo* spleen IFN-γ ELISpot after stimulation with the peptides Pb9, P15 and eGFP$_{200-208}$ showed that the adjuvant seemed to slightly diminish the transgene immunogenicity at increasing 2'3'-cGAMP doses; nevertheless, at the 2'3'-cGAMP doses tested, the diminished immunogenicity never reached statistical significance (Kruskal-Wallis test with Dunn's post hoc, $p>0.05$) (**Figure 5.3**).

A



B



C

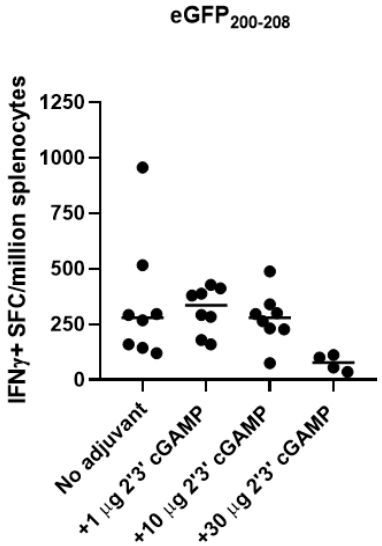
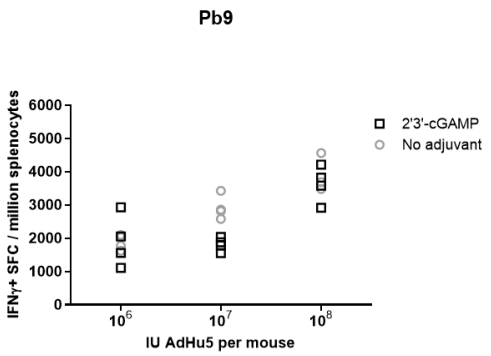


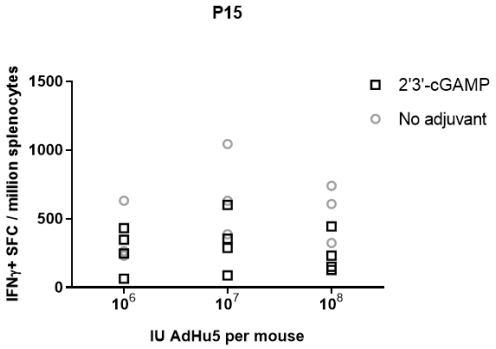
Figure 5.3. Dose-dependent effect of 2'3'-cGAMP on the transgene immunogenicity of AdHu5. *The effect on the transgene immunogenicity of AdHu5 of increasing doses of 2'3'-cGAMP (1-30 μg) was evaluated using a spleen ex-vivo IFN-γ ELISpot after stimulation with peptides Pb9 (A), P15 (B) and* $eGFP_{200-208}$ *(C). The AdHu5 was employed in a dose of* 10^7 *IU per BALB/c mouse. Statistical analysis: Kruskal-Wallis test with Dunn's post hoc, p>0.05. Black horizontal lines represent the group medians.*

The effect of the adjuvant was also evaluated using different doses of AdHu5. For this, increasing doses of the viral vector were mixed with a single dose of 10 µg of 2'3'-cGAMP per BALB/c mouse. The IFN-γ ELISpot results do not show a statistically significant difference between the treatments (adjuvant versus no adjuvant vaccination) for each viral vector dose (Mann-Whitney, p>0.05) (**Figure 5.4**).

A



B



C

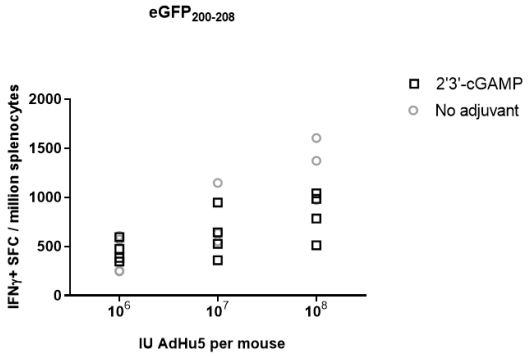


Figure 5.4. Effect of 2'3'-cGAMP using increasing doses of AdHu5. *A. IFNγ⁺ CD8⁺ T cell responses against the peptide Pb9. B. IFNγ⁺ CD4⁺ T cell responses against the peptide P15. C. IFNγ⁺ CD8⁺ T cell responses against the peptide eGFP$_{200-208}$. Statistical test: Mann-Whitney test between the adjuvant and no adjuvant treatments for each viral vector dose used (p>0.05).*

5.2.3 2’3’-cGAMP does not enhance the immunogenicity of MVA-encoded antigens by the intramuscular route of vaccination

In order to test whether 2’3’-cGAMP could be used to enhance the transgene immunogenicity of poxvirus-vectored vaccines, the previously described dose of 2’3’-cGAMP (10 µg per mouse) was mixed with 10^6 PFU of MVA-TIPeGFP. The adjuvanted vaccine was administered intramuscularly and the IFNγ$^+$ T cell responses were measured at day 7 after vaccination from spleens (peak of response). The negative control consisted of the MVA virus alone injected by the same route of administration (no adjuvant).

Although the experiment was small (n=4), the *ex-vivo* IFN-γ ELISpot showed neither a statistically significant effect nor, indeed, any trend to an effect of the adjuvant upon the T cell immunogenicity against the encoded peptides (Pb9, P15 and $eGFP_{200\text{-}208}$) (**Figure 5.5** A-C).

In a similar experiment, the effect of the adjuvant upon immunogenicity of an MVA expressing the spike (S) glycoprotein of the Middle East Respiratory Syndrome coronavirus (MERS) was also analysed. Again, doses used were 10^6 pfu of MVA and 10 µg 2’3’-cGAMP, and T cell immunogenicity against the antigen was measured by splenocyte IFN-γ ELISpot 7 days after vaccination. The ELISpot results showed no statistically significant effect on the T cell immunogenicity against the encoded antigen as compared to the negative control group (**Figure 5.5** D).

Although both of these experiments were small and so a small effect could not be confidently excluded, it was decided not to pursue further studies with MVA as a large beneficial effect seemed unlikely.

In summary, from the previous experiments concerning AdHu5 and MVA, a trend to a detrimental effect on the transgene immunogenicity of AdHu5 due to the adjuvant was observed. Regarding MVA, no obvious effect on the transgene immunogenicity was observed.

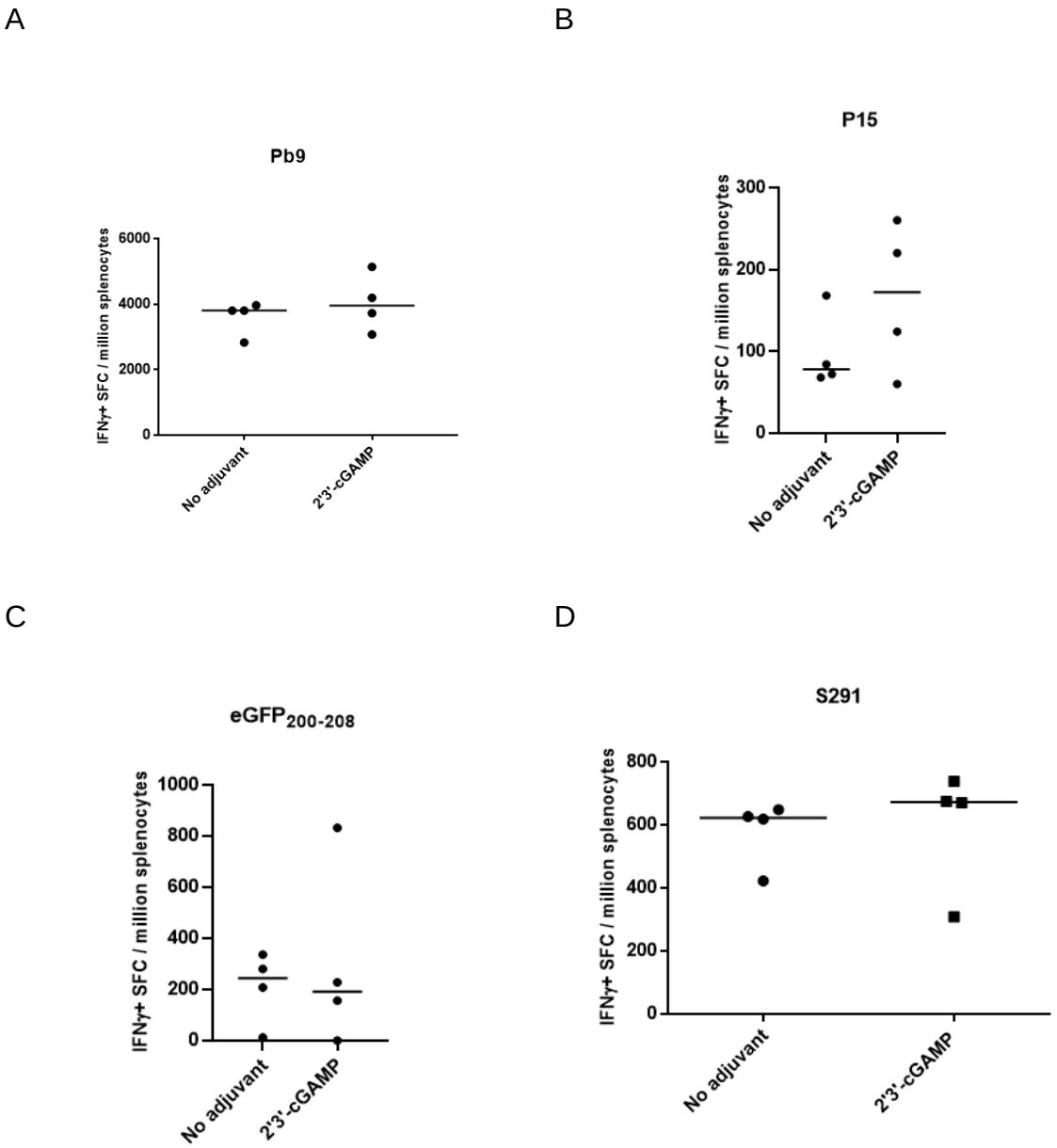


Figure 5.5. Effect of 2'3'-cGAMP on the cellular immunogenicity against the transgene-encoded antigens of MVA. *A. IFNγ$^+$ CD8$^+$ T cell responses against the peptide Pb9. B. IFNγ$^+$ CD4$^+$ T cell responses against the peptide P15. C. IFNγ$^+$ CD8$^+$ T cell responses against the peptide eGFP$_{200-208}$. D. IFNγ$^+$ CD8$^+$ T cell responses against the peptide S291, an immunodominant*

peptide of the S glycoprotein of MERS coronavirus. Statistical analysis: Mann-Whitney test, p>0.05. Black horizontal lines for each mouse group represent the group medians.

5.2.4 The intradermal route of vaccination does not help in increasing the immunogenicity against transgene-encoded antigens

It has been previously described that 2'3'-cGAMP has a superior adjuvant effect when it is administered by the intradermal route of vaccination as compared to the intramuscular route. Wang *et al.* (2016) discovered that 2'3'-cGAMP greatly enhanced the T cell immunogenicity of the H1N1 and H5N1 Influenza vaccines only when administered intradermally and not intramuscularly, employing similar 2'3'-cGAMP doses as in the present research [353].

In order to test whether 2'3-cGAMP might enhance the immunogenicity of intradermally-administered viral vectors, AdHu5- and MVA-TIPeGFP were mixed with 2'3'-cGAMP at the doses previously described (10 µg of 2'3'-cGAMP per mouse, 10^7 IU of AdHu5 per mouse and 10^6 PFU of MVA per mouse) and administered by the intradermal route of vaccination. IFNγ$^+$ CD8$^+$ T cell responses against the Pb9 peptide were measured at the peak of responses (14 days for AdHu5 and 7 days for MVA after vaccination) using the spleen *ex-vivo* IFN-γ ELISpot. In the case of AdHu5, an intramuscular vaccination using the same doses as the intradermal vaccination was included for comparison purposes. The effect of the adjuvant upon responses to the P15 and eGFP$_{200\text{-}208}$ epitopes was not evaluated, due to the higher interest in the murine malaria protective peptide Pb9 and the previous finding that the effect of 2'3'-cGAMP on the Pb9 immunogenicity most likely correlated to the immunogenicity of the peptides P15 and eGFP$_{200\text{-}208}$.

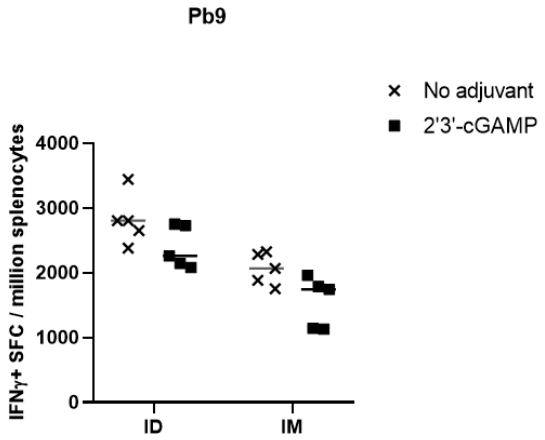
Pb9-stimulated *ex-vivo* splenocyte IFN-γ ELISpot by the intradermal vaccination showed no enhancement of CD8$^+$ T cell immunogenicity to either vector by 2'3'-

cGAMP (**Figure 5.6** A & B). Indeed, a visible detrimental effect on the Pb9-specific $CD8^+$ T cell immunogenicity was observed specifically for AdHu5 (**Figure 5.6** A). This means that the intradermal route of vaccination does not enhance the transgene immunogenicity of AdHu5 and MVA when employing 2'3'-cGAMP at the doses tested.

A two-way ANOVA main effect analysis considering the T cell responses obtained from the intradermal vaccination and the intramuscular vaccination performed side-by-side using AdHu5 showed that the 2'3'-cGAMP does have a significant detrimental effect on the Pb9 immunogenicity (**p=0.0076 for effect of 2'3'-cGAMP, ****p<0.0001 for effect of route, p=0.7812 for interaction).

Data obtained after intradermal vaccination thus provided further evidence that 2'3'-cGAMP can detrimentally affect AdHu5 transgene immunogenicity.

A



B

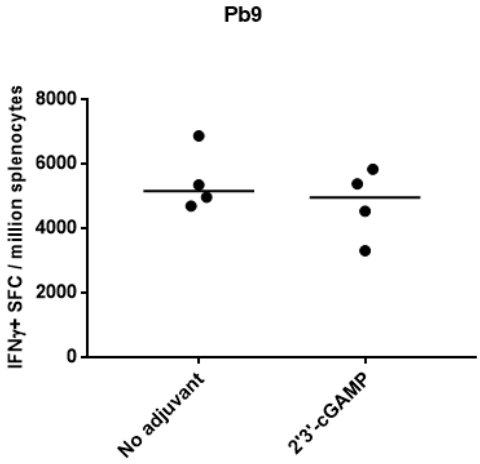


Figure 5.6. Effect on the CD8⁺ T cell responses against the murine malaria protective peptide Pb9 encoded by AdHu5 and MVA by different routes of vaccination. *A. Effect of the adjuvant on the frequency of IFNγ⁺ CD8⁺ T cell responses against Pb9 encoded by AdHu5 (ID=intradermal vaccination, IM=intramuscular vaccination). B. Effect of the adjuvant on the frequency of IFNγ⁺ CD8⁺ T cell responses against Pb9 encoded by MVA by the intradermal vaccination. Statistical analysis: Mann-Whitney test (p>0.05); see text regarding two-way ANOVA analysis. Horizontal lines for each mouse group represent the medians.*

5.3 Effect of 2’3’-cGAMP on the immunogenicity of the structural proteins of AdHu5

5.3.1 Study design

During the performance of the aforementioned studies, it was hypothesised that 2’3’-cGAMP did not enhance the immunogenicity of the transgene-encoded antigens because 2’3’-cGAMP was degraded or moved away from the injection site before transgene expression from the viral vectors. This effect would be less problematic for non-vectored vaccines, with which the antigen is present immediately after injection, explaining the discrepancy between these negative results and previous observations that 2’3’-cGAMP enhanced immunogenicity of inactivated flu vaccine, OVA and other antigens [274], [353], [354]. In support of this hypothesis, 2’3’-cGAMP has a reported half-life of minutes to 1 hour in mouse tissue lysates (including muscle tissue) [353], [362] and it is known that the transgene expression in replication-deficient AdHu5 has a peak of expression around day 1 when injected intramuscularly in BALB/c mice as measured by in-vivo imaging [363]. Therefore, it was tested if the adjuvant would have a positive effect on the immunogenicity of the physical proteins of AdHu5 that do not need to be expressed in the host cells.

For this, the experiment of the intramuscular injection of AdHu5 mixed with 2’3’-cGAMP (10^7 IU and 10 µg per mouse) was repeated. The induced immunogenicity against the AdHu5 hexon (the major capsid protein of AdHu5) was evaluated using an *ex-vivo* IFN-γ ELISpot from spleens harvested 14 days after vaccination (at the peak of response [364]). For this, a T cell peptide pool spanning the whole AdHu5 hexon protein was used to stimulate the cells in the ELISpot assay. In

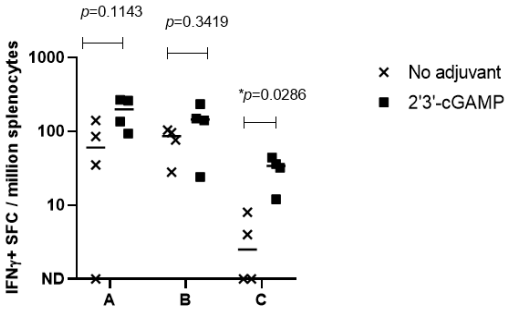
addition, the antibody responses against the AdHu5 hexon were evaluated employing an endpoint IgG serum ELISA from samples taken at the same time point.

5.3.2 2'3'-cGAMP enhances the humoral and cellular immunogenicity against the AdHu5 hexon

In order to test the effect of 2'3'-cGAMP on the immunogenicity of the AdHu5 hexon, both AdHu5 and 2'3'-cGAMP were injected at the doses previously described (10^7 IU and 10 µg per mouse, respectively) and analysed the AdHu5 hexon-specific immunogenicity using the spleen *ex-vivo* IFN-γ ELISpot and a serum IgG anti-hexon ELISA. A positive effect on both the cellular and humoral immunogenicity against the AdHu5 hexon protein for each individual experiment was consistently found when employing 2'3'-cGAMP as compared to the negative control (no adjuvant in the vaccine formulation) (**Figure 5.7**). This was non-significant for most comparisons of individual mouse groups within an experiment by Mann-Whitney, but a two-way ANOVA main effect analysis combining the experiments performed showed that the adjuvant has a statistically significant enhancing effect on the T cell and antibody-mediated immunogenicity (**p=0.0079 and *p=0.0481, respectively).

2'3'-cGAMP can thus enhance the immunogenicity of the structural proteins of the virus, while at the same time diminishing the immunogenicity of the transgene-encoded antigens.

A



B

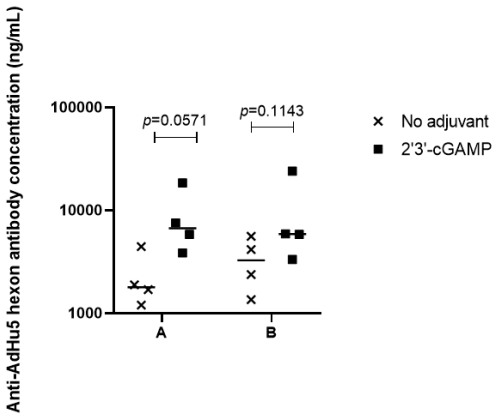


Figure 5.7. Effect of 2'3'-cGAMP on the immunogenicity of the AdHu5 hexon proteins. *A. T cell immunogenicity against the AdHu5 hexon protein as measured by an ex-vivo spleen IFN-γ ELISpot stimulating with an AdHu5 hexon T cell peptide pool. The experiment was performed three times independently (indicated as A, B & C in the X-axis). B. Serum IgG antibody responses against the AdHu5 hexon proteins as measured by an endpoint IgG anti-AdHu5 hexon ELISA. These experiment results were obtained using the same mice as panel A. Mann-Whitney test, *p<0.05. For panels A & B, the horizontal lines represent the group medians.*

5.4 Effect of 2’3’-cGAMP on the immunogenicity of R21, a highly immunogenic virus-like particle

5.4.1 Study design

Since it was shown that 2’3’-cGAMP enhanced the immunogenicity of structural proteins of AdHu5, it was hypothesised that the adjuvant would have similar effects upon other virus-based vaccines that do not require transgene expression for inducing immunogenicity. Therefore, the adjuvant was tested with the virus-like particle R21, a highly immunogenic vaccine for malaria. This vaccine, now in clinical studies in The Jenner, is based on the yeast expression and self-assembling of the Hepatitis B surface antigens fused to the central repeat region and C terminus of the circumsporozoite protein (CSP) of the malaria parasite *Plasmodium falciparum* [141]. This vaccine can be considered as the next generation of the most advanced malaria vaccine to date called RTS’S in that the ratio of CSP to Hepatitis B surface proteins is higher, therefore promoting an immunological reaction towards the malaria-relevant antigen.

The protective efficacy of R21 adjuvanted with Matrix M1 and MF59 in pre-clinical studies has been correlated to both antibody responses and a higher frequency of $IFN\gamma^+$ $CD4^+$ T cell responses against the CSP [141]. Therefore, it was of interest to test the effect of 2’3’-cGAMP upon this type of T cell responses. In addition, the adjuvant was tested for other types of responses, such as antibody titres and $CD8^+$ T cell responses against the CSP, since these are likely to also contribute to the vaccine efficacy.

For these experiments, R21 mixed with 2’3’-cGAMP were injected intramuscularly at doses previously described (0.5 µg per mouse for R21 [141] and 10 µg per

mouse of 2'3'-cGAMP [354]) to BALB/c mice. An already established prime-boost homologous vaccination regimen was employed consisting of the same vaccine formulation injected 3 weeks apart [141]. Immunological responses were measured 3 weeks after boosting (peak of response) employing an *ex-vivo* spleen IFN-γ ELISpot and IgG serum ELISA. In the case of the *ex-vivo* ELISpot, the spleens were stimulated either with a T cell peptide pool spanning the C terminus of the CSP or a $CD4^+$ T cell epitope of the CSP [365]. In the case of the endpoint IgG ELISA, the serum antibody concentrations against the $NANP_6$ polypeptide found in the CSP was measured. The immunological effect of 2'3'-cGAMP was compared to the R21 injected alone (negative control). The effect of 2'3'-cGAMP was compared to the adjuvant Matrix M1 injected at a standard dose of 6 µg per mouse (manufacturer-recommended dose). This adjuvant confers protective efficacy in pre-clinical studies when combined with R21, therefore it was considered as a highly relevant benchmark adjuvant [141]. The effect of 2'3'-cGAMP was then not only evaluated in its capacity to increase the immunogenicity of the already highly immunogenic R21 vaccine but also its performance was compared to one of the most successful adjuvants for the R21 vaccine to date, Matrix M1.

5.4.2 2'3'-cGAMP enhances the cellular immunogenicity of R21

After vaccinating the BALB/c mice with the different vaccine formulations previously described and analysing the results with the *ex-vivo* spleen IFN-γ ELISpot, two-way ANOVA was used to assess whether 2'3'-cGAMP and Matrix M1 had statistically significant independent effects and whether there was any significant interaction (i.e. antagonism or synergism; lack of such an interaction would indicate additive effects).

The results showed that the adjuvant 2'3'-cGAMP mixed with the R21 enhanced the IFNγ$^+$ T cell responses and the IFNγ$^+$ CD4$^+$ T cell responses against the CSP of the R21 vaccine as compared to the R21 vaccine injected alone (**Figure 5.8** B & C).

Nevertheless, this enhancing effect on the T cell immunogenicity did not outperform the adjuvant Matrix M1, as a stronger significant effect was observed for this adjuvant compared to the R21 vaccine injected alone. In addition, unlike Matrix M1, 2'3'-cGAMP did not induce a positive effect on the humoral responses against CSP as compared to the R21 vaccine alone. This was determined using an endpoint IgG serum ELISA (**Figure 5.8** D).

For CD8$^+$ T cell and IgG responses, no significant interactive effects between 2'3'-cGAMP and Matrix M1 were observed (i.e. the two adjuvants appeared to have additive effects) (**Figure 5.9** A & C). CD4$^+$ T cell immunogenicity, in contrast, showed a negative interactive effect (i.e. the effect of 2'3'-cGAMP on the CD4$^+$ T cell immunogenicity of R21 was higher when the Matrix M1 was not included in the vaccine formulation; **Figure 5.9** B).

