
Harnessing the innate immune system for improved VLP-based immunotherapy



Ariane Karla Cruz Gomes
Kellogg College
University of Oxford

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“I must constantly choose among competing and apparently incommensurable goods [...]; - and accept- that an event that simply happens to me may, without my consent, alter my life; that it is equally problematic to entrust one’s good to friends, lovers, or country and try to have a good life without them—all these I take to be not just the material of tragedy, but everyday facts of lived practical reason.”

Alec Nevala-Lee

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List of Abbreviations

A

APCs – Antigen Presenting Cells

AMP – Adenosine Monophosphate

B

BCR – B cell receptor

BMDC – Bone Marrow derived conventional Dendritic cells

BMpDCs – Bone Marrow derived plasmocytic Dendritic cells

BP – Biological processes

C

CAR-T – Chimeric antigen receptor T cell

CC – Cellular compartmen

cGAS – Cyclic GMP-AMP synthase

CpG – 20-deoxyribo (cytidine-phosphateguanosine)

CPM-MM – Counts per Million Multi mapped

CTL – Cytotoxic T lymphocytes

D

DAMPs – Danger Associated Molecular Patterns

DCs – Dendritic Cells

DNA - Deoxyribonucleotides

DEG – Differently Expressed Genes

DLS – Dynamic Light Scattering

dsRNA – double strand

DTT - 1,4-dithiothreitol

DX – differently expressed genes

E

ELISA – Enzyme-Linked Immunosorbent Assay

EM – Electronic microscopy

EU – Endotoxin units

E7 – Envelope Protein 7 (derived from HPV)

F

FC – Fold change

FDR – False Discovery Rate

G

GMP – Good Manufacturing Practices

cGMP – Cyclic Guanosine Monophosphate

GO – Gene ontology

H

HA – Hemagglutinin

HBV – Hepatitis B virus

HBE – Hepatitis E virus

HIV – Human Immunodeficiency Virus

HPV – Human Papilloma Virus

I

IFTI - Interferon induced protein with tetratricopeptide repeats

IFN - Interferon

IgG – Immunoglobulin G

ISG – Interferon Stimulated Genes

i.v. - intravenous

K

KEGG – Kyoto encyclopaedia of genes and genomes

KO – Knockout (gene)

L

LCMV – Lymphocytic Choriomeningitis virus

LD50 – Lethal dose

LN – Lymph nodes

LPS - Lipopolysaccharide

LRR – Leucine rich repeats

M

MAVS – Mitochondrial antiviral signalling protein

MDA5 - Melanoma Differentiation-Associated protein 5

MF – Molecular function

MFI – Median fluorescence intensity

MHC – Major Histocompatibility Complex

MVA – Modified Vaccinia Ankara

N

NA - Neuraminidase

NK – Natural Killer (cells)

NLR – Nod-like receptor

NLRP12 – Nod-like receptor p12

O

ODN - oligodeoxynucleotides

P

PAMPs – Pathogen Associated Molecular Pattern

PASP – Pathogen Associated Structural Pattern

PBMCs – Peripheral Blood Mononuclear Cells.

PCs – Plasma cells

PD-1 – Programmed Death receptor - 1

Poly I:C – Polyinosinic-polycytidilic

PRR – Pattern recognition receptor

Q

Q β – Virus Like Particle derived from bacteriophage Q β

Q β (CpG) – Q β Virus Like Particle packaging CpG

Q β (o) – Q β Virus Like Particle devoid of nucleic acid

R

RIG-I - Retinoic-acid inducible gene

RNA – Ribonucleic acid

S

s.c. – subcutaneous

SEC – Size Exclusion Chromatography

SEM – Standard Error of the mean

ssRNA – single strand ribonucleic acid

STING – Stimulators of Interferon Genes

T

TB – Tuberculosis

TCR – T cell receptors

TD – T helper dependent antigens

TI – T helper independent antigens

TIL – Tumour infiltrating lymphocytes

Th cell – T helper cell

TLR – Toll Like Receptors

V

VLPs – Virus like particles

W

WGS – Whole Genome Studies

WHO – World Health Organisation

List of publications and presentations

1. During PhD studies:

- 1) **Gomes, A.**, Mohsen, M., & Bachmann, M. (2017). **Harnessing Nanoparticles for Immunomodulation and Vaccines.** *Vaccines*, 5(1), 6.
<http://doi.org/10.3390/vaccines501000>
- 2) **Gomes, A. C.**, Flace, A., Saudan, P., Zabel, F., Cabral-Miranda, G., Turabi, A. El, ... Bachmann, M. F. (2017). **Adjusted Particle Size Eliminates the Need of Linkage of Antigen and Adjuvants for Appropriated T Cell Responses in Virus-Like Particle-Based Vaccines.** *Frontiers in Immunology*, 8(March), 1–10.
<http://doi.org/10.3389/fimmu.2017.00226>
- 3) Mohsen, M. O., **Gomes, A. C.**, Cabral-Miranda, G., Krueger, C. C., Leoratti, F. M., Stein, J. V., & Bachmann, M. F. (2017). **Delivering adjuvants and antigens in separate nanoparticles eliminates the need of physical linkage for effective vaccination.** *Journal of Controlled Release*, 251(October), 92–100.
<http://doi.org/10.1016/j.jconrel.2017.02.031>
- 4) Cabral-Miranda, G., Heath, M., **Gomes, A.**, Mohsen, M., Montoya-Diaz, E., Salman, A., ... Bachmann, M. (2017). **Microcrystalline Tyrosine (MCT®): A Depot Adjuvant in Licensed Allergy Immunotherapy Offers New Opportunities in Malaria.** *Vaccines*, 5(4), 32. <http://doi.org/10.3390/vaccines5040032>

5) Doucet, M., El-Turabi, A., Zabel, F., Hunn, B. H. M., Bengoa-Vergniory, N., Cioroch, M., Franziska Zabel, Benjamin H.M. Hunn, Nora Bengoa-Vergniory, Milena Cioroch, Mauricio Ramm, Amy M. Smith, **Ariane C. Gomes**, Gustavo Cabral de Miranda, Bachmann, M. F. (2017). **Preclinical development of a vaccine against oligomeric alpha-synuclein based on virus-like particles.** *PLoS ONE*, 12(8), 1–25.
<http://doi.org/10.1371/journal.pone.0181844>

6) Mona O. Mohsen, **Ariane C. Gomes**, Monique Vogel, Martin F. Bachmann. (2018)
Interaction of virus-like particles (VLPs) with the innate immune system.
Vaccines,

NDM DPhil day 2015 – poster presentation : *Optimization of the immunogenicity of VLPs-based vaccines using TLR ligands.*

4th European Congress of Immunology 2015– Poster presentation: *Optimization of the immunogenicity of VLPs-based vaccines using TLR ligands.*

NDM Student seminar 2017 – Oral presentation: *Harnessing the Immune system for improved VLP-based vaccines*

Virus-like particle & Nanoparticle vaccines 2017 Conference – Oral presentation: *Harnessing the Immune system for improved VLP-based vaccines*

2. Previous to PhD studies

1) Amaral, S. S., Oliveira, A. G., Marques, P. E., Quintão, J. L. D., Pires, D. A., Resende, R. R., ... Menezes, G. B. (2013). **Altered responsiveness to extracellular ATP enhances acetaminophen hepatotoxicity.** *Cell Communication and Signaling*, 11(1), 1–14.

<http://doi.org/10.1186/1478-811X-11-10>

2) Dutra, M. S., Béla, S. R., Peixoto-Rangel, A. L., Fakiola, M., Cruz, A. G., Gazzinelli, A., ... Gazzinelli, R. T. (2013). **Association of a NOD2 gene polymorphism and T-helper 17 cells with presumed ocular toxoplasmosis.** *Journal of Infectious Diseases*, 207(1), 152–163.