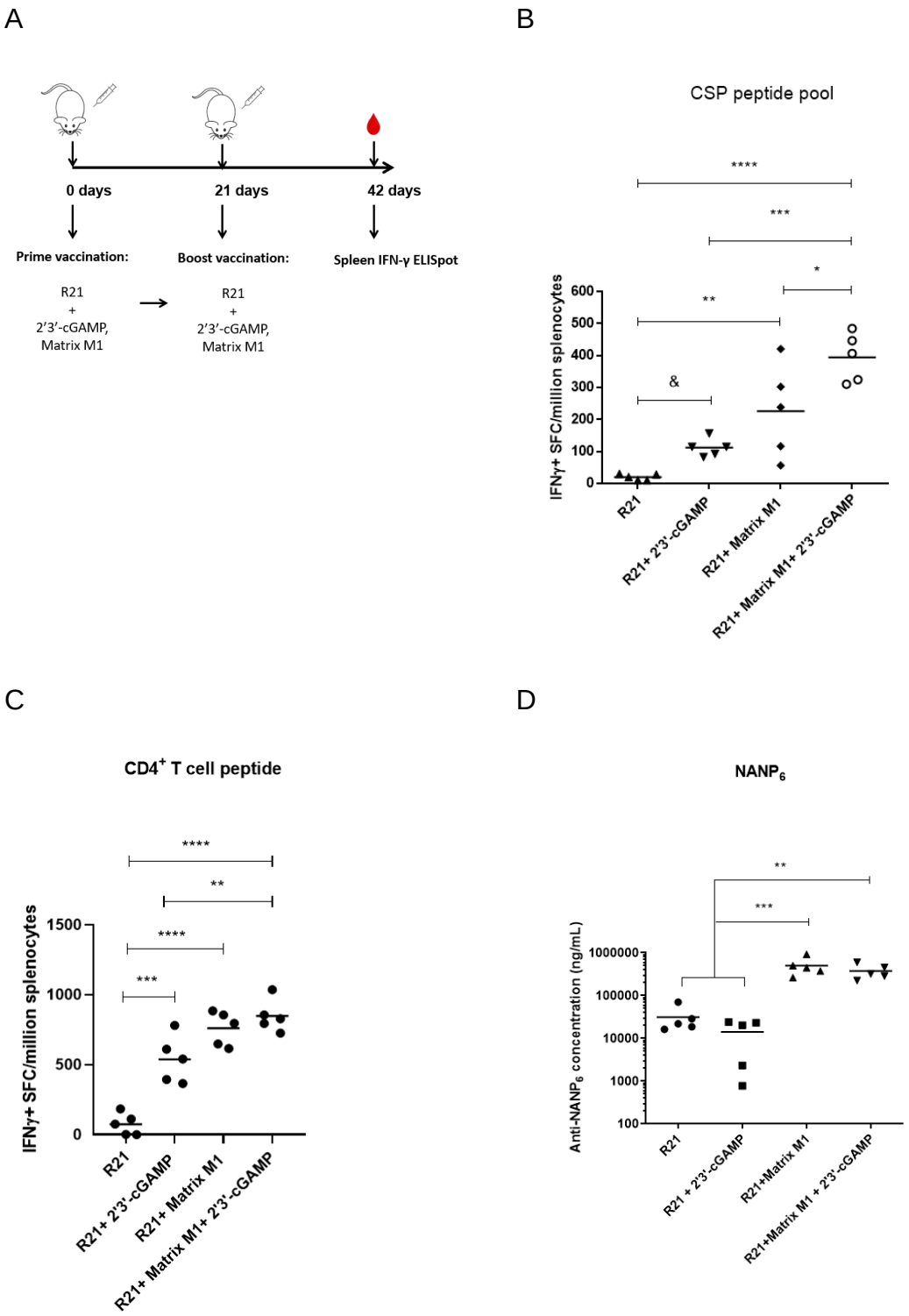


Figure 5.8. Effect of 2'3'-cGAMP on the immunogenicity of R21 and its comparison to the adjuvant Matrix M1. *A. Scheme showing the vaccination regimens with R21 and adjuvants 2'3'-cGAMP and Matrix M1 used in this study. Adjuvanted R21 vaccine formulations (either with 2'3'-cGAMP or Matrix M1 or in combination) were injected intramuscularly to BALB/c mice on day 0 and boosted with the same vaccine formulation 3 weeks after. Immunological responses were*

*measured 3 weeks after boosting. Vaccine doses used: R21 (0.5 µg/mouse), 2'3'-cGAMP (10 µg/mouse) and Matrix M1 (6 µg/mouse). B. IFNγ⁺ T cell responses from spleens against the C-terminus of the CSP of the R21 vaccine (CSP T cell peptide pool). C. IFNγ⁺ CD4⁺ T cell response from spleens using a CD4⁺ T cell peptide from the C terminus of the CSP. D. Endpoint IgG serum ELISA titres against the $NANP_6$ polypeptide of the C terminus of the CSP. Statistical analysis: Two-way ANOVA with Sidak/Tukey post hoc. *p<0.05, **p<0.01, ****p<0.0001. For panel B, &Mann-Whitney test (**p=0.0079). Horizontal lines represent means of mouse groups.*

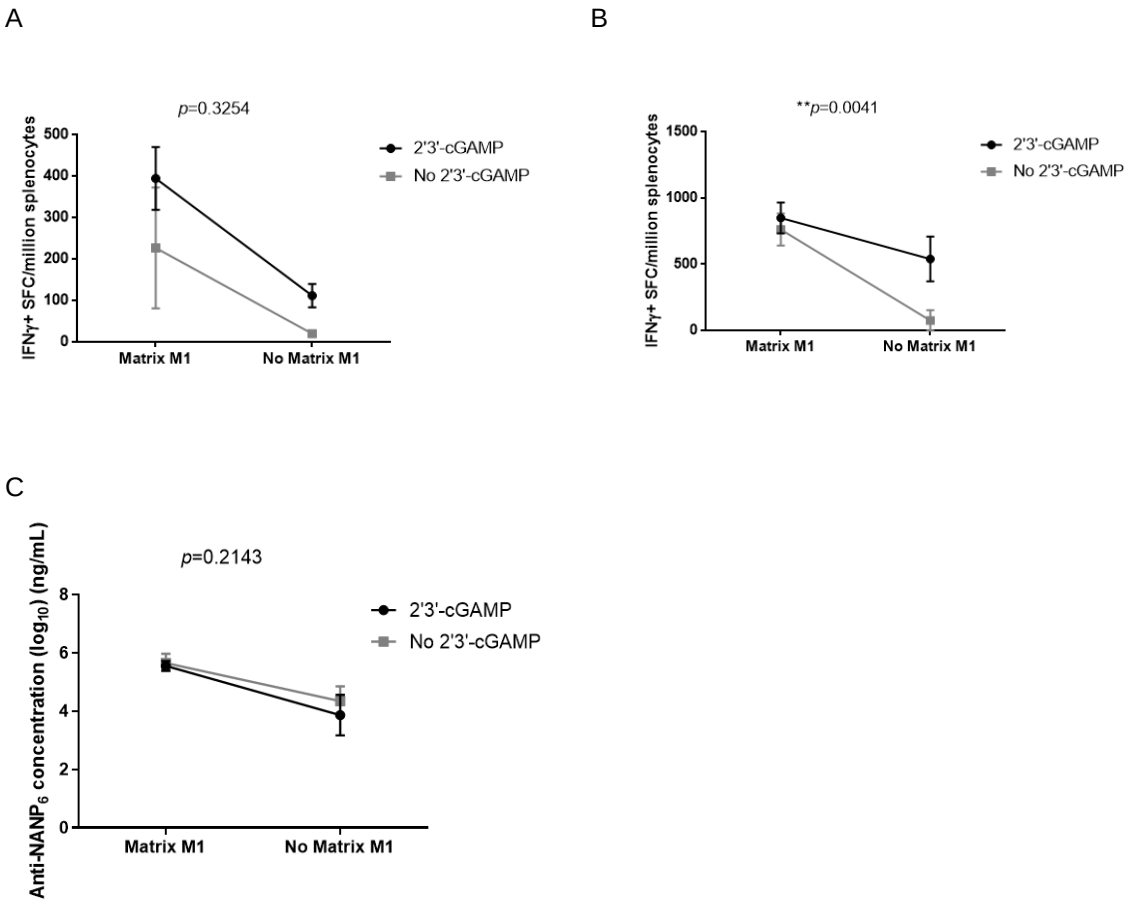
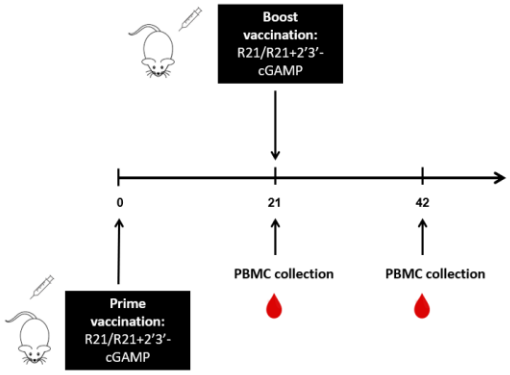


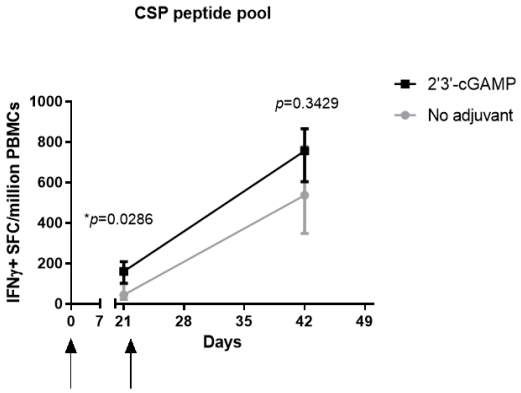
***Figure 5.9. Interactive effect of 2'3'-cGAMP and Matrix M1 on the R21 immunogenicity. A** two-way ANOVA main effect analysis was performed for the IFNγ⁺ T cell and IFNγ⁺ CD4⁺ T cell responses against the R21 vaccine using the data from **Figure 5.8** (panels A and B, respectively). C. Interactive effect of both adjuvants on the IgG antibody responses against R21. Data points represent the medians + range, n=5.*

The enhanced effect of 2'3'-cGAMP on the T cell immunogenicity against R21 was also observed from PBMCs after prime and boosting vaccination (**Figure 5.10**). For this, BALB/c mice were vaccinated as previously described and the PBMCs were analysed 3 weeks after prime vaccination and 3 weeks after boosting vaccination using IFN-γ ELISpot and stimulating the cells with the CSP peptide pool and the $CD4^+$ T cell peptide.

A



B



C

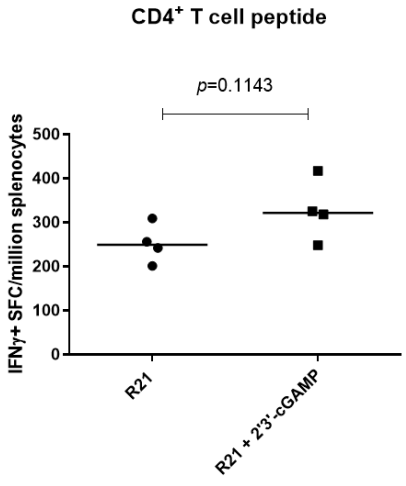


Figure 5.10. Effect of 2'3'-cGAMP on the T cell immunogenicity of R21 from PBMCs. *A. Scheme of the R21 prime-boost vaccination using 2'3'-cGAMP. B. IFNγ⁺ T cell immunogenicity against R21 at different time points after vaccination. Data points represent the medians + range, n=4. Black arrows under the X-axis represent the time points of vaccination. C. IFNγ⁺ CD4⁺ T cell*

responses against R21 measured 3 weeks after boosting vaccination. Statistical analysis: Mann-Whitney test (between the adjuvant and no adjuvant treatments).

These results indicate that 2'3'-cGAMP can be used to enhance CSP-specific T cell immunogenicity with an effect comparable to that of Matrix M1. It does not, however, have a beneficial effect on the B cell immunogenicity, a feature different from Matrix M1 that actually enhances this type of responses.

5.5 Discussion

In the present study, it was shown that 2'3'-cGAMP induced a modest enhancement of the humoral and cellular immunogenicity against the major capsid protein of AdHu5 (the hexon). This contrasted with the observation that 2'3'-cGAMP diminished both the humoral and cellular immunogenicity against the transgene-encoded antigens.

Previously, a similar effect using a 2'3'-cGAMP analogue and STING-agonist (ci-diGMP) was found to enhance the immunogenicity against the structural proteins of AdHu5 but having a detrimental effect against transgene antigens [248].

The mechanisms behind this phenomenon are not yet known, but some hypotheses can be considered, taking into account indirect evidence from published studies and from the experiment results of this chapter.

One of the explanations for the immunological effect of 2'3'-cGAMP on the viral-vectored vaccines has to do with the relatively short half-life of the adjuvant inside mouse tissues [353], [362]. From previously published reports, it is known that 2'3'-cGAMP enhances the immunogenicity of protein-based vaccines (OVA,

Pneumococcal surface protein A) and inactivated viruses (Influenza H5N1/H1N1 vaccines), both of which are vaccines platforms that do not need to express antigens to induce immunogenicity. In addition, from the present studies, it is known that 2'3'-cGAMP enhances the immunogenicity only of the structural proteins of AdHu5, and enhanced responses to the R21 virus-like particle vaccine. Therefore, it can be suggested that the adjuvant might only work with antigens that do not need to be expressed from the host cells since the adjuvant is likely to be removed from the injection site before antigen expression to induce an immunological effect.

Consistent with this, it is known that replication-deficient AdHu5 has a characteristic steep peak of transgene expression at day 1 after vaccination either intramuscularly or intradermally in BALB/c mice (*in vivo* imaging experiments) [363], [366]. On the other side, the production of cytokines and chemokines induced by 2'3'-cGAMP injected by the same routes of vaccination has a peak of expression of 6 hours after injection, as analysed from the site of the injection and from the lymph nodes in BALB/c (levels of these molecules return to baseline 24 hours after 2'3'-cGAMP injection) [353]. This clearly creates a considerable gap between the transgene expression and the effect of 2'3'-cGAMP. This might also explain the null effect of the adjuvant with the MVA vaccine whose transgene expression has a later time peak of transgene expression compared to the 2'3'-cGAMP innate immune effects.

This possible mechanism is also consistent with the finding that 2'3'-cGAMP enhances the immunogenicity against the structural proteins of the AdHu5. In this regard, it is hypothesised that the effect of 2'3'-cGAMP on the immunogenicity of capsid proteins might follow the same mechanism of action as the previously

described effect of 2’3’-cGAMP for protein-based vaccines (dendritic cell activation and enhancement of cross-presentation).

To my knowledge, no information about the immunological effects of 2’3’-cGAMP (or other related cyclic second messengers) as an adjuvant assessing its effect either on the structural proteins or the transgenes of viral-vectored vaccines is publicly available. The present study supports the use of 2’3’-cGAMP for protein-based vaccines, inactivated viruses and virus-like particles while discouraging the use of 2’3’-cGAMP for enhancing the transgene immunogenicity of viral vectors. It is hoped that these findings help into the refinement of the use of 2’3’-cGAMP and related cyclic second messengers for viral-vectored vaccines and other relevant vaccine platforms.

Chapter 6 Mechanism of action of 2’3’-cGAMP for viral-vectored vaccines

6.1 Introduction

In **Chapter 5** it was shown that when the adjuvant 2’3’-cGAMP was mixed with the viral vector AdHu5, the adjuvant modestly enhanced the immunogenicity of the viral capsid proteins while at the same time showing a non-significant trend towards a detrimental effect on the transgene immunogenicity.

Some published studies agree with these findings. It has been described that a 2’3’-cGAMP analogue (ci-diGMP) enhanced the immunogenicity of the capsid proteins of AdHu5 [248]. They found that the *in vivo* synthesis of the 2’3’-cGAMP analogue enhanced the immunogenicity of the structural proteins of AdHu5 but induced detrimental immunogenicity against the transgene antigens. The authors did not investigate any cell biology mechanism driving this phenomenon, except for the measurement of the activation of antigen-presenting cells by the 2’3’-cGAMP analogue being produced by the AdHu5.

On the other hand, Lam *et al*. (2014) and Anghelina *et al*. (2016) focused their research on the impact of the cGAS/STING pathway on the innate and adaptive responses to AdHu5. They discovered that the pathway has a minimal impact on the immunogenicity of the virus when injected alone intramuscularly. Nevertheless, these studies did not address the effect of 2’3’-cGAMP used as an adjuvant on the immunogenicity of the virus.

Since there is, to my knowledge, currently no published information about the possible mechanisms driving the observed effects of the adjuvant 2'3'-cGAMP on the immunogenicity of AdHu5, this chapter aims to provide a possible explanation.

To determine whether the effect of 2'3'-cGAMP on the T-cell and antibody-mediated immunogenicity against the capsid proteins of the AdHu5 was due to the activation of the cGAS/STING pathway *in vivo*, C57BL/6 STING-knockout mice and an analogue of 2'3'-cGAMP that does not activate the cGAS/STING pathway were employed.

Furthermore, and in order to provide an explanation for the detrimental effect upon the immunogenicity of the transgene-encoded antigens, the possibility that 2'3'-cGAMP could be hindering AdHu5 transgene expression via an antiviral effect was investigated. Different components of the cGAS/STING pathway that could potentially have a direct antiviral effect on AdHu5 were identified. The identified antiviral compound was subsequently tested *in vitro* to determine whether it could directly block AdHu5 transgene expression, as antigen expression levels and persistence play a key role in its immunogenicity [367].

Data presented in the previous chapter suggested that simultaneous presence of 2'3'-cGAMP and antigen could enhance immunogenicity, but that presence of 2'3'-cGAMP at the time of adenovirus injection could be detrimental. In order to achieve the simultaneous presence of 2'3'-cGAMP and the transgene antigen, expression of murine cGAS from the adenovirus vector was explored. Therefore, co-expression of the murine cGAS and an antigen transgene was analysed for its effect on the transgene immunogenicity *in vivo*. Finally, and due to the interest in the translational applicability of the viral-vectored vaccines being made for malaria,

the cGAS-expressing AdHu5 was employed in order to increase the immunogenicity of the highly immunogenic virus-like particle called R21.

To my knowledge, no study has explored more deeply into the cell biology mechanisms behind the effect of the adjuvant 2’3’-cGAMP on the immunogenicity of viral vectors, taking into account its potential differential effects on the structural proteins of the virus and antigen(s) expressed by its transgenes. It is hoped that the findings in this chapter help in the refinement of the use of 2’3’-cGAMP as an adjuvant for viral-vectored vaccines and other related vaccines platforms.

6.2 Effect of the adjuvant 2'3'-cGAMP on the immunogenicity of AdHu5 in STING-deficient mice

6.2.1 Study design

Before identifying the possible cell biology mechanisms behind the observed immunological effects of 2'3'-cGAMP on the viral vector AdHu5 (previously described in **Chapter 5**), it was first tested if these immunological effects were dependent on the cGAS/STING pathway *in vivo*. For this, the previously employed doses of AdHu5 and 2'3'-cGAMP that induced higher immunogenicity against the capsid proteins in wild type BALB/c mice were selected. This vaccine formulation was injected by the intramuscular route to C57BL/6 wild type and STING-knockout mice (Tmem173 -/-, The Jackson Laboratory, U.S.A). A comparator consisting of the viral vector alone injected by the same route of vaccination was employed. It was then tested if the enhancement or decrement in IFNγ^{+} T cell immunogenicity against the structural proteins or the transgene antigens observed in wild type mice due to the adjuvant 2'3'-cGAMP was also observed in the knockout mice. Immunogenicity was tested using an *ex-vivo* spleen ELISpot and stimulating the cells with previously characterised peptides corresponding to the capsid proteins and that encoded by the viral vector. Furthermore, for the analysis of the antibody responses against AdHu5 capsid proteins, a whole IgG serum ELISA was used, in which plates were coated with commercially-produced AdHu5 capsid proteins.

6.2.2 The enhancement effect of the adjuvant 2'3'-cGAMP on the AdHu5 capsid immunogenicity is dependent on the cGAS/STING pathway

In order to test if the previously observed 2'3'-cGAMP-induced enhancement in immunogenicity of AdHu5 capsid proteins was dependent on the cGAS/STING pathway, a dose of AdHu5 and 2'3'-cGAMP was injected to wild type and STING-knockout C57BL/6 mice. By the analysis of the IFNγ^{+} T cell responses obtained after employing an *ex-vivo* spleen ELISpot, it was observed that the enhancement in the T cell immunogenicity against the capsid proteins was dependent on the cGAS/STING pathway (**Figure 6.1** A) as the enhancement in T cell immunogenicity was only seen in the wild type mice and not in the STING-knockout mice. The *ex-vivo* spleen ELISpot results also indicated that the basal T cell immunogenicity against the AdHu5 capsid proteins is not dependent on the cGAS/STING pathway as there is no obvious difference between the T cell immunogenicity of the non-adjuvanted wild type and STING-knockout mice.

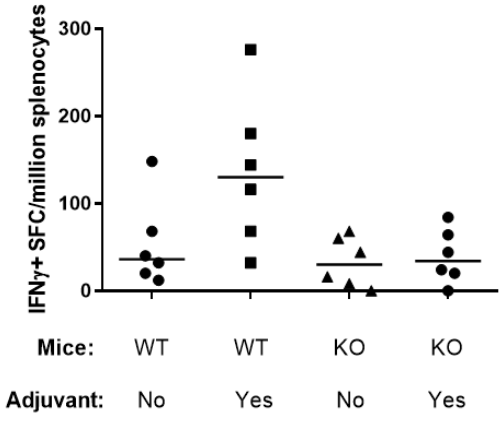
In the case of the antibody responses against the AdHu5 capsid proteins, the IgG serum ELISA indicated that the previously observed enhancement by 2'3'-cGAMP of antibody responses against the AdHu5 capsid proteins was also dependent on the cGAS/STING pathway (**Figure 6.1** B): this enhancement in the IgG antibody titres against the capsid proteins was only observed in the wild type mice and not in the STING-knockout mice.

Furthermore, it was observed that the basal antibody responses against the AdHu5 hexon are influenced by the presence of the cGAS/STING pathway: statistically higher IgG antibody titres were seen in the non-adjuvanted wild type

group as compared to their non-adjuvanted knockout counterparts (Mann-Whitney test, ***p*=0.0022).

These results indicate that even though the T cell immunogenicity against the AdHu5 capsid is not dependent on the cGAS/STING pathway, the effect on the capsid immunogenicity when employing the adjuvant 2'3'-cGAMP is dependent on this pathway. In the case of the antibody responses, both the basal and the adjuvant effect are dependent on the cGAS/STING pathway.

A



B

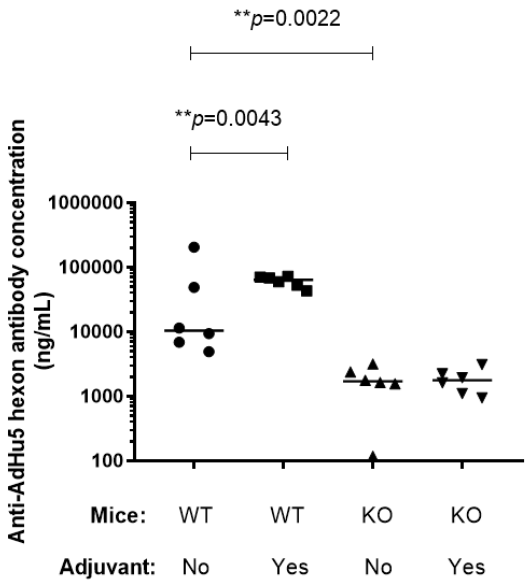


Figure 6.1. Effect of 2'3'-cGAMP on the AdHu5 capsid immunogenicity (hexon) in wild type and STING knockout C57BL/6 mice. *A. IFNγ⁺ T cell immunogenicity against the AdHu5 hexon proteins as measured by an ex-vivo spleen ELISpot and stimulating the cells with a T cell peptide pool spanning the hexon protein. B. Anti-hexon IgG antibody titres from serum as determined by an endpoint ELISA. Mice were vaccinated with 10^7 IU AdHu5 per mouse with or without 2'3'-cGAMP (10 µg per mouse). Samples for both assays were obtained at the peak of the immune*

*response (day 14 after vaccination). Statistical analysis: Mann-Whitney test, **p<0.01. The horizontal lines represent the medians for each mouse group.*

6.2.3 The detrimental effect of 2'3'-cGAMP on the AdHu5 transgene immunogenicity is not apparent in STING-deficient C57BL/6 mice

Work described in previous chapters made use of BALB/c mice and $H2K^d$-restricted epitopes such as Pb9 within the vector-encoded TIP-eGFP antigen. The STING knockout mice, however, were on a C57BL/6 background. In order to analyse the effect of 2'3'-cGAMP upon the T cell immunogenicity of a vector-encoded antigen, responses against the peptide AL11 (an $H\text{-}2D^b$-restricted $CD8^+$ T cell immunodominant peptide coming from the gag protein of the Simian Immunodeficiency Virus [281]) were analysed. This peptide is also present within the TIPeGFP antigen and induces responses in C57BL/6 mice.

AL11 responses were measured in the same mice for which the anti-vector responses were described in the previous section. IFN-γ ELISpot showed that the adjuvant 2'3'-cGAMP at the dose tested did not impact on the immunogenicity of the AL11 peptide in wild type C57BL/6 mice (**Figure 6.2**; Mann-Whitney test, $p>0.05$).

In addition, the non-adjuvanted STING-knockout mice displayed similar levels of $IFN\gamma^+$ T cell responses to the non-adjuvanted wild type mice (Mann-Whitney test, $p>0.05$). This indicates that the basal T cell immunogenicity against this peptide might not be dependent on the cGAS/STING pathway.

The lack of a detrimental effect of 2'3'-cGAMP upon $CD8^+$ T cell responses to the vector-encoded antigen in C57BL/6 mice contrasted with results seen in BALB/c mice using similar viral vector and 2'3'-cGAMP doses (described in **Chapter 5**). It is possible that the effect of 2'3'-cGAMP on the transgene-specific T cell immunogenicity could be strain-dependent. Alternatively, it is possible that 2'3'-cGAMP can exert an effect upon $CD8^+$ T cell responses to vector-encoded antigens in C57BL/6 mice, but this experimental design lacked sensitivity for this effect. Statistical power here was relatively low due to the small group sizes, and a dose-response experiment was not performed which might have clarified whether, for example, doses used were on a plateau of the dose-response curve. It was not therefore possible to draw a conclusion from these knockout mouse experiments about whether the cGAS/STING pathway was responsible for the effect previously seen (in BALB/c mice).

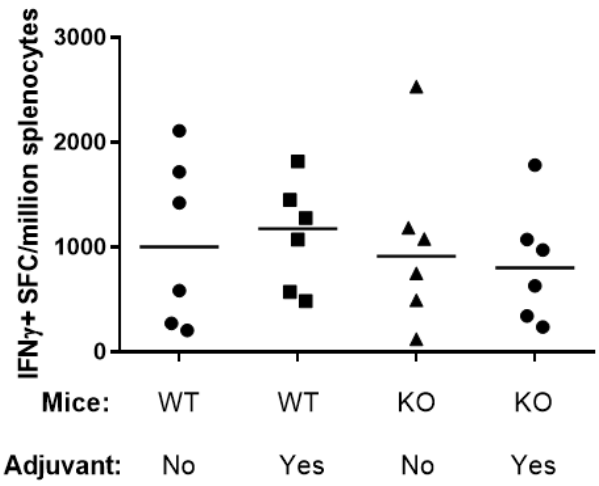


Figure 6.2. Effect of the adjuvant 2'3'-cGAMP on the AdHu5 transgene immunogenicity in wild type and STING-knockout C57BL/6 (B6) mice. *IFNγ^{+} CD8^{+} T cell responses against the AdHu5-encoded peptide AL11 and determined by an ex-vivo spleen ELISpot. Mice were vaccinated with 10^{7} IU AdHu5 per mouse with or without 2'3'-cGAMP (10 μg per mouse). Spleen samples were analysed at the peak of the immune response (day 14 after vaccination). Statistical analysis: Mann-Whitney test, p>0.05. The horizontal lines represent the medians for each mouse group.*

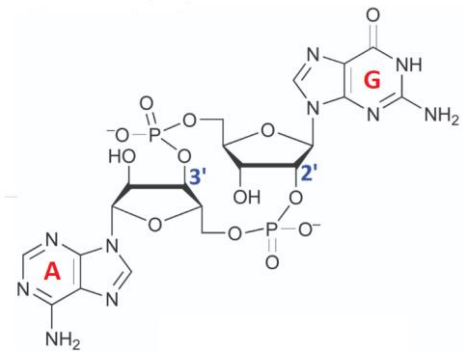
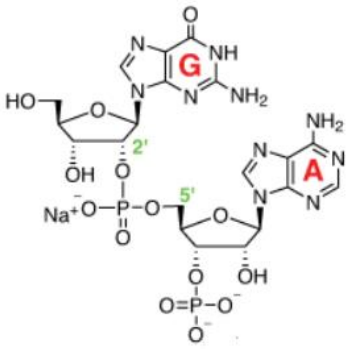
6.2.4 Use of an inactive analogue of 2'3'-cGAMP suggests that its detrimental effect upon transgene immunogenicity in BALB/c mice is due to cGAS-STING activation

In view of the inconclusive results of the above experiment, an alternative means to explore whether 2'3'-cGAMP's detrimental effect upon transgene immunogenicity in BALB/c mice was due to cGAS-STING activation was sought.

A practical way to determine if the physicochemical properties of 2'3'-cGAMP could have a detrimental effect on the AdHu5 transgene immunogenicity irrespective of the activation of the cGAS/STING pathway is to test the effect of its

biologically inactive analogue 2’5’-GpAp. This analogue corresponds to the linearized version of 2’3’-cGAMP product of the hydrolysis of the 3’5’ phosphodiester and does not activate the STING protein *in vitro* [368], [369]. After reviewing the relevant physicochemical properties of both 2’3’-cGAMP and its analogue (**Table 6.1**), it was determined that their properties were highly similar. It is therefore likely that any difference between AdHu5 transgene immunogenicity after formulation with 2’3’-cGAMP and after formulation with 2’5’-GpAp is attributable to cGAS/STING activation, rather than to a non-specific physicochemical effect.

Table 6.1. Computationally-predicted physicochemical properties of 2’3’-cGAMP and its biologically inactive analogue 2’5’-GpAp. *Source: PubChem.*

	2’3’-cGAMP	**2’5’-GpAp**
		
Molecular weight (MW)	674.417	692.432
Net charge	-2	-3
Partition coefficient (XLogP)	-5.9	-6.4
Topological	325	357

Polar Surface Area (A^2)		

Therefore, a dose of the AdHu5 vector (10^7 IU per mouse) mixed with a dose of 14.8 nmol per mouse (~10 μg per mouse) of either 2'3'-cGAMP or its biologically inactive analogue 2'5'-GpAp were injected intramuscularly into BALB/c mice. *Ex-vivo* IFN-γ ELISpot performed from spleens at the peak of the immune response indicated that the levels of T cell responses for all peptides tested when using 2'5'-GpAp trended to be higher than their 2'3'-cGAMP counterparts (**Figure 6.3** A-C). A similar effect was observed for the antibody responses against the encoded eGFP, as analysed using an endpoint IgG serum ELISA (**Figure 6.3** D). Comparing between mouse groups from a single experiment, the difference was not statistically significant (Mann-Whitney test, $p>0.05$). When data from replicate experiments were combined using a two-way ANOVA main effect analysis, the detrimental effect of 2'3'-cGAMP on the T cell responses (as compared to the 2'5'-GpAp treatment) against the peptides Pb9 and $eGFP_{200-208}$ was statistically significant (**$p=0.0095$ and *$p=0.0402$, respectively). The effect against the peptide P15 remained non-significant ($p=0.1195$).

It must be pointed out that for all the peptides tested, the 2'5'-GpAp mouse group tended to show higher T cell responses compared to the AdHu5 injected alone group in one of the two replicate experiments. Even though the conversion of 2'3'-cGAMP to 2'5'-GpAp is promptly performed by cytosolic phosphodiesterases (PDE1-PDE11), the degradation of the 2'5'-phosphodiester of 2'5'-GpAp is performed in specific and scarce sites of the cell, mostly by the enzyme ENPP1 found in the endoplasmic reticulum lumen and the surface of the outer cell

membrane [362]. Therefore, it is possible that the injection of 2’5’-GpAp might support a negative regulation mechanism resulting in higher immunogenicity against the viral vector. This is however highly speculative and, on balance, there is little evidence for such an effect in this experiment. Unlike the difference between 2’3’-cGAMP and 2’5’-GpAp, the trend to a difference between 2’5’-GpAp and ‘no adjuvant’ was seen in only one of the two experiments and did not reach statistical significance when data from the two experiments were combined (two way ANOVA main effect analysis, $p>0.05$).

Although the effect of 2’3’-cGAMP did not reach statistical significance compared to the “no adjuvant” treatment (even with two-way ANOVA main effect analysis, $p>0.05$), the data overall remained consistent with the view that 2’3’-cGAMP is detrimental, and so it remains reasonable to use this experiment to compare 2’3’-cGAMP and 2’5’-GpAp.

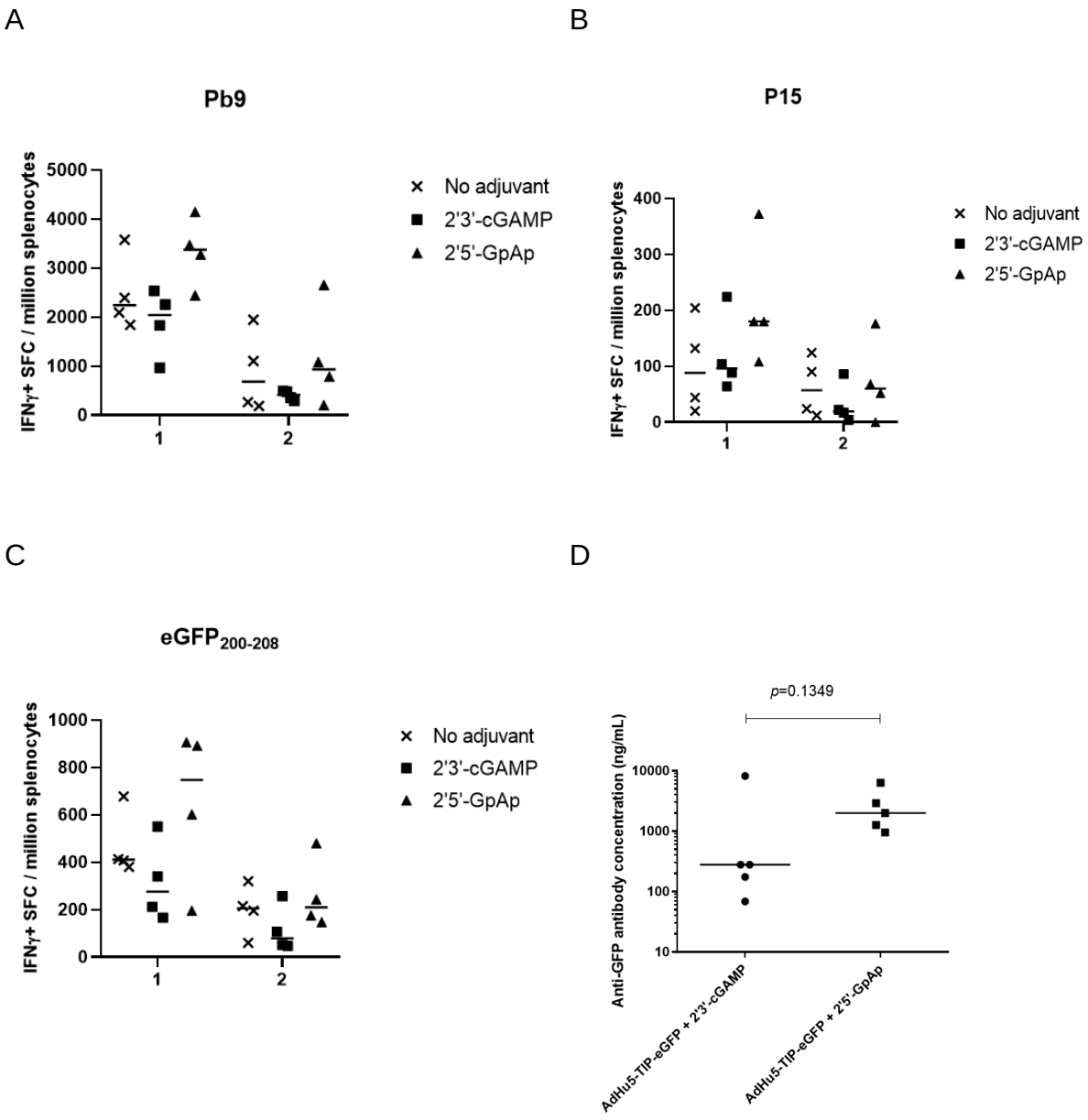


Figure 6.3. Effect of the adjuvant 2'3'-cGAMP on the transgene immunogenicity of AdHu5 compared to its biologically inactive analogue 2'5'-GpAp. *IFNγ⁺ T cell responses against Pb9 (A), $eGFP_{200-208}$ (B) and P15 (C) as measured by an ex-vivo spleen ELISpot at day 14 after vaccination (peak of response) employing a dose of AdHu5 (10^7 IU per mouse) and 14.8 nmol (~10 µg) of either 2'3'-cGAMP or 2'5'-GpAp per BALB/c mouse. 1 & 2 in the X-axis of panels A, B & C represent the individual experiments performed. AdHu5 injected alone is also shown for comparison D. Antibody responses against the encoded protein eGFP, as determined by an endpoint IgG serum ELISA. Statistical analysis: Kruskal-Wallis test with Dunn's post hoc test, p>0.05. For panel D, Mann-Whitney test, p>0.05. For all the panels, horizontal lines represent the group medians for each mouse group.*

6.3 Cell biology mechanisms behind the detrimental effect of 2’3’-cGAMP on the AdHu5 transgene immunogenicity

6.3.1 Study design

Notwithstanding some limitations in the data, the early part of this chapter has provided some support for the belief that the detrimental effect of 2’3’-cGAMP upon the AdHu5 transgene immunogenicity in BALB/c mice is likely to be due to cGAS/STING activation.

Therefore, a mechanism which could explain this phenomenon was investigated, in the hope that understanding this could assist the development of more effective vaccines.

It was hypothesised that a component being expressed during the activation of this pathway could have a direct effect to block the AdHu5 transgene expression, as the levels and persistence of transgene expression is a major determinant of AdHu5 transgene immunogenicity [367]. *In vitro* assays were therefore used to try to link an antiviral response triggered during the activation of the cGAS/STING pathway to a detrimental effect on the AdHu5 transgene expression.

6.3.2 The production of IFN-β triggered during the activation of the cGAS/STING pathway correlates with the blocking of AdHu5 transgene expression *in vitro*

The previously published finding that the induction of type I interferons by the adjuvant poly I:C correlated with a detrimental effect on the AdHu5 transgene expression and immunogenicity in wild type mice [367] led us to hypothesise that the induction of IFNs by 2'3'-cGAMP might also have similar effects.

In this regard, it is known that the activation of the cGAS/STING pathway directly leads to the production of IFN-β [351]. Nevertheless, the effect of IFN-β on the AdHu5 transgene expression has never been directly assessed. Therefore, it was tested if this type of interferon could have a detrimental effect on AdHu5 transgene expression. For this, the human cell line Huh7.5 was used in conjunction with an AdHu5 virus expressing luciferase, a reporter transgene.

Huh7.5 cells were plated in a 96-well plate and incubated overnight. Serial dilutions of human IFN-β and human IFN-α2 (the latter is known to not directly affect AdHu5 transgene expression *in vitro* [370]*)* were added to the wells, along with a single dose of AdHu5 expressing a firefly luciferase gene. The plate was incubated at 37° C for 2 days. Then cell lysates were harvested to measure luciferase levels. An AdHu5 luciferase expression percentage (%) for each IFN dilution was calculated as the percentage of the luciferase signal coming from the AdHu5-only (no IFN) wells.

After plotting the AdHu5 luciferase expression percentages at each IFN serial dilution, it was determined that the human IFN-β affected the AdHu5 transgene expression as the luciferase activity coming from the AdHu5 was significantly

blocked with increasing concentrations of IFN-β (**Figure 6.4** A). This detrimental effect of IFN-β on the AdHu5 transgene expression was also observed using the murine hepatocyte cell line Hepa 1-6 (**Figure 6.4** B).

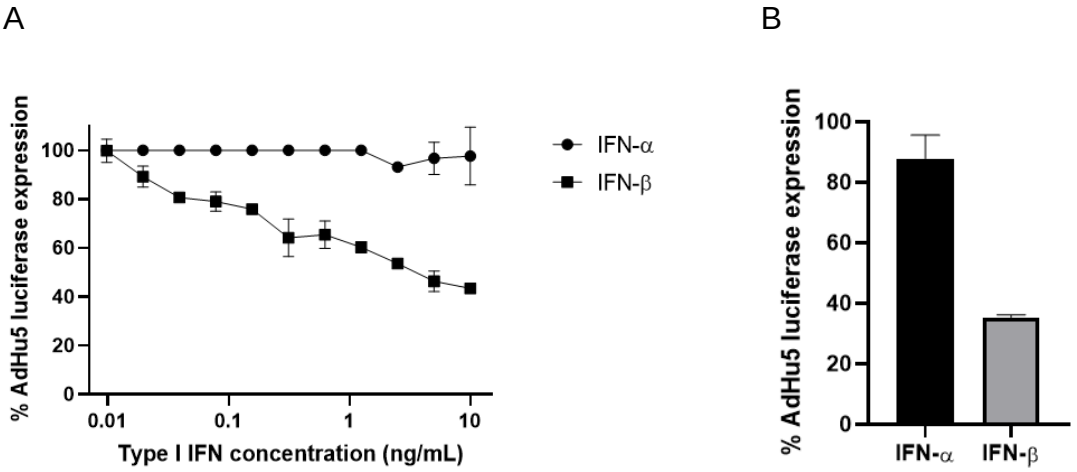
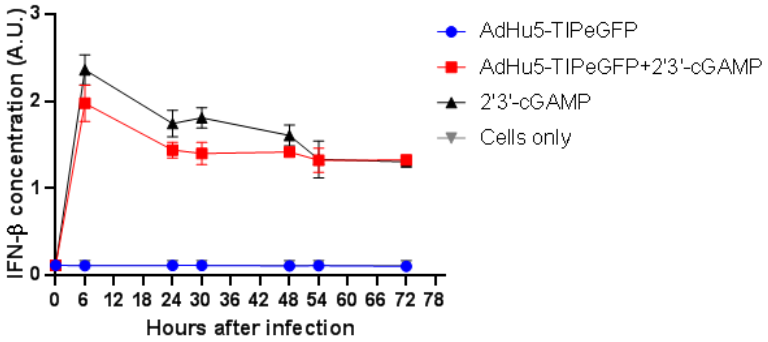


Figure 6.4. IFN-β but not IFN-α (α2) affects AdHu5 transgene expression. *A. Percentage of AdHu5 luciferase expression dependent on human IFN- α/β concentration measured 48 hours after viral infection in the human cell line Huh7.5 cells (MOI 12). B. AdHu5 luciferase expression at a concentration of 5 ng/mL of murine IFN-α/β measured 48 hours after viral infection in the murine cell line Hepa 1-6 (MOI 12). Raw luciferase values coming from the virus at a determined IFN concentration were normalised against the maximum and minimum luciferase values from the assay (virus/cells without IFN and only cells, respectively). Each luciferase value was then converted to a percentage of the maximum possible luciferase value (Y-axis). Values at each time point correspond to the percentage (%) of AdHu5 luciferase expression obtained from the mean value of 3 technical replicates. Standard deviation is also shown for each time point.*

In order to confirm that 2'3'-cGAMP could induce the production of IFN-β and potentially affect AdHu5 transgene expression through the activation of the cGAS/STING pathway, an *in vitro* experiment using the murine cell line Hepa 1-6 was set up. This murine cell line requires the presence of exogenous 2'3'-cGAMP or mouse cGAS expression to trigger the production of IFN-β, most likely due to

the lack of the endogenous expression of cGAS [6]. After cell line exposure to a single dose of 2'3'-cGAMP (83.3 mM), the supernatants were collected at different time-point after exposure and titrated using an anti-mouse IFN-β ELISA. The kinetics of IFN-β production indicated that 2'3'-cGAMP has the capacity to induce the production of IFN-β after activation of the cGAS/STING pathway (**Figure 6.5** A) and that IFN-β production was not triggered by the addition of AdHu5 itself. In a similar experiment, 2'3'-cGAMP significantly affected the transgene expression from the same virus, as measured by the eGFP mean fluorescence intensity at different time points after infection (the virus encodes the eGFP) (**Figure 6.5** B). This effect of 2'3'-cGAMP upon AdHu5 transgene expression is unlikely to be explained by the induction of apoptosis since STING-agonists have been shown to not directly affect cell growth or viability of the Hepa 1-6 cell line [372].

A



B

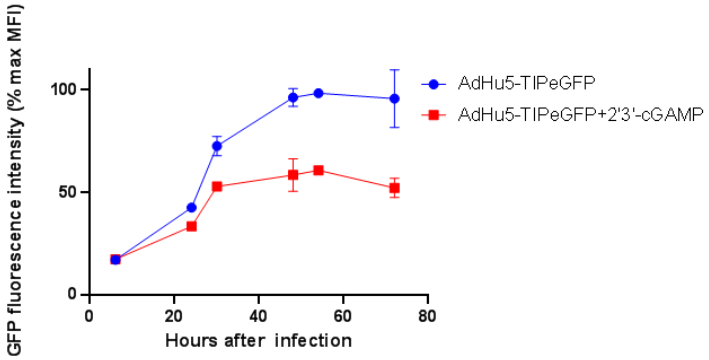


Figure 6.5. Effect of 2'3'-cGAMP on AdHu5 transgene expression in vitro. *Effect of the adjuvant on the kinetics of IFN-β production (A) and the eGFP expression (B) from the replication-deficient AdHu5. The Hepa 1-6 cell line in a 96-well plate was exposed to a single dose of 2'3'-cGAMP (83.3 mM) and AdHu5 expressing eGFP (MOI 1000). The experiment was performed in technical triplicates. Data points represent the means + SD.*

In order to confirm that the 2'3'-cGAMP affected transgene expression and not early stages of the virus infection, the adjuvant was added in serial concentrations after the establishment of virus infection (24 hours after virus infection). The luciferase activity was measured 48 hours after virus infection and luciferase values were normalised as before. The percentages of luciferase expression

dependent on adjuvant concentration confirmed that the adjuvant negatively affected the transgene expression of the virus (**Figure 6.6**).

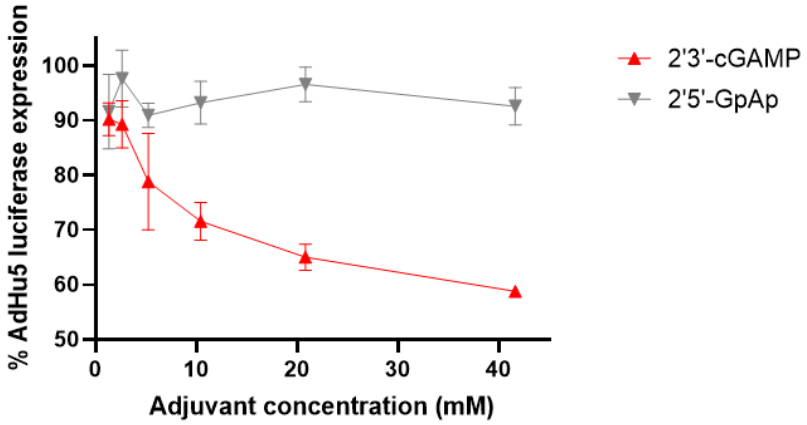


Figure 6.6. Effect of 2'3'-cGAMP on the AdHu5 transgene expression. *Hepa 1-6 cells were plated in a 96-well plate and exposed to serial concentrations of 2'3'-cGAMP 24 hours after AdHu5 infection (MOI 12). Luciferase activity was measured 48 hours after viral infection. Data points correspond to the mean of three technical replicates + SD.*

From these *in vitro* experiments, it can be established that the adjuvant 2'3'-cGAMP has the capacity to induce the production of IFN-β through the activation of the cGAS/STING pathway and that this IFN-β production correlates with a detrimental effect on the AdHu5 transgene expression.

6.4 Effect of the co-expression of cGAS on the transgene immunogenicity of AdHu5

6.4.1 Study design

From previous studies, it was determined that 2’3’-cGAMP mixed with replication-deficient AdHu5 can induce the production of IFN-β through the activation of the cGAS/STING pathway and directly affect the AdHu5 transgene expression and potentially the AdHu5 transgene immunogenicity. In addition, it has been shown that the adjuvant can activate the cGAS/STING pathway in order to enhance the immunogenicity of the AdHu5 capsid proteins. These findings led us to hypothesise that the activation of the cGAS/STING pathway at a later time after AdHu5 immunisation might still be used to enhance the immunogenicity of the transgene antigens, bypassing of the antiviral state otherwise induced by the addition of 2’3’-cGAMP in the vaccine formulation.

Interestingly and somewhat in support of this hypothesis, the administration of 2’3’-cGAMP at a later time after AdHu5 administration (3 days after immunisation) had a less detrimental effect upon the AdHu5 transgene immunogenicity as compared to the co-administration of the AdHu5 and 2’3’-cGAMP, although responses remained no higher than those achieved by AdHu5 alone (**Figure 6.7**).

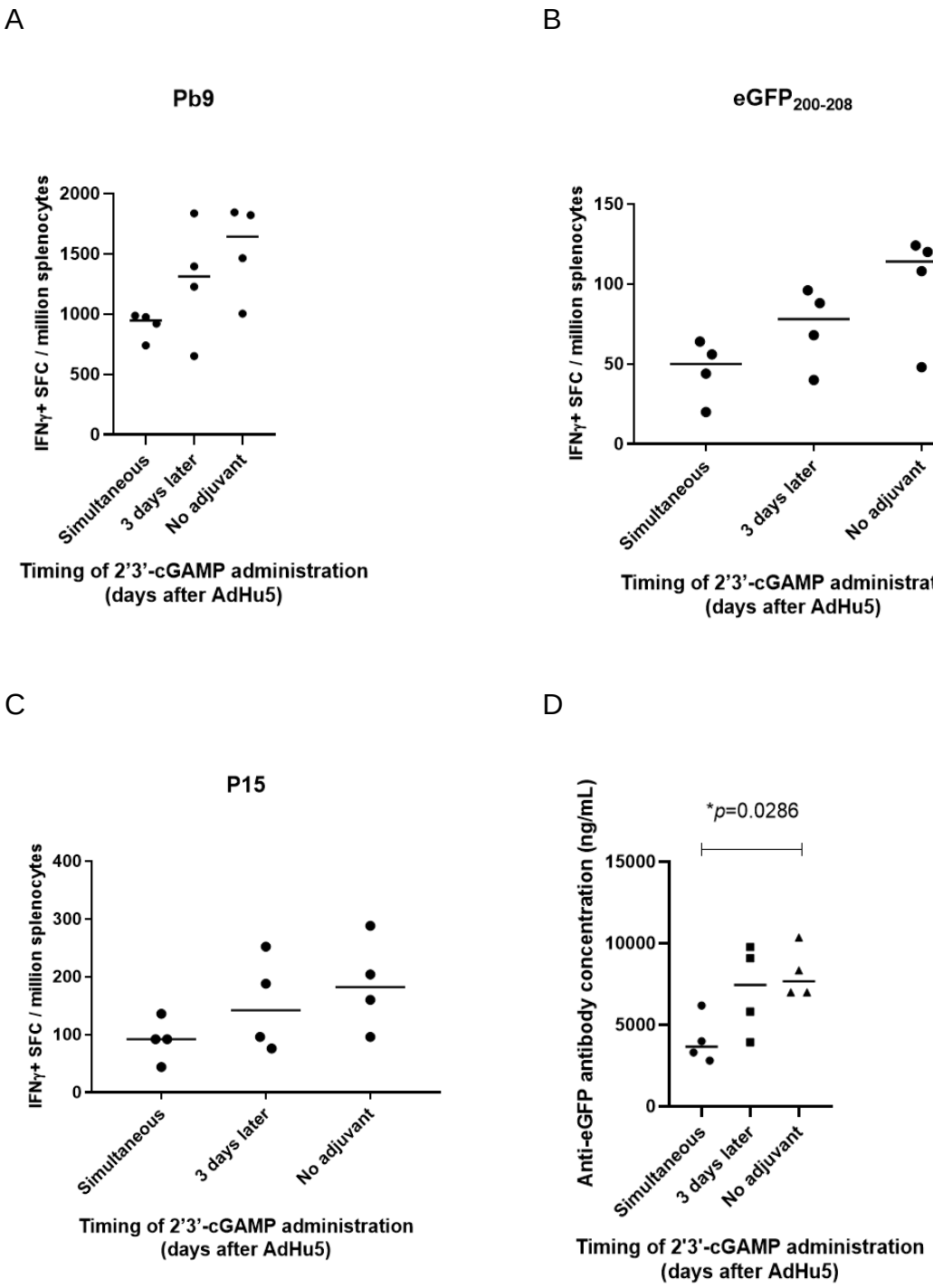


Figure 6.7. Effect of the time of administration of 2'3'-cGAMP on the AdHu5 transgene immunogenicity. *A dose of AdHu5 expressing the antigen TIP-eGFP was administered intramuscularly (10^7 IU per BALB/c mouse) and the adjuvant 2'3'-cGAMP (10 μg per mouse) was co-administered ("Simultaneous") or injected at the same thigh region 3 days after viral vector vaccination ("3 days later"). The immunogenicity of the viral vector injected alone is shown for comparison ("No adjuvant"). Spleen IFNγ⁺ T cell responses against the transgene peptides Pb9*

*(A), eGFP$_{200-208}$ (B) and P15 (C) were evaluated at day 14 after vaccination. D. Serum IgG anti-eGFP antibody responses at the same time point as A-C. Statistical analysis: Mann-Whitney test, *p<0.05. Time points represent the median values and range (n=4).*

1.1.1 Construction of an AdHu5 expressing a catalytic cGAS

In order to test if the activation of the cGAS/STING pathway in a similar time-frame to the expression of the AdHu5 transgenes could enhance the AdHu5 transgene immunogenicity (while avoiding inhibiting the transgene expression by inducing an antiviral state), a replication-deficient AdHu5 co-expressing the mammalian codon-optimised mouse cGAS and the antigen TIP-eGFP under the control of the promoters CMV and human EF-1α, respectively, was constructed (**Figure 6.8**). An AdHu5 co-encoding a catalytically inactive mouse cGAS (created by the mutation of two catalytic amino acids, E211A/D213A [283]) and the TIP-eGFP antigen was created and used as a negative control.

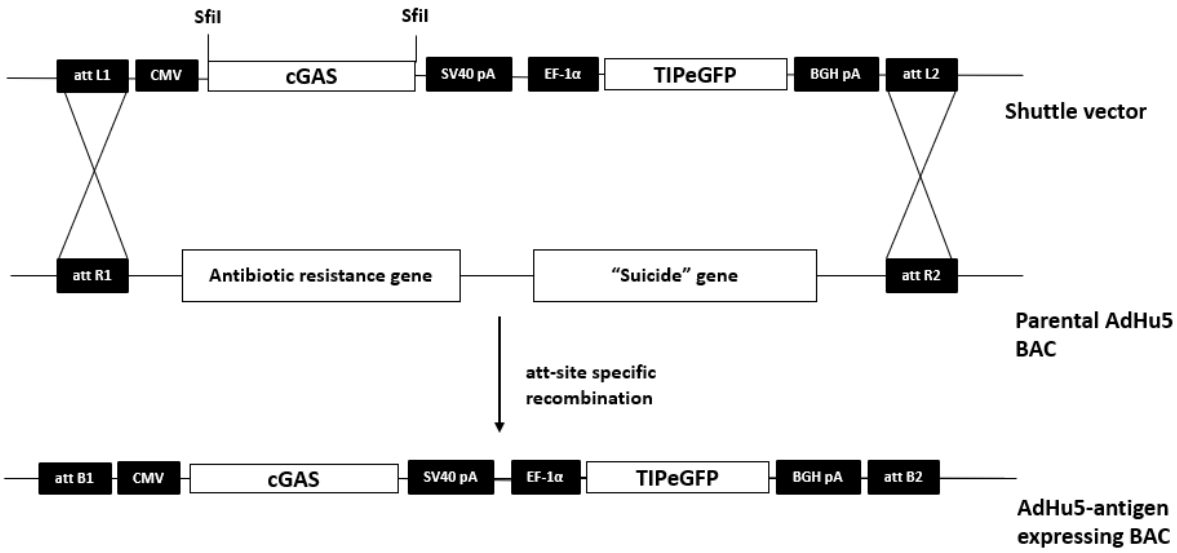


Figure 6.8. Scheme of the molecular cloning for the construction of the AdHu5 expressing the mouse cGAS. *For the construction of the AdHu5 expressing the antigen TIPeGFP and the mammalian codon-optimised mouse cGAS, the cGAS gene was inserted by restriction digestion and ligation to a shuttle vector already containing the antigen TIPeGFP (SfiI restriction sites). The*

cGAS gene was inserted right downstream the human CMV promoter while the TIPeGFP already contained the human EF-1α promoter. Two different polyadenylation signals (SV40 and BGH pA) are used. Both gene cassettes ended up flanked by attL recombination sites. The shuttle vector was then subjected to in vitro directional recombination with a parental AdHu5 BAC genome (Gateway recombination). The complete cassette was inserted in the E1 locus of the AdHu5 genome. The "suicide gene" ccdB (being flanked by attR recombination sites in the parental genome) is a toxic protein for standard E. coli strains used for cloning and it is used for negative selection. The AdHu5 expressing the mutated version of the mouse cGAS and used as a negative control was constructed in the same manner.

The evaluation of the expression of TIP-eGFP from these two viral vectors was performed using fluorescence microscopy of the encoded eGFP (**Figure 6.9** A). In this regard, a negligible eGFP expression from these bicistronic viral vectors was observed, as compared to a monocistronic AdHu5 expressing only the eGFP under the same promoter.

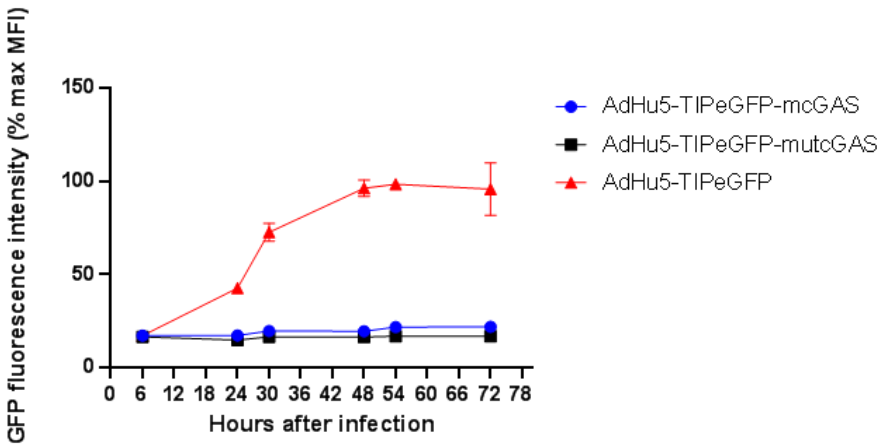
The reason for this lower expression of eGFP from the bicistronic vectors compared to the monocistronic vector cannot be explained by differences in infectivity of the bicistronic viruses. The VVCF of The Jenner Institute (which constructed the viruses) determined that the infectivity particle ratio (P:I) from the bicistronic vectors are low compared to the monocistronic AdHu5 (66-97 versus 39). Nevertheless, this lower P:I ratio would not explain the 5-10-fold reduction in the observed eGFP expression.

A counter-interaction between these two specific genes is also possible, but unlikely. Previously, this technology had been used with other antigens with no described counter-interaction [236]. Therefore, the reason for this diminished eGFP expression from the bicistronic vectors needs further investigation.

On the other hand, the cGAS expression from the bicistronic viral vectors could not be directly evaluated by Western blot most likely due to the low expression of the cGAS genes.

Nevertheless, the catalytic activity of the mouse cGAS compared to its mutated version could be indirectly evaluated by measuring the IFN-β production from Hepa 1-6 cells after viral vector infection (**Figure 6.9** B). As previously shown in **Figure 6.5** A, the Hepa 1-6 cell line does not have the capacity to induce the production of IFN-β in response to AdHu5 infection, most likely due to the lack of the endogenous expression of cGAS. This experiment showed that the AdHu5 expression of mouse cGAS activated the cGAS/STING pathway inducing the production of IFN-β as compared to the expression of the catalytically inactive cGAS, which did not induce detectable IFN-β. Furthermore, the peak of expression of IFN-β was at 24 hours after infection, in agreement with a transgene expression pattern (the adjuvant 2'3'-cGAMP induces a peak of IFN-β at 6 hours after exposure in the same cell line model and conditions).

A



B

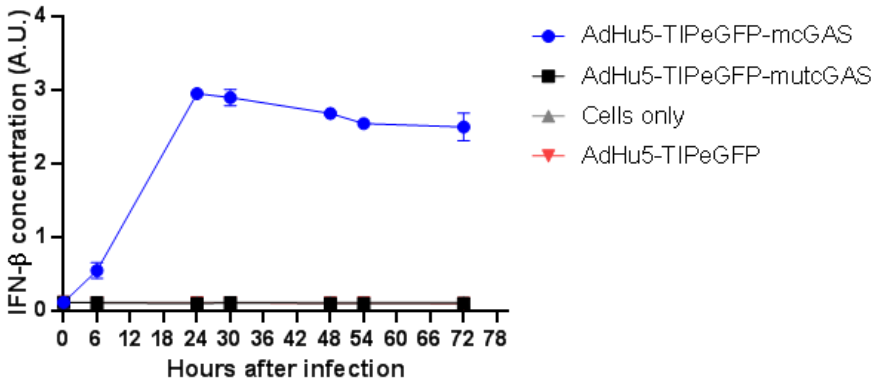


Figure 6.9. Evaluation of cGAS and TIP-eGFP expression from the bicistronic AdHu5s. *A. eGFP expression kinetics after infection of Hepa 1-6 cells with the AdHu5s (MOI 1000). B. IFN-β expression kinetics as determined by an anti-mouse IFN-β ELISA at the same experimental conditions as A. Data points from A and B correspond to the mean values of 3 technical replicates + SD.*

Since both cGAS-expressing viral vectors contain the cGAS and the TIP-eGFP genes under the control of the same promoters and the only difference between them is in their ability to activate the cGAS/STING pathway, it was considered that

this pair of viral vectors could be used for establishing the effect of the activation of the cGAS/STING pathway on the TIP-eGFP transgene immunogenicity.

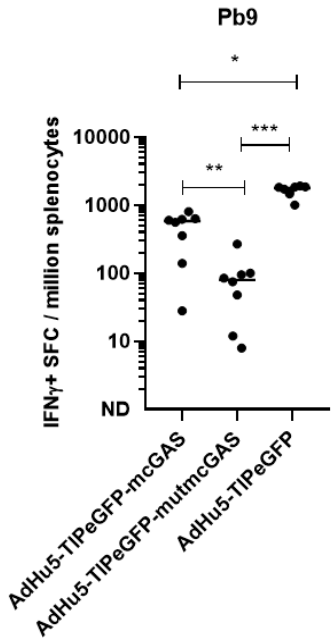
6.4.2 The activation of cGAS/STING pathway at a similar time frame to the AdHu5 transgene expression enhances the AdHu5 transgene immunogenicity

In order to test if the activation of the cGAS/STING pathway at a similar time-frame to the AdHu5 transgene expression could enhance the AdHu5 transgene immunogenicity (and evade the antiviral state otherwise induced by the co-administration of the adjuvant 2'3'-cGAMP), BALB/c mice were vaccinated with different doses of the AdHu5 vector co-expressing the catalytically active mouse cGAS and the TIPeGFP antigen (AdHu5-TIPeGFP-mcGAS). The same bicistronic vector expressing the catalytically inactive mouse cGAS (AdHu5-TIPeGFP-mutcGAS) was used as a negative control. The enhancement on the AdHu5 transgene immunogenicity due to the co-expression of cGAS was determined using an IFN-γ ELISpot after the stimulation of the splenocytes with the $CD8^+$ T cell peptides Pb9 and $eGFP_{200-208}$, and the $CD4^+$ T cell peptide P15 (**Figure 6.10**). The results indicated that the immunogenicity induced by both bicistronic viral vectors was significantly lower than AdHu5-TIPeGFP. This is likely due to the substantially higher expression of eGFP by AdHu5-TIPeGFP compared to the bicistronic viral vectors, as previously discussed.

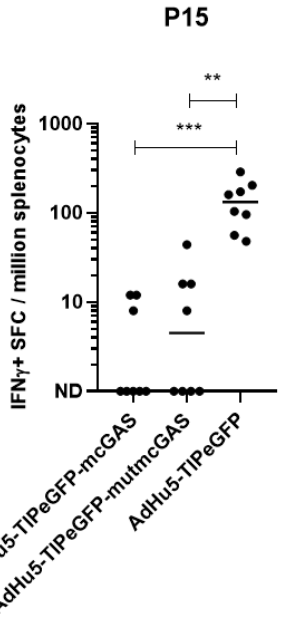
Nevertheless, the ELISpot results also showed that the AdHu5-TIPeGFP-mcGAS induced a significant higher $IFN\gamma^+$ $CD8^+$ T cell immunogenicity against the peptide

Pb9 and $eGFP_{200\text{-}208}$, as compared to the AdHu5-TIPeGFP-mutcGAS (Mann-Whitney test, $^{**}p=0.0070$ and $^{*}p=0.0467$, respectively). This means that the activation of the cGAS/STING pathway could be employed to enhance the immunogenicity against transgene antigens.

A



B



C

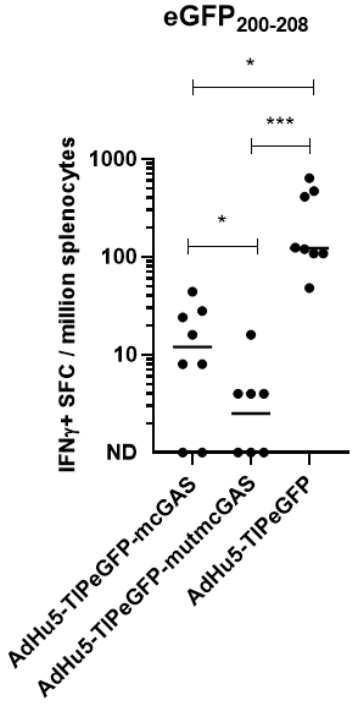


Figure 6.10. Effect on the AdHu5 transgene immunogenicity of the activation of the cGAS/STING pathway in a time-frame similar to the TIPeGFP antigen expression. *Spleen IFNγ⁺ T cell responses against the peptides Pb9 (A), P15 (B) and eGFP$_{200-208}$* (C) as measured by ELISpot at day 14 after vaccination (10^7 IU per BALB/c mouse). Statistical analysis: Kruskal-Wallis

test with Dunn's post hoc, *p<0.05, **p<0.01, ***p<0.001, n=8. For differences in immunogenicity between the bicistronic vectors: Mann-Whitney test: *p<0.05, ** p<0.01. Horizontal lines represent the median values of the mouse groups.

Since it has been hypothesised that intradermal vaccination might help to potentiate the adjuvant effect of 2'3'-cGAMP [353], the previously described doses of cGAS-expressing viral-vectors were administered by this route of vaccination in order to test if this route of vaccination could further help in the enhancement of the transgene immunogenicity. The ELISpot results show that this route of vaccination replicates the previous results using the intramuscular administration of vaccination for Pb9 (difference between the bicistronic viral vectors: Mann-Whitney test, *p=0.0286) (**Figure 6.11**).

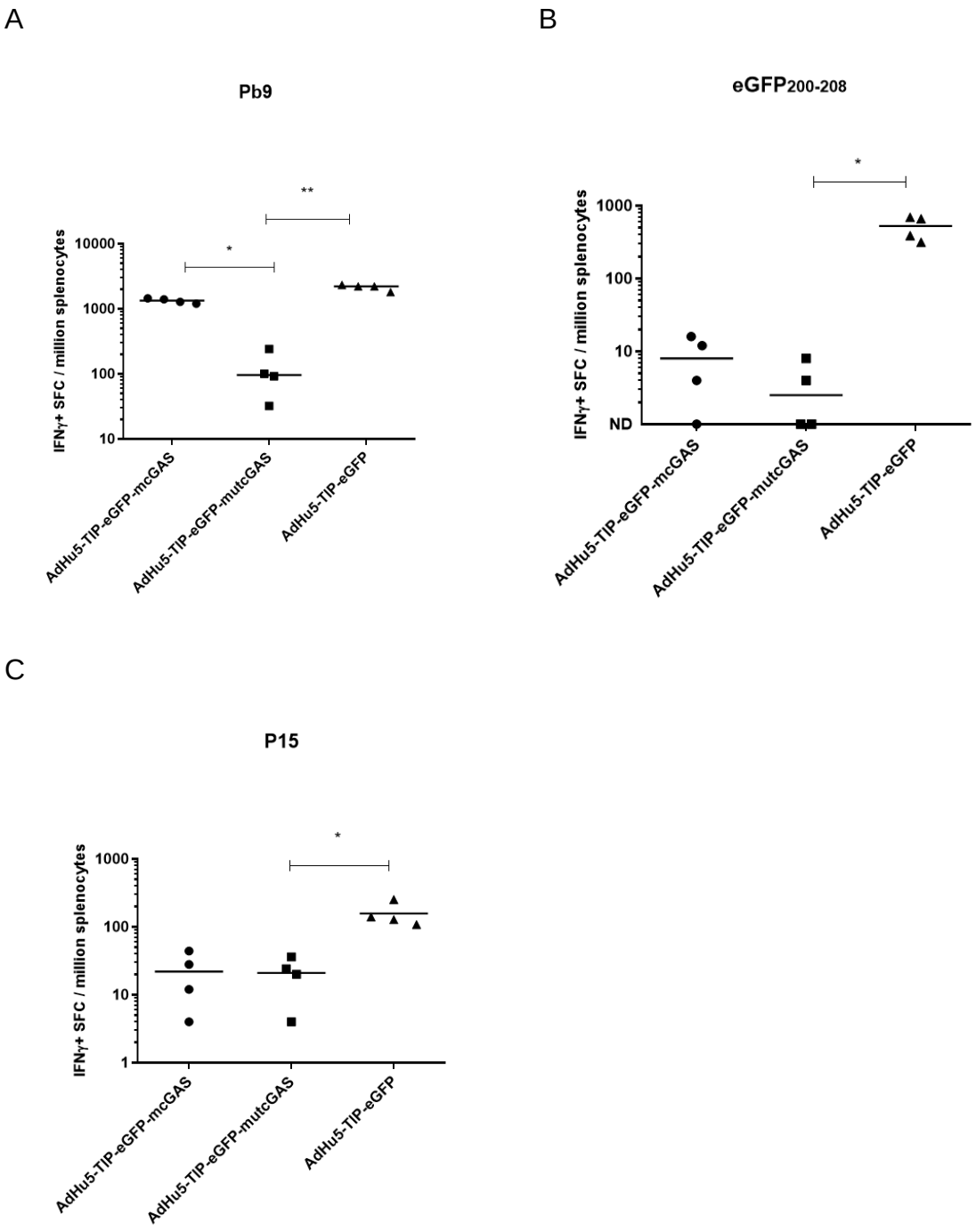


Figure 6.11. Effect on the AdHu5 transgene immunogenicity of the expression of cGAS by the intradermal route of vaccination. *A. IFNγ⁺ CD8⁺ T cell responses against the peptide Pb9 (A) and eGFP$_{200-208}$ (B) and IFNγ⁺ CD4⁺ T cell responses against the peptide P15 (C)* as measured by spleen ELISpot at day 14 after vaccination (10^7 IU per BALB/c mouse). *Statistical analysis: Kruskal-Wallis test with Dunn's post hoc, *p<0.05, **p<0.01, n=4.* For the difference in

immunogenicity between the bicistronic vectors: Mann-Whitney test, *p<0.05. *Horizontal lines represent the group medians for each mouse group.*

A dose-response assay using intramuscular administration showed that the optimal effect on the AdHu5 transgene immunogenicity due to the activation of the cGAS/STING pathway is at the dose of 10^7 IU per mouse of AdHu5-TIPeGFP-mcGAS (**Figure 6.12**). This dose-response assay was performed only for the Pb9 peptide, as the peptides P15 and $eGFP_{200-208}$ already had shown low values at a dose 10^7 IU per mouse, which were considered suboptimal for this type of assay.

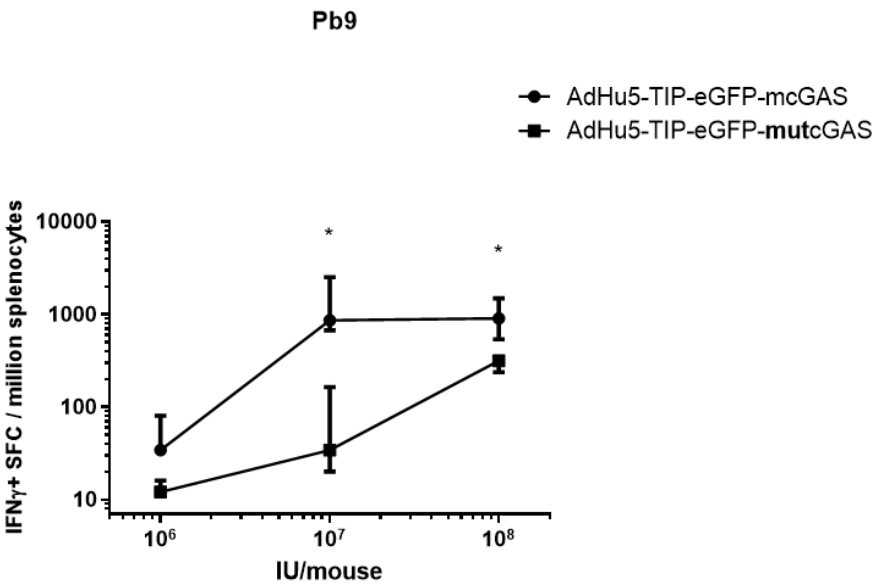


Figure 6.12. Effect of the cGAS/STING pathway activation on the AdHu5 transgene immunogenicity at different doses of the viral vectors. *BALB/c mice were vaccinated intramuscularly and Pb9-specific IFNγ⁺ CD8⁺ T cell responses were measured 14 days after vaccination by spleen ELISpot.* Statistical analysis: Mann-Whitney test for each viral vector dose, *p<0.05, n=4. Data-points represent the median values + range.

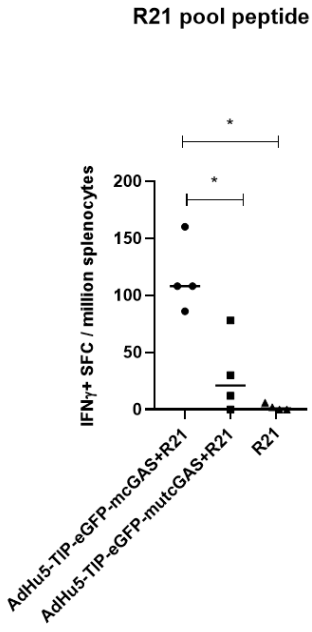
6.4.3 Viral vector expression of cGAS enhances the immunogenicity of the highly immunogenic virus-like particle R21

It has previously been published that an AdHu5 vector expressing the *Vibrio cholerae* diguanylate cyclase that catalyses the formation of the second messenger c-di-GMP (a 2'3'-cGAMP analogue and STING-agonist) has the capacity to enhance the immunogenicity of OVA protein when it is mixed in the vaccine formulation along with the viral vector [248]. Nevertheless, this effect was only shown for immunogenicity against the soluble antigen being mixed with the viral vector and not to any viral vector transgene. Therefore, the viral vector expressing the mouse cGAS in this study was used to not only enhance the immunogenicity against a co-expressed antigen but also to enhance the immunogenicity of a relevant vaccine mixed with the viral vector.

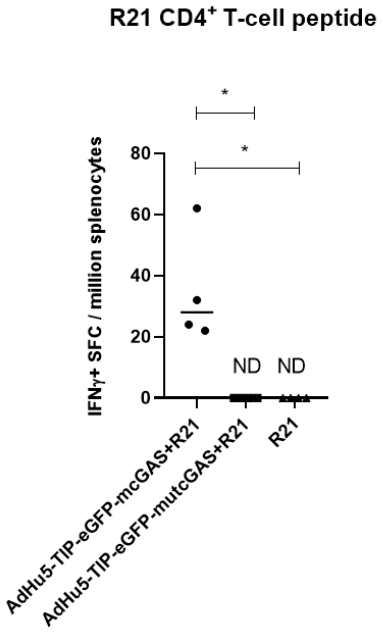
Since the enhancement in immunogenicity of R21 by the inclusion of the 2'3'-cGAMP adjuvant in the vaccine formulation had been previously shown (**Chapter 5**), the co-expression of the mouse cGAS with the TIP-eGFP antigen from AdHu5 was tested for ability to both enhance the immunogenicity of the R21 VLP and to conserve the immunogenicity of the TIP-eGFP transgene product. Therefore, a dose of AdHu5-TIP-eGFP-mcGAS mixed with a dose of R21 was injected by the intramuscular route. The viral vector AdHu5-TIP-eGFP-mutcGAS mixed with the R21 at the same doses was used as the negative control. The effect on the immunogenicity of the co-expressed antigen TIPeGFP and the R21 was determined by using a spleen IFN-γ ELISpot at the peak of the immune response of the viral vector. The IFN-γ ELISpot results showed that the expression of the

cGAS significantly enhanced the T cell immunogenicity of R21 without compromising the immunogenicity of the co-expressed TIPeGFP peptide (**Figure 6.13**).

A



B



C

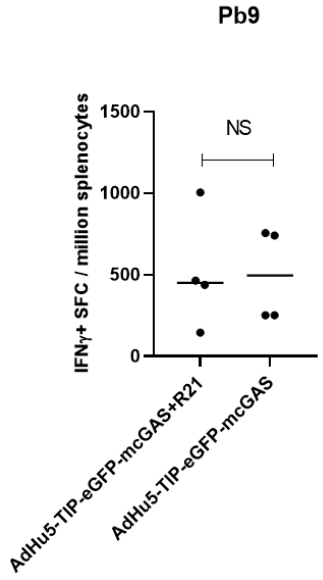


Figure 6.13. Effect of the viral vector expression of cGAS on the R21 immunogenicity. *IFN-γ ELISpot results after stimulation of the splenocytes with a T cell peptide pool comprising the CSP of the R21 vaccine (A), a CD4⁺ T cell peptide coming from the CSP of the R21 vaccine (B), and the peptide Pb9 co-expressed by the AdHu5 (C). BALB/c mice were vaccinated with 10^7 IU of AdHu5 per mouse and 0.5 µg of R21 per mouse. Spleen ELISpot was performed at day 14 after*

*vaccination. Statistical test: Kruskal-Wallis test with Dunn's post hoc, *p<0.05. Horizontal lines represent the group medians. ND=not detectable. NS=not significant.*

6.5 Discussion

The present study suggests that the activation of the cGAS/STING pathway by 2'3'-cGAMP has a detrimental effect on the AdHu5 transgene immunogenicity due to the production of IFN-β that directly affects AdHu5 transgene expression. Nevertheless, the activation of the same pathway enhances the immunogenicity against the capsid proteins of the virus. These results are compatible with the previously published finding that the cGAS/STING pathway has a modest but significant positive effect on the neutralising anti-vector antibodies against the replication-deficient AdHu5 [373]. Nevertheless, the results published in that study using cGAS/STING-deficient mice also argue that this pathway does not have an effect on the AdHu5 transgene expression *in vivo*, which might seem to contradict the present results.

During the mouse immunisation with AdHu5, its DNA is sensed by the cGAS/STING pathway inducing an antiviral state that includes the production of type I IFNs α & β [367]. Nevertheless, in the case of AdHu5, these type I IFNs are believed to be present at a level too low to have a substantial impact on the AdHu5 transgene expression and immunogenicity [367]. Therefore, it is possible that studies concerning cGAS/STING knockout mice fail to detect a difference on the AdHu5 transgene expression and immunogenicity, simply because such difference in type I IFN production is too low to have an impact.

The situation differs when adding an adjuvant that enhances the production of type I IFNs through the activation of the same pathway, as in the case of 2'3'-

cGAMP. In this case, the induction of type I IFNs can reach a level that is enough to cause an effect on the AdHu5 transgene expression and immunogenicity. This is also exemplified by the previously published finding that the adjuvant poly I:C mixed with AdHu5 triggers the induction of type I IFNs affecting AdHu5 transgene expression and immunogenicity in a STING-dependent manner [367].

The capacity of IFN-β to directly block AdHu5 transgene expression has not, to my knowledge, been previously described. Nevertheless, previous studies indirectly support this finding. For example, it is known that, in IFNAR-deficient mice (mice lacking the receptor for the type I IFNs α & β), the detrimental effect of adjuvant-induced type I IFNs on the AdHu5 expression and immunogenicity are eliminated [367]. Since IFN-α is not thought to directly affect AdHu5 transgene expression and immunogenicity [370], it seems likely that IFN-β is responsible for this detrimental phenomenon. Also, in support of this hypothesis, the human IFN-β has been described to have a detrimental effect on the replication of different adenoviral serotypes *in vitro* but experiments with AdHu5 were not performed [124].

Other *in vitro* studies also support the notion of a detrimental effect of IFN-β on transgene expression. It is known that liposomes carrying DNA induce IFN-β that in turn affects transgene expression [374]. On the other hand, transduction of DNA encoding IFN-β decreases herpes simplex virus type 1 (HSV-1) infection and viral expression [375]. Furthermore, this type I IFN being encoded by a vaccinia viral vector strongly affects its replication [376].

The finding in this study that the immunogenicity of all transgene peptides and antigens encoded by the viral vector were affected by the addition of 2’3’-cGAMP

in the vaccine formulation in BALB/c mice further suggests a broadly-acting mechanism. The peptides employed in the study Pb9, $eGFP_{200-208}$ and P15 are all processed and presented differently activating different immunological components. For example, the peptide $eGFP_{200-208}$ is an MHC Class I peptide product of the processing of an encoded eGFP that is secreted by the host cells and, therefore, it can also be cross-presented. On the other hand, the peptide Pb9 is directly presented in an MHC Class I complex by the viral-infected myocytes (intramuscular vaccination) and the peptide P15 is presented by the MHC Class II class complex. Therefore, a pleiotropic innate immune mechanism that could affect all these processes is more likely to exist such as the blocking of transgene expression.

With further work, this information might be used to enhance the transgene immunogenicity of adenovirus-based vaccines in a translationally-relevant manner. For example, it is known that ChAd63 induces the production of type I IFN affecting its transgene expression and immunogenicity during immunisation of wild type mice [367], therefore, to be able to block such effects such as with the use of anti-IFNs antibodies mixed with the viral vector in the vaccine formulation could potentially be used to enhance the immunogenicity of this viral vector. Nevertheless, more studies are needed in this regard.

The present study also suggests that the activation of the cGAS/STING pathway in a time frame similar to the AdHu5 transgene expression can be used to enhance its immunogenicity. Two reasons were hypothesised for this. The first one is because by inducing the activation of the cGAS/STING in a later time after AdHu5 immunisation abrogates the initial exacerbated antiviral state otherwise

induced by the addition of 2'3'-cGAMP in the vaccine formulation, allowing more AdHu5 transgene expression. The second reason requires a deeper explanation.

It is known that the enhancement of immunogenicity on protein-based vaccines by the use of the adjuvant 2'3'-cGAMP is linked to the activation of dendritic cells during the initiation of the antiviral state [354], [357]. It is known that the adjuvant directly enhances the dendritic cell activation and therefore the processing and presentation of antigens coming from the vaccines in a STING-dependent manner. This enhanced T cell immunogenicity is more likely to be a product of MHC Class II antigen presentation and cross-priming by dendritic cells and macrophages that subsequently will induce the activation of both $CD4^+$ and $CD8^+$ T cell responses. Therefore, it can be hypothesised that the same mechanism might be playing a role against the capsid and encoded proteins of the AdHu5 and the R21 vaccine.

Unfortunately, the translation applicability of the cGAS-expressing AdHu5 (AdHu5-TIP-eGFP-mcGAS) was compromised since its immunogenicity was considerably lower than the one induced by the AdHu5 only expressing the TIP-eGFP antigen (AdHu5-TIPeGFP). From the *in vitro* studies, it can be inferred that this lower immunogenicity is due to the lower antigen expression of AdHu5-TIP-eGFP-mcGAS compared to AdHu5-TIPeGFP and that this is an inherent property of the bicistronic viral vectors constructed (the eGFP expression of both the AdHu5-TIP-eGFP-mcGAS and the AdHu5-TIP-eGFP-mutcGAS are both negligible).

Overall, the present study describes for the first time the translational implications in vaccine development of the activation of the cGAS/STING pathway for adenoviral vectors, making a differentiation between structural proteins versus transgene antigens and the time of activation of the pathway. The present study

also sheds light on the cell biology mechanisms driving the observed immunogenicity against the AdHu5 during the activation of the cGAS/STING pathway. It is hoped that these findings help in the development of more effective and safe vaccines for malaria and other intracellular infectious diseases.

Chapter 7 Concluding remarks

7.1 Overview

Viral-vectored vaccines represent a highly promising tool for the eradication of global infectious diseases where a highly effective vaccine is not yet available. Recent advancements in the use of vectored vaccines were explored in order to create highly immunogenic vaccines for the global infectious diseases MERS coronavirus and malaria.

In the case of MERS coronavirus, a highly immunogenic prime-boost vaccination regimen was developed. This was based on a chimpanzee adenovirus (ChAdOx1) followed by an MVA vaccination. A tissue-Plasminogen Activator leader sequence (tPA-LS) fused to the transgene antigen allowed the induction of higher immune responses in ChAdOx1, specifically neutralising antibodies. These neutralising antibody responses were further boosted with the MVA vaccination encoding the same MERS S glycoprotein antigen. The MVA vaccination also allowed the boosting of T cell responses induced by ChAdOx1.

In the case of malaria, two chemical adjuvants were tested in order to enhance the immunogenicity of adenovirus and poxviruses vaccines. The adjuvant spermidine (a polyamine) increased the peak of effector $CD8^+$ T cell responses to a model antigen when administered during adenovirus vaccination. Nevertheless, the adjuvant failed to influence the persistence and memory of these $CD8^+$ T cell responses, and it was not possible to deliver effective doses of the adjuvant by a practical route.

On the other hand, the adjuvant 2'3'-cGAMP had different immunological effects upon different elements of the vectored vaccine. The adjuvant induced higher anti-vector immunological responses but reduced the transgene immunogenicity of the vaccine. A deeper analysis showed that these immunological effects were dependent on the cGAS/STING pathway.

The reduction in transgene immunogenicity was linked to the induction of IFN-β. A mechanism to limit the detrimental immunological effects of IFN-β was explored. The expression of cGAS (the endogenous enzyme that synthesises 2'3'-cGAMP) allowed the enhancement of transgene-specific immune responses of an adenovirus-based vaccine. This technique also allowed the enhancement of T cell immunogenicity of the virus-like particle vaccine R21.

These results highlight the potential but also the limitations in trying to enhance the immunogenicity of vectored vaccines. It is hoped that the findings in this thesis help into the development of highly effective vaccines for the eradication of these two global infectious diseases.

7.2 Conclusions and further directions

7.2.1 Generation of a viral-vectored vaccination regimen for MERS coronavirus based on adenovirus

The chimpanzee adenovirus ChAdOx1 was used during the creation of a highly immunogenic viral-vectored vaccination regimen for MERS coronavirus. This adenovirus has two advantages over other adenovirus-based vaccines: firstly, the avoidance of human anti-vector immunity; and secondly, its high production yield [220].

ChAdOx1 allowed the induction of high levels of cellular and humoral responses against the encoded MERS S glycoprotein. ChAdOx1 had been also successfully employed with other infectious diseases [221], [224], [225], [377]–[381]. The use of the human tissue Plasminogen Activator leader sequence (tPA-LS) allowed the higher induction of neutralising antibodies against the transgene. This agrees with other findings using this genetic design modulating transgene expression [213], [308]. Both cellular and humoral responses induced by ChAdOx1 were successfully increased following MVA boost vaccination. The effect of two promoters (F11 versus mH5) driving MVA transgene expression was also evaluated. The endogenous F11 promoter showed a higher capacity to boost the T cell responses induced by ChAdOx1 prime.

The ChAdOx1-MVA heterologous prime-boost vaccination regimen exemplifies another recent improvement in the development of adenoviruses. The technique circumvents the undesired effects of anti-vector immunity induced during priming by using another virus as a boosting agent and has shown promising immunogenicity and efficacy in clinical trials targeting a number of diseases [142]–[145]. Furthermore, according to the neutralising assay also developed in this thesis, the induction of MERS-CoV-neutralising antibodies induced by the viral-vectored vaccination regimen is comparable to the levels found in naturally exposed camels. Considering that neutralising antibodies are potent mediators of protection in MERS coronavirus, this makes this vaccination regimen a good candidate for further testing in humans.

The contribution of humoral and cellular responses to the induction of immunity in MERS coronavirus is not yet clear. It is known that antibodies might play a role. Nevertheless, the participation of $CD8^+$ and $CD4^+$ T cell responses against the

MERS S glycoprotein might also be important, potentially conferring longer-term protection [382], [383]. Therefore, the viral vector regimen created here might have advantages over other vaccine platforms in their capacity to induce high T cell responses.

The ChAdOx1 vaccine developed here is under Phase I clinical testing in England (NCT03399578). This ChAdOx1 vaccine is also under investigation in camels in Saudi Arabia. It is hoped that these studies will further help in the development of safe and highly effective vaccines for the Middle East Respiratory Syndrome.

7.2.2 Spermidine as a chemical adjuvant for an adenovirus-based vaccination regimen

Spermidine is a polyamine substance that has been shown to enhance the memory $CD8^+$ T cell responses in elderly mice [286]. In the present research, it was investigated if the same molecule could enhance the immunogenicity of an adenovirus-based vaccine (human adenovirus type 5; AdHu5). The adjuvant, administered in the drinking water or by intramuscular injection, failed to enhance the effector $CD8^+$ T cell responses analysed from PBMCs. Furthermore, the adjuvant failed to influence the memory $CD8^+$ T cell responses. Nevertheless, spermidine enhanced the peak of effector $CD8^+$ T cell responses analysed from the spleens.

The effect of spermidine on the antigen-specific $CD8^+$ T cell responses in old mice has been associated with the increased level of autophagy [286]. Due to the finding that these autophagy levels can also be improved in young mice using spermidine [326], [341], [342], it is likely that this mechanism of action is behind

the enhanced immunogenicity seen in the current work. Nevertheless, more experiments would be needed to clarify this.

The finding that spermidine did not influence memory T cell responses (either from spleens or PBMCs) induced by AdHu5 does not encourage further experimentation: it would be desirable, instead, to enhance this type of T cell responses since they will play a major role in challenge studies.

Despite the failure in the use of spermidine, the present experiments might be of high value for the refinement of experimentation concerning spermidine. The present experiments show that it is possible for adjuvants acting via mechanisms like spermidine to influence the immunological responses induced by viral vectors. Nevertheless, the finding that spermidine enhanced effector $CD8^+$ T cell responses but no memory $CD8^+$ T cell responses highlights the need for a broader assessment of the adjuvant. This includes the investigation of more potent and practical versions of the adjuvant.

7.2.3 2’3’-cGAMP as a chemical adjuvant for an adenovirus-based vaccine

2’3’-cGAMP is a second messenger from mammalian cells, synthesised in response to foreign DNA [351]. This cyclic dinucleotide has been successfully used as an adjuvant for other vaccine platforms [274], [353]–[355]. This thesis section aimed to explore the immunological properties of 2’3’-cGAMP on an adenovirus-based vaccine (AdHu5).

This thesis showed, for the first time, that 2'3'-cGAMP has the capacity to enhance the anti-vector immunogenicity of AdHu5, while at the same time negatively affecting its transgene immunogenicity. The enhancing effect on the anti-vector immunogenicity can be attributed to the activation of the cGAS/STING pathway that results in the subsequent activation of dendritic cells [355], [358]. This is supported by the finding in this thesis that the anti-vector immunogenicity against AdHu5 is dependent on the cGAS/STING pathway.

The detrimental effect on the transgene immunogenicity of AdHu5 by 2'3'-cGAMP was further investigated. This effect was linked to the induction of IFN-β by the adjuvant. It was found that IFN-β has the capacity to negatively affect the transgene expression from this adenovirus-based vaccine. To my knowledge, this effect on adenovirus-based vaccines has not been previously described. These results can potentially help in the development of better adjuvants for adenoviruses. For example, chemical adjuvants that do not induce IFN-β should be considered.

In order to attempt to direct the adjuvating effect of 2'3'-cGAMP towards the transgene rather than the vector, ongoing production of 2'3'-cGAMP (by genetically-encoded cGAS) at the same time as antigen production was explored. Synchronous production of 2'3'-cGAMP and the transgene antigens allowed an enhancement in the $CD8^+$ T cell transgene immunogenicity of ~10 fold. Although the mechanisms for this effect are not yet fully defined, this is one of the few examples of the success of a rational development of chemical adjuvants for adenovirus-based vaccines.

An assessment of the effect of the cGAS expression on the protection levels induced by AdHu5 could not be performed. Nevertheless, the adjuvant is extremely promising in this crucial effect. Recently, the activation of the cGAS/STING pathway by adenovirus-based vaccines was found to be indispensable for the induction of $CD8^+$ T cell memory [367]. Therefore, the use of an adjuvant that further increases the activation of this pathway could be extremely beneficial. Therefore, different from spermidine, 2'3'-cGAMP is highly recommended for further development for human vaccination.

7.2.4 Final remarks

This thesis examines the immunological properties of recent trends in the development of vectored vaccines for two global infectious diseases. The results found in this thesis highlight the potential but also the limitations in considering such recent trends. It is hoped that the vaccines, adjuvants and techniques developed here help in the eradication of MERS coronavirus and malaria.

References

[1] A. M. Zaki, van BS, T. M. Bestebroer, A. D. Osterhaus, and R. A. Fouchier, “Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia,” *N Engl J Med*, vol. 367, 2012.

[2] A. Bermingham, M. A. Chand, C. S. Brown, E. Aarons, C. Tong, and C. Langrish, “Severe respiratory illness caused by a novel coronavirus, in a patient transferred to the United Kingdom from the Middle East, September 2012,” *Euro Surveill*, vol. 17, 2012.

[3] S. F. Fagbo, L. Skakni, D. K. Chu, M. A. Garbati, M. Joseph, and M. Peiris, “Molecular Epidemiology of Hospital Outbreak of Middle East,” *Respir. Syndr. Riyadh, Saudi Arab.*, vol. 21, 2014.

[4] A. Assiri, J. A. Al-Tawfiq, A. A. Al-Rabeeah, F. A. Al-Rabiah, S. Al-Hajjar, and A. Al-Barrak, “Epidemiological, demographic, and clinical characteristics of 47 cases of Middle East respiratory syndrome coronavirus disease from Saudi Arabia: a descriptive study,” *Lancet Infect Dis*, vol. 13, 2013.

[5] WHO, “Middle East respiratory syndrome coronavirus (MERS-CoV),” 2019. [Online]. Available: https://www.who.int/en/news-room/fact-sheets/detail/middle-east-respiratory-syndrome-coronavirus-(mers-cov). [Accessed: 09-Jul-2019].

[6] J. Choi, M.-G. Kim, Y.-K. Oh, and Y. B. Kim, “Progress of Middle East respiratory syndrome coronavirus vaccines: a patent review,” *Expert Opin. Ther. Pat.*, vol. 27, no. 6, pp. 721–731, Jun. 2017.

[7] S. Boheemen, M. Graaf, C. Lauber, T. M. Bestebroer, V. S. Raj, and A. M. Zaki, “Genomic characterization of a newly discovered coronavirus associated with acute respiratory distress syndrome in humans,” *MBio*, vol. 3, 2012.

[8] M. Cotten, T. T. Lam, S. J. Watson, A. L. Palser, V. Petrova, and P. Grant, “Full-genome deep sequencing and phylogenetic analysis of novel human betacoronavirus,” *Emerg Infect Dis*, vol. 19, 2013.

[9] Y. Chen *et al.*, “A sensitive and specific antigen detection assay for Middle East respiratory syndrome coronavirus,” *Emerg. Microbes Infect.*, vol. 4, no. 4, p. e26, Apr. 2015.

[10] K. G. Frey *et al.*, “Full-Genome Sequence of Human Betacoronavirus 2c Jordan-N3/2012 after Serial Passage in Mammalian Cells,” *Genome Announc.*, vol. 2, no. 3, pp. e00324-14, Jun. 2014.

[11] V. D. Menachery *et al.*, “MERS-CoV Accessory ORFs Play Key Role for Infection and Pathogenesis,” *MBio*, vol. 8, no. 4, pp. e00665-17, Sep. 2017.

[12] Y. Yang *et al.*, “The structural and accessory proteins M, ORF 4a, ORF 4b, and ORF 5 of Middle East respiratory syndrome coronavirus (MERS-CoV) are potent interferon antagonists,” *Protein Cell*, vol. 4, no. 12, pp. 951–961, 2013.

[13] J. M. Thornbrough *et al.*, “Middle East Respiratory Syndrome Coronavirus NS4b Protein Inhibits Host RNase L Activation,” *MBio*, vol. 7, no. 2, pp. e00258-16, May 2016.

[14] H. H. Rabouw *et al.*, “Middle East Respiratory Coronavirus Accessory

Protein 4a Inhibits PKR-Mediated Antiviral Stress Responses," *PLOS Pathog.*, vol. 12, no. 10, p. e1005982, Oct. 2016.

[15] J. K. Millet and G. R. Whittaker, "Host cell proteases: Critical determinants of coronavirus tropism and pathogenesis," *Virus Res.*, vol. 202, pp. 120–134, 2015.

[16] A. I. Zumla and Z. A. Memish, "Middle East respiratory syndrome coronavirus: epidemic potential or a storm in a teacup?," *Eur Respir J*, vol. 43, 2014.

[17] J. Lei, Y. Kusov, and R. Hilgenfeld, "Nsp3 of coronaviruses: Structures and functions of a large multi-domain protein," *Antiviral Res.*, vol. 149, pp. 58–74, 2018.

[18] Y. M. Báez-Santos, S. E. St. John, and A. D. Mesecar, "The SARS-coronavirus papain-like protease: Structure, function and inhibition by designed antiviral compounds," *Antiviral Res.*, vol. 115, pp. 21–38, 2015.

[19] A. C. Galasiti Kankanamalage *et al.*, "Structure-guided design of potent and permeable inhibitors of MERS coronavirus 3CL protease that utilize a piperidine moiety as a novel design element," *Eur. J. Med. Chem.*, vol. 150, pp. 334–346, 2018.

[20] M. Kandeel and A. Altaher, "Synonymous and biased codon usage by MERS CoV papain-like and 3CL-proteases," *Biol. Pharm. Bull.*, vol. advpub, 2017.

[21] D. Forni *et al.*, "The heptad repeat region is a major selection target in MERS-CoV and related coronaviruses," *Sci. Rep.*, vol. 5, p. 14480, Sep.

2015.

[22] M. A. Müller *et al.*, “MERS coronavirus neutralizing antibodies in camels, Eastern Africa, 1983-1997,” *Emerg. Infect. Dis.*, vol. 20, no. 12, pp. 2093–2095, Dec. 2014.

[23] R. A. Perera *et al.*, “Seroepidemiology for MERS coronavirus using microneutralisation and pseudoparticle virus neutralisation assays reveal a high prevalence of antibody in dromedary camels in Egypt, June 2013.,” *Euro Surveill. Bull. Eur. sur les Mal. Transm. = Eur. Commun. Dis. Bull.*, vol. 18, no. 36, p. pii=20574, 2013.

[24] M. G. Hemida, R. A. Perera, R. A. Jassim, G. Kayali, L. Y. Siu, and P. Wang, “Seroepidemiology of Middle East respiratory syndrome (MERS) coronavirus in Saudi Arabia (1993) and Australia (2014) and characterisation of assay specificity,” *Euro Surveill*, vol. 19, 2014.

[25] C. Gutierrez, M. T. Tejedor-Junco, M. Gonzalez, E. Lattwein, and S. Renneker, “Presence of antibodies but no evidence for circulation of MERS-CoV in dromedaries on the Canary Islands, 2015.,” *Euro Surveill. Bull. Eur. sur les Mal. Transm. = Eur. Commun. Dis. Bull.*, vol. 20, no. 37, 2015.

[26] M. A. Ali *et al.*, “Systematic, active surveillance for Middle East respiratory syndrome coronavirus in camels in Egypt,” *Emerg. Microbes Infect.*, vol. 6, no. 1, pp. e1–e1, Jan. 2017.

[27] N. van Doremalen *et al.*, “High Prevalence of Middle East Respiratory Coronavirus in Young Dromedary Camels in Jordan,” *Vector Borne Zoonotic Dis.*, vol. 17, no. 2, pp. 155–159, Feb. 2017.

[28] J.-A. Cui, F. Chen, and S. Fan, “Effect of Intermediate Hosts on Emerging Zoonoses.,” *Vector Borne Zoonotic Dis.*, vol. 17, no. 8, pp. 599–609, Aug. 2017.

[29] M. G. Hemida, “Middle East Respiratory Syndrome Coronavirus and the One Health concept,” *PeerJ*, vol. 7, pp. e7556–e7556, Aug. 2019.

[30] B. M. Alraddadi, J. T. Watson, G. R. A. Almarashi, A. Turkistani, M. Sadran, and A. Housa, “Risk Factors for Primary Middle East Respiratory Syndrome Coronavirus Illness in Humans, Saudi Arabia, 2014,” *Emerg Infect Dis*, vol. 22, 2016.

[31] A. L. Funk *et al.*, “MERS-CoV at the Animal-Human Interface: Inputs on Exposure Pathways from an Expert-Opinion Elicitation,” *Front. Vet. Sci.*, vol. 3, p. 88, Oct. 2016.

[32] C. Gossner *et al.*, “Human-Dromedary Camel Interactions and the Risk of Acquiring Zoonotic Middle East Respiratory Syndrome Coronavirus Infection.,” *Zoonoses Public Health*, vol. 63, no. 1, pp. 1–9, Feb. 2016.

[33] C. Reusken, H. Mou, G. J. Godeke, van der HL, B. Meyer, and M. A. Muller, “Specific serology for emerging human coronaviruses by protein microarray,” *Euro Surveill*, vol. 18, 2013.

[34] A. N. Alagaili, T. Briese, N. Mishra, V. Kapoor, S. C. Sameroff, and P. D. Burbelo, “Middle East respiratory syndrome coronavirus infection in dromedary camels in Saudi Arabia,” *M Bio*, vol. 5, 2014.

[35] J. Vergara-Alert *et al.*, “Livestock Susceptibility to Infection with Middle East Respiratory Syndrome Coronavirus,” *Emerg. Infect. Dis.*, vol. 23, no. 2, pp.

232–240, Feb. 2017.

[36] D. R. Adney, van DN, V. R. Brown, T. Bushmaker, D. Scott, and de WE, “Replication and shedding of MERS-CoV in upper respiratory tract of inoculated dromedary camels,” *Emerg Infect Dis*, vol. 20, 2014.

[37] I. M. Mackay and K. E. Arden, “MERS coronavirus: diagnostics, epidemiology and transmission,” *Virol. J.*, vol. 12, no. 1, p. 222, 2015.

[38] M. Letko *et al.*, “Adaptive Evolution of MERS-CoV to Species Variation in DPP4,” *Cell Rep.*, vol. 24, no. 7, pp. 1730–1737, Aug. 2018.

[39] S. Xia *et al.*, “Middle East respiratory syndrome coronavirus (MERS-CoV) entry inhibitors targeting spike protein,” *Virus Res.*, vol. 194, pp. 200–210, 2014.

[40] N. Wang *et al.*, “Structure of MERS-CoV spike receptor-binding domain complexed with human receptor DPP4,” *Cell Res.*, vol. 23, p. 986, Jul. 2013.

[41] S. R. Weiss and S. Navas-Martin, “Coronavirus Pathogenesis and the Emerging Pathogen Severe Acute Respiratory Syndrome Coronavirus,” *Microbiol. Mol. Biol. Rev.*, vol. 69, no. 4, p. 635 LP-664, Dec. 2005.

[42] L. Du, Y. He, Y. Zhou, S. Liu, B.-J. Zheng, and S. Jiang, “The spike protein of SARS-CoV — a target for vaccine and therapeutic development,” *Nat. Rev. Microbiol.*, vol. 7, no. 3, pp. 226–236, 2009.

[43] A. S. Agrawal *et al.*, “Generation of a Transgenic Mouse Model of Middle East Respiratory Syndrome Coronavirus Infection and Disease,” *J. Virol.*, vol. 89, no. 7, p. 3659 LP-3670, Apr. 2015.

[44] file:///C:/Users/eriko/Downloads/PMC4547275.ris, “Identification of human

neutralizing antibodies against MERS-CoV and their role in virus adaptive evolution," *Proc. Natl. Acad. Sci.* , vol. 111, no. 19, pp. E2018–E2026, May 2014.

[45] file:///C:/Users/eriko/Downloads/PMC4547275.ris, "Potent neutralization of MERS-CoV by human neutralizing monoclonal antibodies to the viral spike glycoprotein.," *Sci. Transl. Med.*, vol. 6, no. 234, p. 234ra59, Apr. 2014.

[46] file:///C:/Users/eriko/Downloads/PMC4547275.ris, "Exceptionally potent neutralization of Middle East respiratory syndrome coronavirus by human monoclonal antibodies," *J. Virol.*, vol. 88, no. 14, pp. 7796–7805, Jul. 2014.

[47] D. Corti *et al.*, "Prophylactic and postexposure efficacy of a potent human monoclonal antibody against MERS coronavirus," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 112, no. 33, pp. 10473–10478, Aug. 2015.

[48] K. E. Pascal *et al.*, "Pre- and postexposure efficacy of fully human antibodies against Spike protein in a novel humanized mouse model of MERS-CoV infection," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 112, no. 28, pp. 8738–8743, Jul. 2015.

[49] J. Zhao, R. A. P. M. Perera, G. Kayali, D. Meyerholz, S. Perlman, and M. Peiris, "Passive immunotherapy with dromedary immune serum in an experimental animal model for Middle East respiratory syndrome coronavirus infection," *J. Virol.*, vol. 89, no. 11, pp. 6117–6120, Jun. 2015.

[50] J. Mair-Jenkins *et al.*, "The effectiveness of convalescent plasma and hyperimmune immunoglobulin for the treatment of severe acute respiratory infections of viral etiology: a systematic review and exploratory meta-analysis," *J. Infect. Dis.*, vol. 211, no. 1, pp. 80–90, Jan. 2015.

[51] E. Kim *et al.*, “Immunogenicity of an adenoviral-based Middle East Respiratory Syndrome coronavirus vaccine in BALB/c mice.,” *Vaccine*, vol. 32, no. 45, pp. 5975–5982, Oct. 2014.

[52] A. Volz *et al.*, “Protective Efficacy of Recombinant Modified Vaccinia Virus Ankara Delivering Middle East Respiratory Syndrome Coronavirus Spike Glycoprotein.,” *J. Virol.*, vol. 89, no. 16, pp. 8651–8656, Aug. 2015.

[53] A. H. Malczyk *et al.*, “A Highly Immunogenic and Protective Middle East Respiratory Syndrome Coronavirus Vaccine Based on a Recombinant Measles Virus Vaccine Platform.,” *J. Virol.*, vol. 89, no. 22, pp. 11654–11667, Nov. 2015.

[54] B. L. Haagmans *et al.*, “An orthopoxvirus-based vaccine reduces virus excretion after MERS-CoV infection in dromedary camels,” *Science (80-.).*, vol. 351, no. 6268, p. 77 LP-81, Dec. 2015.

[55] X. Guo *et al.*, “Systemic and mucosal immunity in mice elicited by a single immunization with human adenovirus type 5 or 41 vector-based vaccines carrying the spike protein of Middle East respiratory syndrome coronavirus,” *Immunology*, vol. 145, no. 4, pp. 476–484, Aug. 2015.

[56] K. Muthumani *et al.*, “A synthetic consensus anti-spike protein DNA vaccine induces protective immunity against Middle East respiratory syndrome coronavirus in nonhuman primates.,” *Sci. Transl. Med.*, vol. 7, no. 301, p. 301ra132, Aug. 2015.

[57] L. Wang *et al.*, “Evaluation of candidate vaccine approaches for MERS-CoV,” *Nat Commun*, vol. 6, Jul. 2015.

[58] A. Cerwenka, T. M. Morgan, A. G. Harmsen, and R. W. Dutton, “Migration kinetics and final destination of type 1 and type 2 CD8 effector cells predict protection against pulmonary virus infection,” *J. Exp. Med.*, vol. 189, no. 2, pp. 423–434, 1999.

[59] B. Saha, S. Jyothi Prasanna, B. Chandrasekar, and D. Nandi, “Gene modulation and immunoregulatory roles of interferon gamma,” *Cytokine*, vol. 50, no. 1, pp. 1–14, 2010.

[60] F. Almazan *et al.*, “Engineering a replication-competent, propagation-defective Middle East respiratory syndrome coronavirus as a vaccine candidate.,” *MBio*, vol. 4, no. 5, pp. e00650-13, 2013.

[61] C. Wirblich *et al.*, “One-Health: a Safe, Efficient, Dual-Use Vaccine for Humans and Animals against Middle East Respiratory Syndrome Coronavirus and Rabies Virus,” *J. Virol.*, vol. 91, no. 2, pp. e02040-16, Jan. 2017.

[62] B. L. Haagmans *et al.*, “An orthopoxvirus-based vaccine reduces virus excretion after MERS-CoV infection in dromedary camels.,” *Science*, vol. 351, no. 6268, pp. 77–81, Jan. 2016.

[63] N. Zhang, J. Tang, L. Lu, S. Jiang, and L. Du, “Receptor-binding domain-based subunit vaccines against MERS-CoV.,” *Virus Res.*, vol. 202, pp. 151–159, Apr. 2015.

[64] J. Lan *et al.*, “Recombinant Receptor Binding Domain Protein Induces Partial Protective Immunity in Rhesus Macaques Against Middle East Respiratory Syndrome Coronavirus Challenge.,” *EBioMedicine*, vol. 2, no. 10, pp. 1438–1446, Oct. 2015.

[65] C. M. Coleman *et al.*, “MERS-CoV spike nanoparticles protect mice from MERS-CoV infection,” *Vaccine*, vol. 35, no. 12, pp. 1586–1589, Mar. 2017.

[66] C. M. Coleman *et al.*, “Purified coronavirus spike protein nanoparticles induce coronavirus neutralizing antibodies in mice,” *Vaccine*, vol. 32, no. 26, pp. 3169–3174, May 2014.

[67] C. C. Bergmann, T. E. Lane, and S. A. Stohlman, “Coronavirus infection of the central nervous system: host-virus stand-off.,” *Nat. Rev. Microbiol.*, vol. 4, no. 2, pp. 121–132, Feb. 2006.

[68] S. Perlman and R. Vijay, “Middle East respiratory syndrome vaccines.,” *Int. J. Infect. Dis.*, vol. 47, pp. 23–28, Jun. 2016.

[69] J. S. Williamson and S. A. Stohlman, “Effective clearance of mouse hepatitis virus from the central nervous system requires both CD4+ and CD8+ T cells,” *J. Virol.*, vol. 64, no. 9, pp. 4589–4592, Sep. 1990.

[70] A. F. Bradburne and B. A. Somerset, “Coronative antibody tires in sera of healthy adults and experimentally infected volunteers,” *J. Hyg. (Lond).*, vol. 70, no. 2, pp. 235–244, Jun. 1972.

[71] K. A. Callow, H. F. Parry, M. Sergeant, and D. A. Tyrrell, “The time course of the immune response to experimental coronavirus infection of man,” *Epidemiol. Infect.*, vol. 105, no. 2, pp. 435–446, Oct. 1990.

[72] F. Tang *et al.*, “Lack of peripheral memory B cell responses in recovered patients with severe acute respiratory syndrome: a six-year follow-up study.,” *J. Immunol.*, vol. 186, no. 12, pp. 7264–7268, Jun. 2011.

[73] Y.-Y. Fan *et al.*, “Characterization of SARS-CoV-specific memory T cells

from recovered individuals 4 years after infection," *Arch. Virol.*, vol. 154, no. 7, pp. 1093–1099, 2009.

[74] L.-T. Yang *et al.*, "Long-lived effector/central memory T-cell responses to severe acute respiratory syndrome coronavirus (SARS-CoV) S antigen in recovered SARS patients.," *Clin. Immunol.*, vol. 120, no. 2, pp. 171–178, Aug. 2006.

[75] WHO, "Malaria," 2019. [Online]. Available: https://www.who.int/news-room/fact-sheets/detail/malaria. [Accessed: 16-Jul-2019].

[76] M. C. de Souza, "Multiple Organ Dysfunction During Severe Malaria: The Role of the Inflammatory Response," T. A. Pádua, Ed. Rijeka: IntechOpen, 2016, p. Ch. 5.

[77] N. J. White, "Determinants of relapse periodicity in Plasmodium vivax malaria.," *Malar. J.*, vol. 10, p. 297, Oct. 2011.

[78] N. M. Anstey, N. M. Douglas, J. R. Poespoprodjo, and R. N. Price, "Chapter Three - Plasmodium vivax: Clinical Spectrum, Risk Factors and Pathogenesis," in *Advances in Parasitology*, vol. 80, S. I. Hay, R. Price, and J. K. B. T.-A. in P. Baird, Eds. Academic Press, 2012, pp. 151–201.

[79] WHO, "WHO-Malaria report," 2018. [Online]. Available: https://www.who.int/malaria/en/. [Accessed: 20-Aug-2019].

[80] P.-C. Burda, R. Caldelari, and V. T. Heussler, "Manipulation of the Host Cell Membrane during Plasmodium Liver Stage Egress," *MBio*, vol. 8, no. 2, pp. e00139-17, May 2017.

[81] K. Baer, C. Klotz, S. H. I. Kappe, T. Schnieder, and U. Frevert, "Release of

Hepatic Plasmodium yoelii Merozoites into the Pulmonary Microvasculature," *PLOS Pathog.*, vol. 3, no. 11, p. e171, Nov. 2007.

[82] M. J. Boyle, D. W. Wilson, and J. G. Beeson, "New approaches to studying Plasmodium falciparum merozoite invasion and insights into invasion biology," *Int. J. Parasitol.*, vol. 43, no. 1, pp. 1–10, 2013.

[83] B. M. Greenwood *et al.*, "Malaria: progress, perils, and prospects for eradication.," *J. Clin. Invest.*, vol. 118, no. 4, pp. 1266–1276, Apr. 2008.

[84] K. Gunalan *et al.*, "Role of Plasmodium vivax Duffy-binding protein 1 in invasion of Duffy-null Africans," *Proc. Natl. Acad. Sci.*, vol. 113, no. 22, p. 6271 LP-6276, May 2016.

[85] M. R. Galinski, C. C. Medina, P. Ingravallo, and J. W. Barnwell, "A reticulocyte-binding protein complex of Plasmodium vivax merozoites.," *Cell*, vol. 69, no. 7, pp. 1213–1226, Jun. 1992.

[86] K. Gunalan, X. Gao, S. S. L. Yap, X. Huang, and P. R. Preiser, "The role of the reticulocyte-binding-like protein homologues of Plasmodium in erythrocyte sensing and invasion," *Cell. Microbiol.*, vol. 15, no. 1, pp. 35–44, Jan. 2013.

[87] L. Jiang, S. Duriseti, P. Sun, and L. H. Miller, "Molecular basis of binding of the Plasmodium falciparum receptor BAEBL to erythrocyte receptor glycophorin C," *Mol. Biochem. Parasitol.*, vol. 168, no. 1, pp. 49–54, 2009.

[88] A. Chakraborty, "Understanding the biology of the Plasmodium falciparum apicoplast; an excellent target for antimalarial drug development.," *Life Sci.*, vol. 158, pp. 104–110, Aug. 2016.

[89] D. S. Guttery, A. A. Holder, and R. Tewari, “Sexual development in Plasmodium: lessons from functional analyses.,” *PLoS Pathog.*, vol. 8, no. 1, p. e1002404, Jan. 2012.

[90] I. S. and control programme for W. nile virus and M. in Greece, “Plasmodium life cycle,” 2016. [Online]. Available: http://www.malwest.gr/en-us/malaria/informationforhealthcareprofessionals/plasmodiumlifecycle.aspx.

[91] CDC, “Malaria,” 2018. [Online]. Available: https://www.cdc.gov/malaria/about/biology/index.html. [Accessed: 16-Jul-2019].

[92] D. L. Doolan, C. Dobano, and J. K. Baird, “Acquired immunity to malaria.,” *Clin. Microbiol. Rev.*, vol. 22, no. 1, p. 13–36, Table of Contents, Jan. 2009.

[93] G. Dobaño, Carlota; Moncunill, “Naturally Acquired Immunity (NAI),” *Encyclopedia of Malaria*. SpringerLink, 2018.

[94] J. D. Smith *et al.*, “Switches in expression of Plasmodium falciparum var genes correlate with changes in antigenic and cytoadherent phenotypes of infected erythrocytes.,” *Cell*, vol. 82, no. 1, pp. 101–110, Jul. 1995.

[95] X. Z. Su *et al.*, “The large diverse gene family var encodes proteins involved in cytoadherence and antigenic variation of Plasmodium falciparum-infected erythrocytes.,” *Cell*, vol. 82, no. 1, pp. 89–100, Jul. 1995.

[96] J. Langhorne, F. M. Ndungu, A.-M. Sponaas, and K. Marsh, “Immunity to malaria: more questions than answers.,” *Nat. Immunol.*, vol. 9, no. 7, pp. 725–732, Jul. 2008.

[97] W. H. H. Reece *et al.*, “A CD4(+) T-cell immune response to a conserved

epitope in the circumsporozoite protein correlates with protection from natural Plasmodium falciparum infection and disease.," *Nat. Med.*, vol. 10, no. 4, pp. 406–410, Apr. 2004.

[98] K. Marsh, L. Otoo, R. J. Hayes, D. C. Carson, and B. M. Greenwood, "Antibodies to blood stage antigens of Plasmodium falciparum in rural Gambians and their relation to protection against infection.," *Trans. R. Soc. Trop. Med. Hyg.*, vol. 83, no. 3, pp. 293–303, 1989.

[99] P. C. Bull, B. S. Lowe, M. Kortok, C. S. Molyneux, C. I. Newbold, and K. Marsh, "Parasite antigens on the infected red cell surface are targets for naturally acquired immunity to malaria.," *Nat. Med.*, vol. 4, no. 3, pp. 358–360, Mar. 1998.

[100] H. Bouharoun-Tayoun, P. Attanath, A. Sabchareon, T. Chongsuphajaisiddhi, and P. Druilhe, "Antibodies that protect humans against Plasmodium falciparum blood stages do not on their own inhibit parasite growth and invasion in vitro, but act in cooperation with monocytes.," *J. Exp. Med.*, vol. 172, no. 6, pp. 1633–1641, Dec. 1990.

[101] M. J. Boyle *et al.*, "Human antibodies fix complement to inhibit Plasmodium falciparum invasion of erythrocytes and are associated with protection against malaria.," *Immunity*, vol. 42, no. 3, pp. 580–590, Mar. 2015.

[102] M. J. Stewart, R. J. Nawrot, S. Schulman, and J. P. Vanderberg, "Plasmodium berghei sporozoite invasion is blocked in vitro by sporozoite-immobilizing antibodies.," *Infect. Immun.*, vol. 51, no. 3, pp. 859–864, Mar. 1986.

[103] K. Peng *et al.*, "Breadth of humoral response and antigenic targets of

sporozoite-inhibitory antibodies associated with sterile protection induced by controlled human malaria infection.," *Cell. Microbiol.*, vol. 18, no. 12, pp. 1739–1750, Dec. 2016.

[104] O. C. Finney, G. J. Keitany, H. Smithers, A. Kaushansky, S. Kappe, and R. Wang, "Immunization with genetically attenuated P. falciparum parasites induces long-lived antibodies that efficiently block hepatocyte invasion by sporozoites.," *Vaccine*, vol. 32, no. 19, pp. 2135–2138, Apr. 2014.

[105] A. S. Ishizuka *et al.*, "Protection against malaria at 1 year and immune correlates following PfSPZ vaccination.," *Nat. Med.*, vol. 22, no. 6, pp. 614–623, Jun. 2016.

[106] I. Zenklusen *et al.*, "Immunization of Malaria-Preexposed Volunteers With PfSPZ Vaccine Elicits Long-Lived IgM Invasion-Inhibitory and Complement-Fixing Antibodies," *J. Infect. Dis.*, vol. 217, no. 10, pp. 1569–1578, Apr. 2018.

[107] A. M. Moormann, C. C. John, P. O. Sumba, D. Tisch, P. Embury, and J. W. Kazura, "Stability of interferon-gamma and interleukin-10 responses to Plasmodium falciparum liver stage antigen-1 and thrombospondin-related adhesive protein in residents of a malaria holoendemic area.," *Am. J. Trop. Med. Hyg.*, vol. 74, no. 4, pp. 585–590, Apr. 2006.

[108] C. C. John, P. O. Sumba, J. H. Ouma, B. L. Nahlen, C. L. King, and J. W. Kazura, "Cytokine responses to Plasmodium falciparum liver-stage antigen 1 vary in rainy and dry seasons in highland Kenya," *Infect. Immun.*, vol. 68, no. 9, pp. 5198–5204, Sep. 2000.

[109] J. M. O. Ong'echa *et al.*, "Association of interferon-gamma responses to pre-

erythrocytic stage vaccine candidate antigens of Plasmodium falciparum in young Kenyan children with improved hemoglobin levels: XV. Asembo Bay Cohort Project.," *Am. J. Trop. Med. Hyg.*, vol. 68, no. 5, pp. 590–597, May 2003.

[110] A. J. Luty *et al.*, "Interferon-gamma responses are associated with resistance to reinfection with Plasmodium falciparum in young African children.," *J. Infect. Dis.*, vol. 179, no. 4, pp. 980–988, Apr. 1999.

[111] C. C. John, A. M. Moormann, P. O. Sumba, A. V Ofulla, D. C. Pregibon, and J. W. Kazura, "Gamma interferon responses to Plasmodium falciparum liver-stage antigen 1 and thrombospondin-related adhesive protein and their relationship to age, transmission intensity, and protection against malaria.," *Infect. Immun.*, vol. 72, no. 9, pp. 5135–5142, Sep. 2004.

[112] S. L. Hoffman *et al.*, "Human lymphocyte proliferative response to a sporozoite T cell epitope correlates with resistance to falciparum malaria.," *J. Immunol.*, vol. 142, no. 4, pp. 1299–1303, Feb. 1989.

[113] J. May, B. Lell, A. J. Luty, C. G. Meyer, and P. G. Kremsner, "HLA-DQB1*0501-restricted Th1 type immune responses to Plasmodium falciparum liver stage antigen 1 protect against malaria anemia and reinfections.," *J. Infect. Dis.*, vol. 183, no. 1, pp. 168–172, Jan. 2001.

[114] D. L. Doolan and S. L. Hoffman, "The complexity of protective immunity against liver-stage malaria.," *J. Immunol.*, vol. 165, no. 3, pp. 1453–1462, Aug. 2000.

[115] M. G. Overstreet, Y.-C. Chen, I. A. Cockburn, S.-W. Tse, and F. Zavala, "CD4+ T cells modulate expansion and survival but not functional properties

of effector and memory CD8+ T cells induced by malaria sporozoites.," *PLoS One*, vol. 6, no. 1, p. e15948, Jan. 2011.

[116] M. G. Overstreet, I. A. Cockburn, Y.-C. Chen, and F. Zavala, "Protective CD8 T cells against Plasmodium liver stages: immunobiology of an 'unnatural' immune response.," *Immunol. Rev.*, vol. 225, pp. 272–283, Oct. 2008.

[117] E. M. Bijker *et al.*, "Cytotoxic markers associate with protection against malaria in human volunteers immunized with Plasmodium falciparum sporozoites.," *J. Infect. Dis.*, vol. 210, no. 10, pp. 1605–1615, Nov. 2014.

[118] B. Mordmuller *et al.*, "Sterile protection against human malaria by chemoattenuated PfSPZ vaccine.," *Nature*, vol. 542, no. 7642, pp. 445–449, Feb. 2017.

[119] S. Waki, "Antibody-dependent neutrophil-mediated parasite killing in non-lethal rodent malaria.," *Parasite Immunol.*, vol. 16, no. 11, pp. 587–591, Nov. 1994.

[120] G. A. Butcher and I. A. Clark, "The inhibition of Plasmodium falciparum growth in vitro by sera from mice infected with malaria or treated with TNF.," *Parasitology*, vol. 101 Pt 3, pp. 321–326, Dec. 1990.

[121] C. F. Ockenhouse, S. Schulman, and H. L. Shear, "Induction of crisis forms in the human malaria parasite Plasmodium falciparum by gamma-interferon-activated, monocyte-derived macrophages.," *J. Immunol.*, vol. 133, no. 3, pp. 1601–1608, Sep. 1984.

[122] K. R. Jones, B. J. Cottrell, G. A. Targett, and J. H. Playfair, "Killing of

Plasmodium falciparum by human monocyte-derived macrophages.," *Parasite Immunol.*, vol. 11, no. 6, pp. 585–592, Nov. 1989.

[123] T. King and T. Lamb, "Interferon-gamma: The Jekyll and Hyde of Malaria.," *PLoS Pathog.*, vol. 11, no. 10, p. e1005118, Oct. 2015.

[124] E. Uchio, H. Inoue, A. Fuchigami, and K. Kadonosono, "Anti-adenoviral effect of interferon-β and interferon-γ in serotypes that cause acute keratoconjunctivitis," *Clin. Experiment. Ophthalmol.*, vol. 39, no. 4, pp. 358–363, May 2011.

[125] M. C. D'Ombrain *et al.*, "Association of early interferon-gamma production with immunity to clinical malaria: a longitudinal study among Papua New Guinean children.," *Clin. Infect. Dis.*, vol. 47, no. 11, pp. 1380–1387, Dec. 2008.

[126] L. J. Robinson *et al.*, "Cellular tumor necrosis factor, gamma interferon, and interleukin-6 responses as correlates of immunity and risk of clinical Plasmodium falciparum malaria in children from Papua New Guinea.," *Infect. Immun.*, vol. 77, no. 7, pp. 3033–3043, Jul. 2009.

[127] R. E. Sinden, "Developing transmission-blocking strategies for malaria control," *PLoS Pathog.*, vol. 13, no. 7, pp. e1006336–e1006336, Jul. 2017.

[128] J. Kubler-Kielb *et al.*, "Long-lasting and transmission-blocking activity of antibodies to Plasmodium falciparum elicited in mice by protein conjugates of Pfs25," *Proc. Natl. Acad. Sci.*, vol. 104, no. 1, p. 293 LP-298, Jan. 2007.

[129] J. G. Kublin *et al.*, "Complete attenuation of genetically engineered

Plasmodium falciparum sporozoites in human subjects.," *Sci. Transl. Med.*, vol. 9, no. 371, Jan. 2017.

[130] S. A. Mikolajczak *et al.*, "A next-generation genetically attenuated Plasmodium falciparum parasite created by triple gene deletion," *Mol. Ther.*, vol. 22, no. 9, pp. 1707–1715, Sep. 2014.

[131] J. E. Epstein *et al.*, "Live attenuated malaria vaccine designed to protect through hepatic CD8(+) T cell immunity.," *Science*, vol. 334, no. 6055, pp. 475–480, Oct. 2011.

[132] J. E. Epstein *et al.*, "Protection against Plasmodium falciparum malaria by PfSPZ Vaccine," *JCI insight*, vol. 2, no. 1, pp. e89154–e89154, Jan. 2017.

[133] R. A. Seder *et al.*, "Protection against malaria by intravenous immunization with a nonreplicating sporozoite vaccine.," *Science*, vol. 341, no. 6152, pp. 1359–1365, Sep. 2013.

[134] K. E. Lyke *et al.*, "Attenuated PfSPZ Vaccine induces strain-transcending T cells and durable protection against heterologous controlled human malaria infection," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 114, no. 10, pp. 2711–2716, Mar. 2017.

[135] A. Olotu *et al.*, "Seven-Year Efficacy of RTS,S/AS01 Malaria Vaccine among Young African Children," *N. Engl. J. Med.*, vol. 374, no. 26, pp. 2519–2529, Jun. 2016.

[136] M. S. Sissoko *et al.*, "Safety and efficacy of PfSPZ Vaccine against Plasmodium falciparum via direct venous inoculation in healthy malaria-exposed adults in Mali: a randomised, double-blind phase 1 trial.," *Lancet.*

Infect. Dis., vol. 17, no. 5, pp. 498–509, May 2017.

[137] S. J. Draper *et al.*, “Malaria Vaccines: Recent Advances and New Horizons.,” *Cell Host Microbe*, vol. 24, no. 1, pp. 43–56, Jul. 2018.

[138] S. C. T. P. RTS, “Efficacy and safety of RTS,S/AS01 malaria vaccine with or without a booster dose in infants and children in Africa: final results of a phase 3, individually randomised, controlled trial,” *Lancet (London, England)*, vol. 386, no. 9988, pp. 31–45, Jul. 2015.

[139] S. C. T. P. (2014) RTS, “Efficacy and Safety of the RTS,S/AS01 Malaria Vaccine during 18 Months after Vaccination: A Phase 3 Randomized, Controlled Trial in Children and Young Infants at 11 African Sites,” *PLoS Med*, vol. 11, no. 7, p. e1001685, Jul. 2014.

[140] M. T. White *et al.*, “Immunogenicity of the RTS,S/AS01 malaria vaccine and implications for duration of vaccine efficacy: secondary analysis of data from a phase 3 randomised controlled trial,” *Lancet. Infect. Dis.*, vol. 15, no. 12, pp. 1450–1458, Dec. 2015.

[141] K. A. Collins, R. Snaith, M. G. Cottingham, S. C. Gilbert, and A. V. S. Hill, “Enhancing protective immunity to malaria with a highly immunogenic virus-like particle vaccine,” *Sci. Rep.*, vol. 7, p. 46621, Apr. 2017.

[142] K. J. Ewer *et al.*, “Protective CD8(+) T-cell immunity to human malaria induced by chimpanzee adenovirus-MVA immunisation,” *Nat. Commun.*, vol. 4, p. 2836, Nov. 2013.

[143] C. Ogwang *et al.*, “Prime-boost vaccination with chimpanzee adenovirus and modified vaccinia Ankara encoding TRAP provides partial protection against

Plasmodium falciparum infection in Kenyan adults," *Sci. Transl. Med.*, vol. 7, no. 286, p. 286re5-286re5, May 2015.

[144] V. A. Mensah *et al.*, "Safety, Immunogenicity and Efficacy of Prime-Boost Vaccination with ChAd63 and MVA Encoding ME-TRAP against Plasmodium falciparum Infection in Adults in Senegal," *PLoS One*, vol. 11, no. 12, pp. e0167951–e0167951, Dec. 2016.

[145] S. H. Hodgson *et al.*, "Evaluation of the efficacy of ChAd63-MVA vectored vaccines expressing circumsporozoite protein and ME-TRAP against controlled human malaria infection in malaria-naive individuals," *J. Infect. Dis.*, vol. 211, no. 7, pp. 1076–1086, Apr. 2015.

[146] K. Venkatraman, Navin; Sridhar, Saranya; Bowyer, Gerogina; Collins, "Safety, immunogenicity and durability of a novel malaria vaccine candidate, R21 adjuvanted with Matrix-M(TM)," 2017.

[147] C. J. Genito *et al.*, "Liposomes containing monophosphoryl lipid A and QS-21 serve as an effective adjuvant for soluble circumsporozoite protein malaria vaccine FMP013.," *Vaccine*, vol. 35, no. 31, pp. 3865–3874, Jul. 2017.

[148] T. Ura, K. Okuda, and M. Shimada, "Developments in Viral Vector-Based Vaccines," *Vaccines*, vol. 2, no. 3, pp. 624–641, Jul. 2014.

[149] S. Akira, S. Uematsu, and O. Takeuchi, "Pathogen recognition and innate immunity.," *Cell*, vol. 124, no. 4, pp. 783–801, Feb. 2006.

[150] S. Rerks-Ngarm *et al.*, "Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand.," *N. Engl. J. Med.*, vol. 361, no. 23, pp. 2209–

2220, Dec. 2009.

[151] F. Garcia *et al.*, “Safety and immunogenicity of a modified pox vector-based HIV/AIDS vaccine candidate expressing Env, Gag, Pol and Nef proteins of HIV-1 subtype B (MVA-B) in healthy HIV-1-uninfected volunteers: A phase I clinical trial (RISVAC02).,” *Vaccine*, vol. 29, no. 46, pp. 8309–8316, Oct. 2011.

[152] C. E. Gómez *et al.*, “The HIV/AIDS vaccine candidate MVA-B administered as a single immunogen in humans triggers robust, polyfunctional, and selective effector memory T cell responses to HIV-1 antigens,” *J. Virol.*, vol. 85, no. 21, pp. 11468–11478, Nov. 2011.

[153] M. Bakari *et al.*, “Broad and potent immune responses to a low dose intradermal HIV-1 DNA boosted with HIV-1 recombinant MVA among healthy adults in Tanzania,” *Vaccine*, vol. 29, no. 46, pp. 8417–8428, Oct. 2011.

[154] J. S. Cavenaugh, D. Awi, M. Mendy, A. V. S. Hill, H. Whittle, and S. J. McConkey, “Partially randomized, non-blinded trial of DNA and MVA therapeutic vaccines based on hepatitis B virus surface protein for chronic HBV infection.,” *PLoS One*, vol. 6, no. 2, p. e14626, Feb. 2011.

[155] T. K. Berthoud *et al.*, “Potent CD8+ T-cell immunogenicity in humans of a novel heterosubtypic influenza A vaccine, MVA-NP+M1,” *Clin. Infect. Dis.*, vol. 52, no. 1, pp. 1–7, Jan. 2011.

[156] P. Bejon *et al.*, “Extended follow-up following a phase 2b randomized trial of the candidate malaria vaccines FP9 ME-TRAP and MVA ME-TRAP among children in Kenya,” *PLoS One*, vol. 2, no. 8, pp. e707–e707, Aug. 2007.

[157] S. H. Sheehy *et al.*, “Phase Ia clinical evaluation of the safety and immunogenicity of the Plasmodium falciparum blood-stage antigen AMA1 in ChAd63 and MVA vaccine vectors,” *PLoS One*, vol. 7, no. 2, pp. e31208–e31208, 2012.

[158] M. D. Tameris *et al.*, “Safety and efficacy of MVA85A, a new tuberculosis vaccine, in infants previously vaccinated with BCG: a randomised, placebo-controlled phase 2b trial,” *Lancet (London, England)*, vol. 381, no. 9871, pp. 1021–1028, Mar. 2013.

[159] D. M. Roberts *et al.*, “Hexon-chimaeric adenovirus serotype 5 vectors circumvent pre-existing anti-vector immunity.,” *Nature*, vol. 441, no. 7090, pp. 239–243, May 2006.

[160] S. Abe *et al.*, “Adenovirus type 5 with modified hexons induces robust transgene-specific immune responses in mice with pre-existing immunity against adenovirus type 5.,” *J. Gene Med.*, vol. 11, no. 7, pp. 570–579, Jul. 2009.

[161] T. Ura *et al.*, “Designed recombinant adenovirus type 5 vector induced envelope-specific CD8(+) cytotoxic T lymphocytes and cross-reactive neutralizing antibodies against human immunodeficiency virus type 1.,” *J. Gene Med.*, vol. 11, no. 2, pp. 139–149, Feb. 2009.

[162] K. J. Ewer *et al.*, “Progress with viral vectored malaria vaccines: A multi-stage approach involving ‘unnatural immunity,’” *Vaccine*, vol. 33, no. 52, pp. 7444–7451, Dec. 2015.

[163] S. M. Hammer *et al.*, “Efficacy trial of a DNA/rAd5 HIV-1 preventive vaccine,” *N. Engl. J. Med.*, vol. 369, no. 22, pp. 2083–2092, Nov. 2013.

[164] H. Kibuuka *et al.*, “A phase 1/2 study of a multiclade HIV-1 DNA plasmid prime and recombinant adenovirus serotype 5 boost vaccine in HIV-Uninfected East Africans (RV 172),” *J. Infect. Dis.*, vol. 201, no. 4, pp. 600–607, Feb. 2010.

[165] M. Gurwith *et al.*, “Safety and immunogenicity of an oral, replicating adenovirus serotype 4 vector vaccine for H5N1 influenza: a randomised, double-blind, placebo-controlled, phase 1 study,” *Lancet. Infect. Dis.*, vol. 13, no. 3, pp. 238–250, Mar. 2013.

[166] F. Smaill *et al.*, “A human type 5 adenovirus-based tuberculosis vaccine induces robust T cell responses in humans despite preexisting anti-adenovirus immunity.,” *Sci. Transl. Med.*, vol. 5, no. 205, p. 205ra134, Oct. 2013.

[167] I. R. Humphreys and S. Sebastian, “Novel viral vectors in infectious diseases,” *Immunology*, vol. 153, no. 1, pp. 1–9, Jan. 2018.

[168] A. W. Sylwester *et al.*, “Broadly targeted human cytomegalovirus-specific CD4+ and CD8+ T cells dominate the memory compartments of exposed subjects,” *J. Exp. Med.*, vol. 202, no. 5, pp. 673–685, Sep. 2005.

[169] Y. Tsuda *et al.*, “A replicating cytomegalovirus-based vaccine encoding a single Ebola virus nucleoprotein CTL epitope confers protection against Ebola virus,” *PLoS Negl. Trop. Dis.*, vol. 5, no. 8, pp. e1275–e1275, Aug. 2011.

[170] Y. Tsuda *et al.*, “A cytomegalovirus-based vaccine provides long-lasting protection against lethal Ebola virus challenge after a single dose,” *Vaccine*, vol. 33, no. 19, pp. 2261–2266, May 2015.

[171] P. C. L. Beverley *et al.*, “A novel murine cytomegalovirus vaccine vector protects against Mycobacterium tuberculosis,” *J. Immunol.*, vol. 193, no. 5, pp. 2306–2316, Sep. 2014.

[172] I. Dekhtiarenko, M. A. Jarvis, Z. Ruzsics, and L. Cicin-Sain, “The context of gene expression defines the immunodominance hierarchy of cytomegalovirus antigens.,” *J. Immunol.*, vol. 190, no. 7, pp. 3399–3409, Apr. 2013.

[173] S. G. Hansen *et al.*, “Profound early control of highly pathogenic SIV by an effector memory T-cell vaccine,” *Nature*, vol. 473, no. 7348, pp. 523–527, May 2011.

[174] J. D. Fuchs *et al.*, “First-in-Human Evaluation of the Safety and Immunogenicity of a Recombinant Vesicular Stomatitis Virus Human Immunodeficiency Virus-1 gag Vaccine (HVTN 090),” *Open forum Infect. Dis.*, vol. 2, no. 3, pp. ofv082-ofv082, Jun. 2015.

[175] “Evaluating the safety of and immune response to HIV-MAG DNA vaccine with or without plasmid IL-12 adjuvant delivered intramuscularly via electroporation followed by VSV-gag HIV vaccine boost in healthy, HIV-uninfected adults,” *ClinicalTrials.gov/NCT01578889*. .

[176] “Therapeutic vaccine for HIV,” *ClinicalTrials.gov/NCT01859325*. .

[177] “Evaluating the safety, tolerability, and immunogenicity of a prime-boost regimen of HIV-1 Nef/Tat/Vif, Env pDNA vaccine delivered intramuscularly with electroporation and HIV-1 rVSV envC vaccine in healthy, HIV-uninfected adults,” *ClinicalTrials.gov/NCT02654080*. .

[178] C. E. Mire, T. W. Geisbert, H. Feldmann, and A. Marzi, “Ebola virus vaccines - reality or fiction?,” *Expert Rev. Vaccines*, vol. 15, no. 11, pp. 1421–1430, Nov. 2016.

[179] A. M. Henao-Restrepo *et al.*, “Efficacy and effectiveness of an rVSV-vectored vaccine in preventing Ebola virus disease: final results from the Guinea ring vaccination, open-label, cluster-randomised trial (Ebola Ça Suffit!),” *Lancet (London, England)*, vol. 389, no. 10068, pp. 505–518, Feb. 2017.

[180] C. Dahlke *et al.*, “Dose-dependent T-cell Dynamics and Cytokine Cascade Following rVSV-ZEBOV Immunization,” *EBioMedicine*, vol. 19, pp. 107–118, May 2017.

[181] A. Baldo, E. Galanis, F. Tangy, and P. Herman, “Biosafety considerations for attenuated measles virus vectors used in virotherapy and vaccination,” *Hum. Vaccin. Immunother.*, vol. 12, no. 5, pp. 1102–1116, May 2016.

[182] P. Dai *et al.*, “Modified vaccinia virus Ankara triggers type I IFN production in murine conventional dendritic cells via a cGAS/STING-mediated cytosolic DNA-sensing pathway.,” *PLoS Pathog.*, vol. 10, no. 4, p. e1003989, Apr. 2014.

[183] I. G. Ovsyannikova, N. Dhiman, R. M. Jacobson, R. A. Vierkant, and G. A. Poland, “Frequency of measles virus-specific CD4+ and CD8+ T cells in subjects seronegative or highly seropositive for measles vaccine,” *Clin. Diagn. Lab. Immunol.*, vol. 10, no. 3, pp. 411–416, May 2003.

[184] R. C. Howe, N. Dhiman, I. G. Ovsyannikova, and G. A. Poland, “Induction of CD4 T cell proliferation and in vitro Th1-like cytokine responses to measles

virus," *Clin. Exp. Immunol.*, vol. 140, no. 2, pp. 333–342, May 2005.

[185] "Study to evaluate the dosage and safety of two intramuscular injections of an investigational Clade B HIV Vaccine," *ClinicalTrials.gov/NCT01320176*. .

[186] K. Ramsauer *et al.*, "Immunogenicity, safety, and tolerability of a recombinant measles-virus-based chikungunya vaccine: a randomised, double-blind, placebo-controlled, active-comparator, first-in-man trial.," *Lancet. Infect. Dis.*, vol. 15, no. 5, pp. 519–527, May 2015.

[187] "Phase II study to evaluate safety and immunogenicity of a Chikungunya vaccine," *ClinicalTrials.gov/NCT02861586*. .

[188] "Study of a live attenuated Chikungunya vaccine in a previously epidemic area," *ClinicalTrials.gov/NCT03101111*. .

[189] K. S. Slobod *et al.*, "Safety and immunogenicity of intranasal murine parainfluenza virus type 1 (Sendai virus) in healthy human adults.," *Vaccine*, vol. 22, no. 23–24, pp. 3182–3186, Aug. 2004.

[190] E. Adderson *et al.*, "Safety and immunogenicity of an intranasal Sendai virus-based human parainfluenza virus type 1 vaccine in 3- to 6-year-old children," *Clin. Vaccine Immunol.*, vol. 22, no. 3, pp. 298–303, Mar. 2015.

[191] S. A. Brown *et al.*, "Heterologous Prime-Boost HIV-1 Vaccination Regimens in Pre-Clinical and Clinical Trials," *Viruses*, vol. 2, no. 2, pp. 435–467, Feb. 2010.

[192] S. Yu *et al.*, "Potent specific immune responses induced by prime-boost-boost strategies based on DNA, adenovirus, and Sendai virus vectors expressing gag gene of Chinese HIV-1 subtype B.," *Vaccine*, vol. 26, no. 48,

pp. 6124–6131, Nov. 2008.

[193] C. J. Russell and J. L. Hurwitz, “Sendai virus as a backbone for vaccines against RSV and other human paramyxoviruses,” *Expert Rev. Vaccines*, vol. 15, no. 2, pp. 189–200, 2016.

[194] D. E. Zak *et al.*, “Merck Ad5/HIV induces broad innate immune activation that predicts $CD8^+$ T-cell responses but is attenuated by preexisting Ad5 immunity,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 109, no. 50, pp. E3503–E3512, Dec. 2012.

[195] S. O. Pine *et al.*, “Pre-existing adenovirus immunity modifies a complex mixed Th1 and Th2 cytokine response to an Ad5/HIV-1 vaccine candidate in humans,” *PLoS One*, vol. 6, no. 4, pp. e18526–e18526, Apr. 2011.

[196] L. R. Baden *et al.*, “Assessment of the Safety and Immunogenicity of 2 Novel Vaccine Platforms for HIV-1 Prevention: A Randomized Trial,” *Ann. Intern. Med.*, vol. 164, no. 5, pp. 313–322, Mar. 2016.

[197] I. D. Milligan *et al.*, “Safety and Immunogenicity of Novel Adenovirus Type 26- and Modified Vaccinia Ankara-Vectored Ebola Vaccines: A Randomized Clinical Trial.,” *JAMA*, vol. 315, no. 15, pp. 1610–1623, Apr. 2016.

[198] G. Shukarev, B. Callendret, K. Luhn, M. Douoguih, and E. consortium, “A two-dose heterologous prime-boost vaccine regimen eliciting sustained immune responses to Ebola Zaire could support a preventive strategy for future outbreaks,” *Hum. Vaccin. Immunother.*, vol. 13, no. 2, pp. 266–270, Feb. 2017.

[199] M. C. Keefer *et al.*, “A phase I double blind, placebo-controlled, randomized

study of a multigenic HIV-1 adenovirus subtype 35 vector vaccine in healthy uninfected adults," *PLoS One*, vol. 7, no. 8, pp. e41936–e41936, 2012.

[200] J. D. Fuchs *et al.*, "Safety and Immunogenicity of a Recombinant Adenovirus Serotype 35-Vectored HIV-1 Vaccine in Adenovirus Serotype 5 Seronegative and Seropositive Individuals," *J. AIDS Clin. Res.*, vol. 6, no. 5, p. 461, May 2015.

[201] J. Mpendo *et al.*, "A Phase I Double Blind, Placebo-Controlled, Randomized Study of the Safety and Immunogenicity of Electroporated HIV DNA with or without Interleukin 12 in Prime-Boost Combinations with an Ad35 HIV Vaccine in Healthy HIV-Seronegative African Adults," *PLoS One*, vol. 10, no. 8, pp. e0134287–e0134287, Aug. 2015.

[202] J. Nyombayire *et al.*, "First-in-Human Evaluation of the Safety and Immunogenicity of an Intranasally Administered Replication-Competent Sendai Virus-Vectored HIV Type 1 Gag Vaccine: Induction of Potent T-Cell or Antibody Responses in Prime-Boost Regimens," *J. Infect. Dis.*, vol. 215, no. 1, pp. 95–104, Jan. 2017.

[203] K. Ewer *et al.*, "A Monovalent Chimpanzee Adenovirus Ebola Vaccine Boosted with MVA," *N. Engl. J. Med.*, vol. 374, no. 17, pp. 1635–1646, Apr. 2016.

[204] J. E. Ledgerwood *et al.*, "Chimpanzee Adenovirus Vector Ebola Vaccine.," *N. Engl. J. Med.*, vol. 376, no. 10, pp. 928–938, Mar. 2017.

[205] M. D. Tapia *et al.*, "Use of ChAd3-EBO-Z Ebola virus vaccine in Malian and US adults, and boosting of Malian adults with MVA-BN-Filo: a phase 1, single-blind, randomised trial, a phase 1b, open-label and double-blind,

dose-escalation trial, and a nested, randomised, double-bli," *Lancet. Infect. Dis.*, vol. 16, no. 1, pp. 31–42, Jan. 2016.

[206] G. A. O'Hara *et al.*, "Clinical assessment of a recombinant simian adenovirus ChAd63: a potent new vaccine vector," *J. Infect. Dis.*, vol. 205, no. 5, pp. 772–781, Mar. 2012.

[207] M. O. Afolabi *et al.*, "Safety and Immunogenicity of ChAd63 and MVA ME-TRAP in West African Children and Infants," *Mol. Ther.*, vol. 24, no. 8, pp. 1470–1477, Aug. 2016.

[208] M. Osman *et al.*, "A third generation vaccine for human visceral leishmaniasis and post kala azar dermal leishmaniasis: First-in-human trial of ChAd63-KH," *PLoS Negl. Trop. Dis.*, vol. 11, no. 5, pp. e0005527–e0005527, May 2017.

[209] A. Krause, W. Z. Whu, Y. Xu, J. Joh, R. G. Crystal, and S. Worgall, "Protective anti-Pseudomonas aeruginosa humoral and cellular mucosal immunity by AdC7-mediated expression of the P. aeruginosa protein OprF.," *Vaccine*, vol. 29, no. 11, pp. 2131–2139, Mar. 2011.

[210] C. M. Crosby and M. A. Barry, "IIIa deleted adenovirus as a single-cycle genome replicating vector," *Virology*, vol. 462–463, pp. 158–165, Aug. 2014.

[211] C. M. Crosby, P. Nehete, K. J. Sastry, and M. A. Barry, "Amplified and persistent immune responses generated by single-cycle replicating adenovirus vaccines," *J. Virol.*, vol. 89, no. 1, pp. 669–675, Jan. 2015.

[212] C. M. Crosby *et al.*, "Replicating Single-Cycle Adenovirus Vectors Generate Amplified Influenza Vaccine Responses," *J. Virol.*, vol. 91, no. 2, pp.

e00720-16, Jan. 2017.

[213] S. Biswas *et al.*, “Transgene Optimization, Immunogenicity and In Vitro Efficacy of Viral Vectored Vaccines Expressing Two Alleles of Plasmodium falciparum AMA1,” *PLoS One*, vol. 6, no. 6, p. e20977, Jun. 2011.

[214] T. H. B Ondondo, S Abdul-Jawad, Y Roshorm, A Bridgeman, “DeliveryDependant Effect of Human Tissue Plasminogen Activator Signal Peptide on Vaccine Induction of T Cells,” *J HIV AIDS*, vol. 2, no. 4, 2016.

[215] I. F. Ciernik, J. A. Berzofsky, and D. P. Carbone, “Induction of cytotoxic T lymphocytes and antitumor immunity with DNA vaccines expressing single T cell epitopes.,” *J. Immunol.*, vol. 156, no. 7, pp. 2369–2375, Apr. 1996.

[216] J. S. Richardson *et al.*, “Enhanced protection against Ebola virus mediated by an improved adenovirus-based vaccine,” *PLoS One*, vol. 4, no. 4, pp. e5308–e5308, 2009.

[217] S. Sridhar *et al.*, “Single-Dose Protection against Plasmodium berghei by a Simian Adenovirus Vector Using a Human Cytomegalovirus Promoter Containing Intron A,” *J. Virol.*, vol. 82, no. 8, p. 3822 LP-3833, Apr. 2008.

[218] Y. Zhang *et al.*, “Effects of the fusion design and immunization route on the immunogenicity of Ag85A-Mtb32 in adenoviral vectored tuberculosis vaccine,” *Hum. Vaccin. Immunother.*, vol. 11, no. 7, pp. 1803–1813, 2015.

[219] A. von Delft *et al.*, “The generation of a simian adenoviral vectored HCV vaccine encoding genetically conserved gene segments to target multiple HCV genotypes.,” *Vaccine*, vol. 36, no. 2, pp. 313–321, Jan. 2018.

[220] M. D. J. Dicks *et al.*, “A novel chimpanzee adenovirus vector with low human seroprevalence: improved systems for vector derivation and comparative immunogenicity.,” *PLoS One*, vol. 7, no. 7, p. e40385, 2012.

[221] R. D. Antrobus *et al.*, “Clinical assessment of a novel recombinant simian adenovirus ChAdOx1 as a vectored vaccine expressing conserved Influenza A antigens.,” *Mol. Ther.*, vol. 22, no. 3, pp. 668–674, Mar. 2014.

[222] S. Awate, L. A. Babiuk, and G. Mutwiri, “Mechanisms of action of adjuvants,” *Front. Immunol.*, vol. 4, p. 114, May 2013.

[223] A. Milicic, C. S. Rollier, C. K. Tang, R. Longley, A. V. S. Hill, and A. Reyes-Sandoval, “Adjuvanting a viral vectored vaccine against pre-erythrocytic malaria,” *Sci. Rep.*, vol. 7, no. 1, p. 7284, 2017.

[224] G. M. Warimwe *et al.*, “Immunogenicity and efficacy of a chimpanzee adenovirus-vectored Rift Valley fever vaccine in mice.,” *Virol. J.*, vol. 10, no. 1, p. 349, Jan. 2013.

[225] G. M. Warimwe *et al.*, “Chimpanzee Adenovirus Vaccine Provides Multispecies Protection against Rift Valley Fever,” *Sci. Rep.*, vol. 6, p. 20617, Feb. 2016.

[226] M. Li, Y. Jiang, T. Gong, Z. Zhang, and X. Sun, “Intranasal Vaccination against HIV-1 with Adenoviral Vector-Based Nanocomplex Using Synthetic TLR-4 Agonist Peptide as Adjuvant.,” *Mol. Pharm.*, vol. 13, no. 3, pp. 885–894, Mar. 2016.

[227] S. Gogev, K. de Fays, M.-F. Versali, S. Gautier, and E. Thiry, “Glycol chitosan improves the efficacy of intranasally administrated replication

defective human adenovirus type 5 expressing glycoprotein D of bovine herpesvirus 1.," *Vaccine*, vol. 22, no. 15–16, pp. 1946–1953, May 2004.

[228] H. Ç. Arca, M. Günbeyaz, and S. Şenel, "Chitosan-based systems for the delivery of vaccine antigens," *Expert Rev. Vaccines*, vol. 8, no. 7, pp. 937–953, Jul. 2009.

[229] A. J. Spencer *et al.*, "Enhanced Vaccine-Induced CD8+ T Cell Responses to Malaria Antigen ME-TRAP by Fusion to MHC Class II Invariant Chain," *PLoS One*, vol. 9, no. 6, p. e100538, Jun. 2014.

[230] S. Capone *et al.*, "Fusion of HCV Nonstructural Antigen to MHC Class II–associated Invariant Chain Enhances T-cell Responses Induced by Vectored Vaccines in Nonhuman Primates," *Mol. Ther.*, vol. 22, no. 5, pp. 1039–1047, May 2014.

[231] N. Koch, A. D. McLellan, and J. Neumann, "A revised model for invariant chain-mediated assembly of MHC class II peptide receptors.," *Trends Biochem. Sci.*, vol. 32, no. 12, pp. 532–537, Dec. 2007.

[232] P. A. Roche, M. S. Marks, and P. Cresswell, "Formation of a nine-subunit complex by HLA class II glycoproteins and the invariant chain.," *Nature*, vol. 354, no. 6352, pp. 392–394, Dec. 1991.

[233] J. S. Blum, P. A. Wearsch, and P. Cresswell, "Pathways of antigen processing.," *Annu. Rev. Immunol.*, vol. 31, pp. 443–473, 2013.

[234] O. J. B. Landsverk, O. Bakke, and T. F. Gregers, "MHC II and the endocytic pathway: regulation by invariant chain.," *Scand. J. Immunol.*, vol. 70, no. 3, pp. 184–193, Sep. 2009.

[235] F. Duan and P. K. Srivastava, “An invariant road to cross-presentation.,” *Nat. Immunol.*, vol. 13, no. 3, pp. 207–208, Feb. 2012.

[236] A. J. Spencer *et al.*, “4-1BBL enhances CD8+ T cell responses induced by vectored vaccines in mice but fails to improve immunogenicity in rhesus macaques,” *PLoS One*, vol. 9, no. 8, pp. e105520–e105520, Aug. 2014.

[237] S. Pauly, K. Broll, M. Wittmann, G. Giegerich, and H. Schwarz, “CD137 is expressed by follicular dendritic cells and costimulates B lymphocyte activation in germinal centers.,” *J. Leukoc. Biol.*, vol. 72, no. 1, pp. 35–42, Jul. 2002.

[238] J. L. Cannons *et al.*, “4-1BB ligand induces cell division, sustains survival, and enhances effector function of CD4 and CD8 T cells with similar efficacy.,” *J. Immunol.*, vol. 167, no. 3, pp. 1313–1324, Aug. 2001.

[239] W. W. Shuford *et al.*, “4-1BB costimulatory signals preferentially induce CD8+ T cell proliferation and lead to the amplification in vivo of cytotoxic T cell responses.,” *J. Exp. Med.*, vol. 186, no. 1, pp. 47–55, Jul. 1997.

[240] L. Diehl *et al.*, “In vivo triggering through 4-1BB enables Th-independent priming of CTL in the presence of an intact CD28 costimulatory pathway.,” *J. Immunol.*, vol. 168, no. 8, pp. 3755–3762, Apr. 2002.

[241] K. E. Pollok *et al.*, “Inducible T cell antigen 4-1BB. Analysis of expression and function.,” *J. Immunol.*, vol. 150, no. 3, pp. 771–781, Feb. 1993.

[242] S. Kanagavelu *et al.*, “HIV-1 Adenoviral Vector Vaccines Expressing Multi-Trimeric BAFF and 4-1BBL Enhance T Cell Mediated Anti-Viral Immunity,” *PLoS One*, vol. 9, no. 2, p. e90100, Feb. 2014.

[243] M. M. Seya T., Azuma M., “Pattern Recognition by Dendritic Cells and Its Application to Vaccine Adjuvant for Antitumor Immunotherapy.,” *Immunother. Cancer.*, 2016.

[244] D. M. Appledorn *et al.*, “A new adenovirus based vaccine vector expressing an Eimeria tenella derived TLR agonist improves cellular immune responses to an antigenic target.,” *PLoS One*, vol. 5, no. 3, p. e9579, Mar. 2010.

[245] D. M. Appledorn, Y. A. Aldhamen, S. Godbehere, S. S. Seregin, and A. Amalfitano, “Sublingual administration of an adenovirus serotype 5 (Ad5)-based vaccine confirms Toll-like receptor agonist activity in the oral cavity and elicits improved mucosal and systemic cell-mediated responses against HIV antigens despite preexisting Ad5 immuni,” *Clin. Vaccine Immunol.*, vol. 18, no. 1, pp. 150–160, Jan. 2011.

[246] W. Peters *et al.*, “Oral administration of an adenovirus vector encoding both an avian influenza A hemagglutinin and a TLR3 ligand induces antigen specific granzyme B and IFN-gamma T cell responses in humans.,” *Vaccine*, vol. 31, no. 13, pp. 1752–1758, Mar. 2013.

[247] K. A. Hofmeyer *et al.*, “Optimizing Immunization Strategies for the Induction of Antigen-Specific CD4 and CD8 T Cell Responses for Protection against Intracellular Parasites.,” *Clin. Vaccine Immunol.*, vol. 23, no. 9, pp. 785–794, Sep. 2016.

[248] F. S. Alyaqoub *et al.*, “In Vivo Synthesis of Cyclic-di-GMP Using a Recombinant Adenovirus Preferentially Improves Adaptive Immune Responses against Extracellular Antigens,” *J. Immunol.*, vol. 196, no. 4, p. 1741 LP-1752, Feb. 2016.

[249] M. S. Diamond *et al.*, “Type I interferon is selectively required by dendritic cells for immune rejection of tumors,” *J. Exp. Med.*, vol. 208, no. 10, pp. 1989–2003, Sep. 2011.

[250] M. Montoya *et al.*, “Type I interferons produced by dendritic cells promote their phenotypic and functional activation.,” *Blood*, vol. 99, no. 9, pp. 3263–3271, May 2002.

[251] S. Parlato *et al.*, “Expression of CCR-7, MIP-3beta, and Th-1 chemokines in type I IFN-induced monocyte-derived dendritic cells: importance for the rapid acquisition of potent migratory and functional activities.,” *Blood*, vol. 98, no. 10, pp. 3022–3029, Nov. 2001.

[252] R. M. Welsh, K. Bahl, H. D. Marshall, and S. L. Urban, “Type 1 interferons and antiviral CD8 T-cell responses,” *PLoS Pathog.*, vol. 8, no. 1, pp. e1002352–e1002352, Jan. 2012.

[253] M. Alsharifi, M. Lobigs, M. Regner, E. Lee, A. Koskinen, and A. Mullbacher, “Type I interferons trigger systemic, partial lymphocyte activation in response to viral infection.,” *J. Immunol.*, vol. 175, no. 7, pp. 4635–4640, Oct. 2005.

[254] D. Braun, I. Caramalho, and J. Demengeot, “IFN-alpha/beta enhances BCR-dependent B cell responses.,” *Int. Immunol.*, vol. 14, no. 4, pp. 411–419, Apr. 2002.

[255] A. Le Bon *et al.*, “Cutting edge: enhancement of antibody responses through direct stimulation of B and T cells by type I IFN.,” *J. Immunol.*, vol. 176, no. 4, pp. 2074–2078, Feb. 2006.

[256] D. M. Alejo *et al.*, “An adenovirus vectored mucosal adjuvant augments protection of mice immunized intranasally with an adenovirus-vectored foot-and-mouth disease virus subunit vaccine,” *Vaccine*, vol. 31, no. 18, pp. 2302–2309, 2013.

[257] D. Drane and M. J. Pearse, “12 - The ISCOMATRIX™ adjuvant,” V. E. J. C. Schijns and D. T. B. T.-I. in M. V. O’Hagan, Eds. London: Academic Press, 2006, pp. 191–215.

[258] Invivogen, “AddaVax.” [Online]. Available: https://www.invivogen.com/sites/default/files/invivogen/old/PDF/AddaVax.pdf. [Accessed: 16-Jul-2019].

[259] C. Wang *et al.*, “A simian-adenovirus-vectored rabies vaccine suitable for thermostabilisation and clinical development for low-cost single-dose pre-exposure prophylaxis,” *PLoS Negl. Trop. Dis.*, vol. 12, no. 10, p. e0006870, Oct. 2018.

[260] R. Bodewes *et al.*, “A Single Immunization with CoVaccine HT-Adjuvanted H5N1 Influenza Virus Vaccine Induces Protective Cellular and Humoral Immune Responses in Ferrets,” *J. Virol.*, vol. 84, no. 16, p. 7943 LP-7952, Aug. 2010.

[261] N. Lakhan, N. E. Stevens, K. R. Diener, and J. D. Hayball, “CoVaccine HT™ adjuvant is superior to Freund’s adjuvants in eliciting antibodies against the endogenous alarmin HMGB1,” *J. Immunol. Methods*, vol. 439, pp. 37–43, 2016.

[262] S. Ascarateil, A. Puget, and M.-E. Koziol, “Safety data of Montanide ISA 51 VG and Montanide ISA 720 VG, two adjuvants dedicated to human

therapeutic vaccines," *J. Immunother. Cancer*, vol. 3, no. Suppl 2, pp. P428–P428, Nov. 2015.

[263] C. Cubillos-Zapata *et al.*, "Differential Effects of Viral Vectors on Migratory Afferent Lymph Dendritic Cells In Vitro Predict Enhanced Immunogenicity In Vivo," *J. Virol.*, vol. 85, no. 18, p. 9385 LP-9394, Sep. 2011.

[264] E. Guzman, G. Taylor, J. Hope, R. Herbert, C. Cubillos-Zapata, and B. Charleston, "Transduction of skin-migrating dendritic cells by human adenovirus 5 occurs via an actin-dependent phagocytic pathway.," *J. Gen. Virol.*, vol. 97, no. 10, pp. 2703–2718, Oct. 2016.

[265] V. B. Joshi, S. M. Geary, and A. K. Salem, "Biodegradable particles as vaccine antigen delivery systems for stimulating cellular immune responses," *Hum. Vaccin. Immunother.*, vol. 9, no. 12, pp. 2584–2590, Dec. 2013.

[266] X. Li, Man; Juang, Yuhong; Gong, Tao; Zhang, Zhirong; Sun, "Intranasal Vaccination against HIV-1 with Adenoviral Vector-Based Nanocomplex Using Synthetic TLR-4 Agonist Peptide as Adjuvant," *Mol. Pharmecutics*, vol. 13, no. 3, pp. 885–894, 2016.

[267] T. Jiang, Yuhong; Li, Man, Zhang, Zhiroing, Gong, "Enhancement of Nasal HIV Vaccination with Adenoviral Vector-Based Nanocomplexes Using Mucoadhesive and DC-Targeting Adjuvants," *Pharm. Res.*, vol. 31, no. 10, pp. 2748–2761, 2014.

[268] T. N. Bullock, "TNF-receptor superfamily agonists as molecular adjuvants for cancer vaccines.," *Curr. Opin. Immunol.*, vol. 47, pp. 70–77, Aug. 2017.

[269] T. K. Sixma *et al.*, “Refined structure of Escherichia coli heat-labile enterotoxin, a close relative of cholera toxin.,” *J. Mol. Biol.*, vol. 230, no. 3, pp. 890–918, Apr. 1993.

[270] E. B. Norton, L. B. Lawson, Z. Mahdi, L. C. Freytag, and J. D. Clements, “The A Subunit of Escherichia coli Heat-Labile Enterotoxin Functions as a Mucosal Adjuvant and Promotes IgG2a, IgA, and Th17 Responses to Vaccine Antigens,” *Infect. Immun.*, vol. 80, no. 7, p. 2426 LP-2435, Jul. 2012.

[271] B. R. Halbroth *et al.*, “Development of a Molecular Adjuvant to Enhance Antigen-Specific CD8+ T Cell Responses,” *Sci. Rep.*, vol. 8, no. 1, p. 15020, 2018.

[272] P. J. Holst, M. R. Sorensen, C. M. Mandrup Jensen, C. Orskov, A. R. Thomsen, and J. P. Christensen, “MHC class II-associated invariant chain linkage of antigen dramatically improves cell-mediated immunity induced by adenovirus vaccines.,” *J. Immunol.*, vol. 180, no. 5, pp. 3339–3346, Mar. 2008.

[273] S. Jensen, M. A. Steffensen, B. A. H. Jensen, D. Schluter, J. P. Christensen, and A. R. Thomsen, “Adenovirus-based vaccine against Listeria monocytogenes: extending the concept of invariant chain linkage.,” *J. Immunol.*, vol. 191, no. 8, pp. 4152–4164, Oct. 2013.

[274] S. M. Blaauboer, S. Mansouri, H. R. Tucker, H. L. Wang, V. D. Gabrielle, and L. Jin, “The mucosal adjuvant cyclic di-GMP enhances antigen uptake and selectively activates pinocytosis-efficient cells in vivo,” *Elife*, vol. 4, p.

e06670, Apr. 2015.

[275] T. Ebensen, K. Schulze, P. Riese, C. Link, M. Morr, and C. A. Guzman, “The bacterial second messenger cyclic diGMP exhibits potent adjuvant properties.,” *Vaccine*, vol. 25, no. 8, pp. 1464–1469, Feb. 2007.

[276] E. K. Forbes *et al.*, “T Cell Responses Induced by Adenoviral Vectored Vaccines Can Be Adjuvanted by Fusion of Antigen to the Oligomerization Domain of C4b-Binding Protein,” *PLoS One*, vol. 7, no. 9, p. e44943, Sep. 2012.

[277] J. K. Dowling and A. Mansell, “Toll-like receptors: the swiss army knife of immunity and vaccine development,” *Clin. Transl. Immunol.*, vol. 5, no. 5, pp. e85–e85, May 2016.

[278] E. Lebedeva *et al.*, “The differences in immunoadjuvant mechanisms of TLR3 and TLR4 agonists on the level of antigen-presenting cells during immunization with recombinant adenovirus vector,” *BMC Immunol.*, vol. 19, no. 1, p. 26, Jul. 2018.

[279] P. Romero, J. L. Maryanski, G. Corradin, R. S. Nussenzweig, V. Nussenzweig, and F. Zavala, “Cloned cytotoxic T cells recognize an epitope in the circumsporozoite protein and protect against malaria.,” *Nature*, vol. 341, no. 6240, pp. 323–326, Sep. 1989.

[280] K. C. Larsen *et al.*, “Expression of tak1 and tram induces synergistic pro-inflammatory signalling and adjuvants DNA vaccines,” *Vaccine*, vol. 27, no. 41, pp. 5589–5598, 2009.

[281] D. H. Barouch *et al.*, “Immunogenicity of Recombinant Adenovirus Serotype

35 Vaccine in the Presence of Pre-Existing Anti-Ad5 Immunity," *J. Immunol.*, vol. 172, no. 10, p. 6290 LP-6297, May 2004.

[282] A. Gambotto *et al.*, "Immunogenicity of enhanced green fluorescent protein (EGFP) in BALB/c mice: identification of an H2-Kd-restricted CTL epitope.," *Gene Ther.*, vol. 7, no. 23, pp. 2036–2040, Dec. 2000.

[283] L. Sun, J. Wu, F. Du, X. Chen, and Z. J. Chen, "Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway," *Science (80-.).*, vol. 339, 2013.

[284] N. K. Alharbi *et al.*, "Enhancing cellular immunogenicity of MVA-vectored vaccines by utilizing the F11L endogenous promoter.," *Vaccine*, vol. 34, no. 1, pp. 49–55, Jan. 2016.

[285] M. G. Cottingham *et al.*, "Preventing spontaneous genetic rearrangements in the transgene cassettes of adenovirus vectors," *Biotechnol. Bioeng.*, vol. 109, no. 3, pp. 719–728, Mar. 2012.

[286] D. J. Puleston *et al.*, "Autophagy is a critical regulator of memory CD8+ T cell formation," *Elife*, vol. 3, p. e03706, 2014.

[287] J.-D. Sauer *et al.*, "The N-ethyl-N-nitrosourea-induced Goldenticket mouse mutant reveals an essential function of Sting in the in vivo interferon response to Listeria monocytogenes and cyclic dinucleotides.," *Infect. Immun.*, vol. 79, no. 2, pp. 688–694, Feb. 2011.

[288] S. J. Draper *et al.*, "Effective induction of high-titer antibodies by viral vector vaccines.," *Nat. Med.*, vol. 14, no. 8, pp. 819–821, Aug. 2008.

[289] M. G. Cottingham *et al.*, "Recombination-mediated genetic engineering of a

bacterial artificial chromosome clone of modified vaccinia virus Ankara (MVA).," *PLoS One*, vol. 3, no. 2, p. e1638, 2008.

[290] C. Parra-Lopez *et al.*, "Major histocompatibility complex and T cell interactions of a universal T cell epitope from Plasmodium falciparum circumsporozoite protein.," *J. Biol. Chem.*, vol. 281, no. 21, pp. 14907–14917, May 2006.

[291] K. Grehan, F. Ferrara, and N. Temperton, "An optimised method for the production of MERS-CoV spike expressing viral pseudotypes.," *MethodsX*, vol. 2, pp. 379–384, 2015.

[292] M. D. J. Dicks *et al.*, "Differential immunogenicity between HAdV-5 and chimpanzee adenovirus vector ChAdOx1 is independent of fiber and penton RGD loop sequences in mice," *Sci. Rep.*, vol. 5, p. 16756, Nov. 2015.

[293] J. Lessler *et al.*, "Estimating the Severity and Subclinical Burden of Middle East Respiratory Syndrome Coronavirus Infection in the Kingdom of Saudi Arabia.," *Am. J. Epidemiol.*, vol. 183, no. 7, pp. 657–663, Apr. 2016.

[294] A. N. Alshukairi *et al.*, "High Prevalence of MERS-CoV Infection in Camel Workers in Saudi Arabia," *MBio*, vol. 9, no. 5, pp. e01985-18, Nov. 2018.

[295] B. Meyer, C. Drosten, and M. A. Muller, "Serological assays for emerging coronaviruses: Challenges and pitfalls," *Virus Res*, vol. 194, 2014.

[296] C. B. Reusken, B. L. Haagmans, M. A. Muller, C. Gutierrez, G. J. Godeke, and B. Meyer, "Middle East respiratory syndrome coronavirus neutralising serum antibodies in dromedary camels: a comparative serological study," *Lancet Infect Dis*, vol. 13, 2013.

[297] M. G. Hemida, R. A. Perera, P. Wang, M. A. Alhammadi, L. Y. Siu, and M. Li, “Middle East Respiratory Syndrome (MERS) coronavirus seroprevalence in domestic livestock in Saudi Arabia, 2010 to 2013,” *Euro Surveill*, vol. 18, 2013.

[298] N. K. Alharbi, “Vaccines against Middle East respiratory syndrome coronavirus for humans and camels.,” *Rev. Med. Virol.*, vol. 27, no. 2, Mar. 2017.

[299] S. J. Draper and J. L. Heeney, “Viruses as vaccine vectors for infectious diseases and cancer,” *Nat Rev Micro*, vol. 8, no. 1, pp. 62–73, Jan. 2010.

[300] S. C. Gilbert, “Clinical development of Modified Vaccinia virus Ankara vaccines.,” *Vaccine*, vol. 31, no. 39, pp. 4241–4246, Sep. 2013.

[301] Z. Qian, S. R. Dominguez, and K. V Holmes, “Role of the Spike Glycoprotein of Human Middle East Respiratory Syndrome Coronavirus (MERS-CoV) in Virus Entry and Syncytia Formation,” *PLoS One*, vol. 8, no. 10, p. e76469, Oct. 2013.

[302] et al Kapp K, Schrempf S, Lemberg MK, “Targeting Functions of Signal Peptides,” *Austin (TX): Landes Bioscience*. [Online]. Available: https://www.ncbi.nlm.nih.gov/books/NBK6322/.

[303] M. Paetzel, A. Karla, N. C. J. Strynadka, and R. E. Dalbey, “Signal peptidases.,” *Chem. Rev.*, vol. 102, no. 12, pp. 4549–4580, Dec. 2002.

[304] G. von Heijne, “Signal sequences. The limits of variation.,” *J. Mol. Biol.*, vol. 184, no. 1, pp. 99–105, Jul. 1985.

[305] A. Wallace, K. West, A. L. Rothman, F. A. Ennis, S. Lu, and S. Wang, “Post-

translational intracellular trafficking determines the type of immune response elicited by DNA vaccines expressing Gag antigen of Human Immunodeficiency Virus Type 1 (HIV-1).," *Hum. Vaccin. Immunother.*, vol. 9, no. 10, pp. 2095–2102, Oct. 2013.

[306] M. Luo, P. Tao, J. Li, S. Zhou, D. Guo, and Z. Pan, "Immunization with plasmid DNA encoding influenza A virus nucleoprotein fused to a tissue plasminogen activator signal sequence elicits strong immune responses and protection against H5N1 challenge in mice.," *J. Virol. Methods*, vol. 154, no. 1–2, pp. 121–127, Dec. 2008.

[307] G. Delogu, A. Li, C. Repique, F. Collins, and S. L. Morris, "DNA Vaccine Combinations Expressing Either Tissue Plasminogen Activator Signal Sequence Fusion Proteins or Ubiquitin-Conjugated Antigens Induce Sustained Protective Immunity in a Mouse Model of Pulmonary Tuberculosis," *Infect. Immun.*, vol. 70, no. 1, p. 292 LP-302, Jan. 2002.

[308] Y. Kou *et al.*, "Tissue plasminogen activator (tPA) signal sequence enhances immunogenicity of MVA-based vaccine against tuberculosis.," *Immunol. Lett.*, vol. 190, pp. 51–57, Oct. 2017.

[309] D. Haddad *et al.*, "Comparative study of DNA-based immunization vectors: effect of secretion signals on the antibody responses in mice.," *FEMS Immunol. Med. Microbiol.*, vol. 18, no. 3, pp. 193–202, Jul. 1997.

[310] N. K. Alharbi *et al.*, "Enhancing cellular immunogenicity of MVA-vectored vaccines by utilizing the F11L endogenous promoter," *Vaccine*, vol. 34, no. 1, pp. 49–55, Jan. 2016.

[311] T. Orubu, N. K. Alharbi, T. Lambe, S. C. Gilbert, and M. G. Cottingham,

"Expression and Cellular Immunogenicity of a Transgenic Antigen Driven by Endogenous Poxviral Early Promoters at Their Authentic Loci in MVA," *PLoS One*, vol. 7, no. 6, p. e40167, Jun. 2012.

[312] M. E. Perkus, K. Limbach, and E. Paoletti, "Cloning and expression of foreign genes in vaccinia virus, using a host range selection system," *J. Virol.*, vol. 63, no. 9, pp. 3829–3836, Sep. 1989.

[313] F. G. Falkner and B. Moss, "Transient dominant selection of recombinant vaccinia viruses.," *J. Virol.*, vol. 64, no. 6, pp. 3108–3111, Jun. 1990.

[314] A. Algaissi and A. M. Hashem, "Evaluation of MERS-CoV Neutralizing Antibodies in Sera Using Live Virus Microneutralization Assay.," *Methods Mol. Biol.*, vol. 2099, pp. 107–116, 2020.

[315] A. Reyes-Sandoval *et al.*, "Prime-Boost Immunization with Adenoviral and Modified Vaccinia Virus Ankara Vectors Enhances the Durability and Polyfunctionality of Protective Malaria CD8+ T-Cell Responses ," *Infect. Immun.* , vol. 78, no. 1, pp. 145–153.

[316] Y. Caì *et al.*, "CD26/DPP4 cell-surface expression in bat cells correlates with bat cell susceptibility to Middle East respiratory syndrome coronavirus (MERS-CoV) infection and evolution of persistent infection," *PLoS One*, vol. 9, no. 11, pp. e112060–e112060, Nov. 2014.

[317] ClinicalTrials.gov, "Safety and Immunogenicity of a Candidate MERS-CoV Vaccine (MERS001)," 2018. [Online]. Available: https://clinicaltrials.gov/ct2/show/NCT03399578. [Accessed: 17-Mar-2019].

[318] C. S. Rollier, A. Reyes-Sandoval, M. G. Cottingham, K. Ewer, and A. V. S.

Hill, “Viral vectors as vaccine platforms: deployment in sight,” *Curr. Opin. Immunol.*, vol. 23, no. 3, pp. 377–382, 2011.

[319] S. E. Magnusson *et al.*, “Matrix-M™ adjuvant enhances immunogenicity of both protein- and modified vaccinia virus Ankara-based influenza vaccines in mice,” *Immunol. Res.*, vol. 66, no. 2, pp. 224–233, 2018.

[320] N. W. Schmidt *et al.*, “Memory CD8 T cell responses exceeding a large but definable threshold provide long-term immunity to malaria.,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 105, no. 37, pp. 14017–14022, Sep. 2008.

[321] S. Bardócz *et al.*, “The importance of dietary polyamines in cell regeneration and growth,” *Br. J. Nutr.*, vol. 73, no. 6, pp. 819–828, 1995.

[322] K. Igarashi and K. Kashiwagi, “Modulation of cellular function by polyamines,” *Int. J. Biochem. Cell Biol.*, vol. 42, no. 1, pp. 39–51, 2010.

[323] A. E. Pegg, “Functions of Polyamines in Mammals.,” *J. Biol. Chem.*, vol. 291, no. 29, pp. 14904–14912, Jul. 2016.

[324] F. Madeo, M. A. Bauer, D. Carmona-Gutierrez, and G. Kroemer, “Spermidine: a physiological autophagy inducer acting as an anti-aging vitamin in humans?,” *Autophagy*, vol. 15, no. 1, pp. 165–168, Jan. 2019.

[325] T. Eisenberg, S. Büttner, G. Kroemer, and F. Madeo, “The mitochondrial pathway in yeast apoptosis,” *Apoptosis*, vol. 12, no. 5, pp. 1011–1023, 2007.

[326] T. Eisenberg *et al.*, “Cardioprotection and lifespan extension by the natural polyamine spermidine,” *Nat. Med.*, vol. 22, no. 12, pp. 1428–1438, Dec. 2016.

[327] T. Eisenberg *et al.*, “Induction of autophagy by spermidine promotes longevity,” *Nat. Cell Biol.*, vol. 11, no. 11, pp. 1305–1314, 2009.

[328] F. Yue *et al.*, “Spermidine Prolongs Lifespan and Prevents Liver Fibrosis and Hepatocellular Carcinoma by Activating MAP1S-Mediated Autophagy,” *Cancer Res.*, vol. 77, no. 11, p. 2938 LP-2951, Jun. 2017.

[329] Q. Yang *et al.*, “Spermidine alleviates experimental autoimmune encephalomyelitis through inducing inhibitory macrophages,” *Cell Death Differ.*, vol. 23, no. 11, pp. 1850–1861, 2016.

[330] I.-F. Wang *et al.*, “Autophagy activators rescue and alleviate pathogenesis of a mouse model with proteinopathies of the TAR DNA-binding protein 43,” *Proc. Natl. Acad. Sci.*, vol. 109, no. 37, p. 15024 LP-15029, Sep. 2012.

[331] V. K. Gupta *et al.*, “Restoring polyamines protects from age-induced memory impairment in an autophagy-dependent manner,” *Nat. Neurosci.*, vol. 16, no. 10, pp. 1453–1460, 2013.

[332] Z. Yin, C. Pascual, and D. J. Klionsky, “Autophagy: machinery and regulation.,” *Microb. cell (Graz, Austria)*, vol. 3, no. 12, pp. 588–596, Dec. 2016.

[333] D. Carmona-Gutierrez, A. L. Hughes, F. Madeo, and C. Ruckenstuhl, “The crucial impact of lysosomes in aging and longevity.,” *Ageing Res. Rev.*, vol. 32, pp. 2–12, Dec. 2016.

[334] D. C. Rubinsztein, G. Marino, and G. Kroemer, “Autophagy and aging.,” *Cell*, vol. 146, no. 5, pp. 682–695, Sep. 2011.

[335] R. A. Frake, T. Ricketts, F. M. Menzies, and D. C. Rubinsztein, “Autophagy

and neurodegeneration.," *J. Clin. Invest.*, vol. 125, no. 1, pp. 65–74, Jan. 2015.

[336] A. M. K. Choi, S. W. Ryter, and B. Levine, "Autophagy in human health and disease.," *N. Engl. J. Med.*, vol. 368, no. 7, pp. 651–662, Feb. 2013.

[337] C. Zhang and A. M. Cuervo, "Restoration of chaperone-mediated autophagy in aging liver improves cellular maintenance and hepatic function.," *Nat. Med.*, vol. 14, no. 9, pp. 959–965, Sep. 2008.

[338] F. Pietrocola *et al.*, "Spermidine induces autophagy by inhibiting the acetyltransferase EP300.," *Cell Death Differ.*, vol. 22, no. 3, pp. 509–516, Mar. 2015.

[339] G. Marino *et al.*, "Regulation of autophagy by cytosolic acetyl-coenzyme A.," *Mol. Cell*, vol. 53, no. 5, pp. 710–725, Mar. 2014.

[340] F. Madeo, T. Eisenberg, F. Pietrocola, and G. Kroemer, "Spermidine in health and disease," *Science (80-.).*, vol. 359, no. 6374, p. eaan2788, Jan. 2018.

[341] P. K. Sacitharan, S. Lwin, G. B. Gharios, and J. R. Edwards, "Spermidine restores dysregulated autophagy and polyamine synthesis in aged and osteoarthritic chondrocytes via EP300," *Exp. Mol. Med.*, vol. 50, no. 9, p. 123, 2018.

[342] M. Chrisam *et al.*, "Reactivation of autophagy by spermidine ameliorates the myopathic defects of collagen VI-null mice," *Autophagy*, vol. 11, no. 12, pp. 2142–2152, Nov. 2015.

[343] A. Amalfitano, M. A. Hauser, H. Hu, D. Serra, C. R. Begy, and J. S.

Chamberlain, “Production and characterization of improved adenovirus vectors with the E1, E2b, and E3 genes deleted.,” *J. Virol.*, vol. 72, no. 2, pp. 926–933, Feb. 1998.

[344] M. Plebanski *et al.*, “Protection from Plasmodium berghei infection by priming and boosting T cells to a single class I-restricted epitope with recombinant carriers suitable for human use,” *Eur. J. Immunol.*, vol. 28, no. 12, pp. 4345–4355, Dec. 1998.

[345] C. L. Hutchings, A. J. Birkett, A. C. Moore, and A. V. S. Hill, “Combination of protein and viral vaccines induces potent cellular and humoral immune responses and enhanced protection from murine malaria challenge.,” *Infect. Immun.*, vol. 75, no. 12, pp. 5819–5826, Dec. 2007.

[346] A. Reyes-Sandoval, J. T. Harty, and S. M. Todryk, “Viral vector vaccines make memory T cells against malaria,” *Immunology*, vol. 121, no. 2. Blackwell Science Inc, pp. 158–165, Jun-2007.

[347] A. Reyes-Sandoval *et al.*, “Single-dose immunogenicity and protective efficacy of simian adenoviral vectors against Plasmodium berghei.,” *Eur. J. Immunol.*, vol. 38, no. 3, pp. 732–741, Mar. 2008.

[348] C. S. Rollier, A. V. S. Hill, and A. Reyes-Sandoval, “Influence of adenovirus and MVA vaccines on the breadth and hierarchy of T cell responses,” *Vaccine*, vol. 34, no. 38, pp. 4470–4474, Aug. 2016.

[349] H. Zhang *et al.*, “Translational control of TFEB and autophagy via eIF5A rejuvenates B cell immunity,” *bioRxiv*, p. 360503, Jan. 2018.

[350] P. K. Sacitharan, S. Lwin, G. B. Gharios, and J. R. Edwards, “Spermidine

restores dysregulated autophagy and polyamine synthesis in aged and osteoarthritic chondrocytes via EP300," *Exp. Mol. Med.*, vol. 50, no. 9, p. 123, 2018.

[351] Q. Chen, L. Sun, and Z. J. Chen, "Regulation and function of the cGAS–STING pathway of cytosolic DNA sensing," *Nat. Immunol.*, vol. 17, p. 1142, Sep. 2016.

[352] J. Tao, X. Zhou, and Z. Jiang, "cGAS-cGAMP-STING: The three musketeers of cytosolic DNA sensing and signaling.," *IUBMB Life*, vol. 68, no. 11, pp. 858–870, Nov. 2016.

[353] J. Wang, P. Li, and M. X. Wu, "Natural STING Agonist as an 'Ideal' Adjuvant for Cutaneous Vaccination.," *J. Invest. Dermatol.*, vol. 136, no. 11, pp. 2183–2191, Nov. 2016.

[354] X.-D. Li, J. Wu, D. Gao, H. Wang, L. Sun, and Z. J. Chen, "Pivotal roles of cGAS-cGAMP signaling in antiviral defense and immune adjuvant effects.," *Science*, vol. 341, no. 6152, pp. 1390–1394, Sep. 2013.

[355] I. Skrnjug, C. A. Guzman, and C. Rueckert, "Cyclic GMP-AMP displays mucosal adjuvant activity in mice.," *PLoS One*, vol. 9, no. 10, p. e110150, 2014.

[356] H. Wang *et al.*, "cGAS is essential for the antitumor effect of immune checkpoint blockade.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 114, no. 7, pp. 1637–1642, Feb. 2017.

[357] T. Li *et al.*, "Antitumor Activity of cGAMP via Stimulation of cGAS-cGAMP-STING-IRF3 Mediated Innate Immune Response," *Sci. Rep.*, vol. 6, p.

19049, Jan. 2016.

[358] A. Gutjahr *et al.*, “The STING ligand cGAMP potentiates the efficacy of vaccine-induced CD8+ T cells.,” *JCI insight*, vol. 4, no. 7, Apr. 2019.

[359] O. Denis *et al.*, “Vaccination with plasmid DNA encoding mycobacterial antigen 85A stimulates a CD4+ and CD8+ T-cell epitopic repertoire broader than that stimulated by Mycobacterium tuberculosis H37Rv infection.,” *Infect. Immun.*, vol. 66, no. 4, pp. 1527–1533, Apr. 1998.

[360] H. McShane, S. Behboudi, N. Goonetilleke, R. Brookes, and A. V. S. Hill, “Protective immunity against Mycobacterium tuberculosis induced by dendritic cells pulsed with both CD8(+)- and CD4(+)-T-cell epitopes from antigen 85A.,” *Infect. Immun.*, vol. 70, no. 3, pp. 1623–1626, Mar. 2002.

[361] O. Kalyuzhniy *et al.*, “Adenovirus serotype 5 hexon is critical for virus infection of hepatocytes in vivo,” *Proc. Natl. Acad. Sci.*, vol. 105, no. 14, p. 5483 LP-5488, Apr. 2008.

[362] L. Li *et al.*, “Hydrolysis of 2’3’-cGAMP by ENPP1 and design of nonhydrolyzable analogs,” *Nat. Chem. Biol.*, vol. 10, no. 12, pp. 1043–1048, Dec. 2014.

[363] R. Geiben-Lynn, J. R. Greenland, K. Frimpong-Boateng, and N. L. Letvin, “Kinetics of recombinant adenovirus type 5, vaccinia virus, modified vaccinia ankara virus, and DNA antigen expression in vivo and the induction of memory T-lymphocyte responses,” *Clin. Vaccine Immunol.*, vol. 15, no. 4, pp. 691–696, Apr. 2008.

[364] R. Schirmbeck, J. Reimann, S. Kochanek, and F. Kreppel, “The

Immunogenicity of Adenovirus Vectors Limits the Multispecificity of CD8 T-cell Responses to Vector-encoded Transgenic Antigens," *Mol. Ther.*, vol. 16, no. 9, pp. 1609–1616, Sep. 2008.

[365] U. Frevert, A. Moreno, J. M. Calvo-Calle, C. Klotz, and E. Nardin, "Imaging effector functions of human cytotoxic CD4+ T cells specific for Plasmodium falciparum circumsporozoite protein.," *Int. J. Parasitol.*, vol. 39, no. 1, pp. 119–132, Jan. 2009.

[366] J. B. Carey, A. Vrdoljak, C. O'Mahony, A. V. S. Hill, S. J. Draper, and A. C. Moore, "Microneedle-mediated immunization of an adenovirus-based malaria vaccine enhances antigen-specific antibody immunity and reduces anti-vector responses compared to the intradermal route," *Sci. Rep.*, vol. 4, p. 6154, Aug. 2014.

[367] K. M. Quinn *et al.*, "Antigen expression determines adenoviral vaccine potency independent of IFN and STING signaling," *J. Clin. Invest.*, vol. 125, no. 3, pp. 1129–1146, Mar. 2015.

[368] J. Gao *et al.*, "Identification and characterization of phosphodiesterases that specifically degrade 3'3'-cyclic GMP-AMP.," *Cell Res.*, vol. 25, no. 5, pp. 539–550, May 2015.

[369] InvivoGen, "2'3'-cGAMP Control," 2018. [Online]. Available: https://www.invivogen.com/23cgamp-control#details. [Accessed: 19-Feb-2019].

[370] E. Huarte *et al.*, "Recombinant Adenoviral Vectors Turn on the Type I Interferon System without Inhibition of Transgene Expression and Viral Replication," *Mol. Ther.*, vol. 14, no. 1, pp. 129–138, 2006.

[371] F. Guo *et al.*, “Activation of Stimulator of Interferon Genes in Hepatocytes Suppresses the Replication of Hepatitis B Virus,” *Antimicrob. Agents Chemother.*, vol. 61, no. 10, pp. e00771-17, Oct. 2017.

[372] C.-H. A. Tang *et al.*, “Agonist-Mediated Activation of STING Induces Apoptosis in Malignant B Cells,” *Cancer Res.*, vol. 76, no. 8, pp. 2137–2152, Apr. 2016.

[373] D. Anghelina, E. Lam, and E. Falck-Pedersen, “Diminished Innate Antiviral Response to Adenovirus Vectors in cGAS/STING-Deficient Mice Minimally Impacts Adaptive Immunity,” *J. Virol.* , vol. 90, no. 13, pp. 5915–5927, Jul. 2016.

[374] K. Sellins, L. Fradkin, D. Liggitt, and S. Dow, “Type I interferons potently suppress gene expression following gene delivery using liposome(-)DNA complexes.,” *Mol. Ther.*, vol. 12, no. 3, pp. 451–459, Sep. 2005.

[375] P. Härle *et al.*, “Differential effect of murine alpha/beta interferon transgenes on antagonization of herpes simplex virus type 1 replication,” *J. Virol.*, vol. 76, no. 13, pp. 6558–6567, Jul. 2002.

[376] S. L. Day, I. A. Ramshaw, A. J. Ramsay, and C. Ranasinghe, “Differential Effects of the Type I Interferons α₄, β, and ε on Antiviral Activity and Vaccine Efficacy,” *J. Immunol.*, vol. 180, no. 11, p. 7158 LP-7166, Jun. 2008.

[377] N. van Doremalen *et al.*, “A single-dose ChAdOx1-vectored vaccine provides complete protection against Nipah Bangladesh and Malaysia in Syrian golden hamsters,” *PLoS Negl. Trop. Dis.*, vol. 13, no. 6, p. e0007462, Jun. 2019.

[378] C. M. Tully *et al.*, “Novel Bivalent Viral-Vectored Vaccines Induce Potent Humoral and Cellular Immune Responses Conferring Protection against Stringent Influenza A Virus Challenge,” *J. Immunol.*, vol. 199, no. 4, p. 1333 LP-1341, Aug. 2017.

[379] C. López-Camacho *et al.*, “A Zika vaccine based on chimpanzee adenovirus ChAdOx1 elicits lineage-transcending sterile immunity and prevents colonisation of brain and ovaries,” *bioRxiv*, p. 514877, Jan. 2019.

[380] E. Stylianou *et al.*, “Identification and Evaluation of Novel Protective Antigens for the Development of a Candidate Tuberculosis Subunit Vaccine,” *Infect. Immun.*, vol. 86, no. 7, pp. e00014-18, Jul. 2018.

[381] V. J. Munster *et al.*, “Protective efficacy of a novel simian adenovirus vaccine against lethal MERS-CoV challenge in a transgenic human DPP4 mouse model,” *npj Vaccines*, vol. 2, no. 1, p. 28, 2017.

[382] H.-S. Shin *et al.*, “Immune Responses to Middle East Respiratory Syndrome Coronavirus During the Acute and Convalescent Phases of Human Infection,” *Clin. Infect. Dis.*, vol. 68, no. 6, pp. 984–992, Jul. 2018.

[383] J. Zhao *et al.*, “Recovery from the Middle East respiratory syndrome is associated with antibody and T cell responses,” *Sci. Immunol.*, vol. 2, no. 14, p. eaan5393, Aug. 2017.