<http://doi.org/10.1093/infdis/jis640>

3) Guabiraba, R., Russo, R. C., Coelho, A. M., Ferreira, M. A. N. D., Lopes, G. A. O., Gomes, A. K. C., ... Teixeira, M. M. (2013). **Blockade of cannabinoid receptors reduces inflammation, leukocyte accumulation and neovascularization in a model of sponge-induced inflammatory angiogenesis.** *Inflammation Research*, 62(8), 811–821.

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Harnessing the innate Immune system for improved Virus-Like Particle based immunotherapy

Abstract

Vaccination is deemed as the most successful intervention in public health¹. As the overall life span of the human population increases and exposure to pathogens strongly decreased due to efficient vaccine programs, the accepted risks associated with prophylactic measures, such as attenuated vaccines, is substantially reduced and efforts need to be focused on the development of safer and better tolerated vaccines. Recombinant, non-replicating vaccines may therefore represent an attractive alternative. Virus-like particles (VLPs) have emerged as a promising alternative due to their excellent safety profile and remarkable immunogenicity.

Recently, the successful concept of educating the immune system to fight infectious diseases has been expanded not only to prevent but also to treat existing chronic and autoimmune diseases. VLPs are increasingly being explored as a platform for vaccines, delivery of drugs and adjuvants, and as a tool to investigate the immune system in the context of infectious and non-infectious diseases.

Here, applications of VLPs as prophylactic and therapeutic vaccines are explored. The immune response raised in response to immunisation is investigated and the findings directly translated and applied to the development of vaccines against tumours and Influenza. First, the impact on the immune response of the size of vaccine components and mode of presentation of heterologous antigens is evaluated in the context of humoral and cellular responses in a HPV tumour model. Second, the initial events in the immune response mediated by dendritic cells that lead to protective T cell responses induced by VLP-based T cell vaccines is investigated. Thirdly, the ability of VLPs to modulate humoral responses is explored and we show that appropriate TLR7 engagement is crucial for the induction of protective antibody responses against influenza.

Finally, this thesis provides insights into the immune response following immunisation with VLP-based vaccines and considerations for vaccine development.

Ariane Cruz Gomes, DPhil in Clinical Medicine, Kellogg College.

Michaelmas term – 2018 (300 words).

Overview of Experimental chapters

Chapter II - VLPs as a platform for T cell based vaccine – Implications of size and mechanism of antigen presentation for vaccine design

HPV is the main cause of cervical cancer worldwide. In the recent years, three vaccines have been developed to prevent infection; however, until immunisation programs reach critical coverage in the population, there is still a large part of subjects at risk of developing HPV-related cancer. To those previously infected subjects, a therapeutic vaccine stands as an attractive solution. Here, we investigate the immune response and the characteristics of a therapeutic VLP-based vaccine against HPV-related tumour.

The trafficking of antigens from the periphery to lymph nodes (LNs) or spleen is governed mainly by the size of the particles^{2,3}. Nanoparticles within the size range of 20-200nm can freely flow through the lymph rapidly reaching LNs where they can encounter relevant APCs, including DCs and B cells that will initiate the immune responses in addition to encountering effector cells such as CD4⁺ and CD8⁺ T cells, that may be engaged in the response depending of the stimuli⁴. Thus, adjusted size of the particle guarantees the rapid spatial distribution of the antigens to the relevant immune centres, such as LNs.

By simply mixing antigens and adjuvants of similar size, it may be possible to target the same individual cells within the LNs avoiding conjugation steps. To test this, we generated particulate adjuvants and antigens of different size and measured humoral and T cell responses. We found that covalent linkage was essential for maximal B cell response regardless of the size of the antigen. In contrast, an admixed formulation of particulate E7 oligomers with CpG-loaded

VLPs of similar size was sufficient to induce optimal CD4⁺ and CD8⁺ T cell responses that proved to be protective as a therapeutic vaccine in an HPV tumour model. These findings have direct implications for vaccine design and can be employed to further understand the requirements for induction of appropriate T and B cell responses upon vaccination.

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Chapter III - Early transcriptional reprogramming in dendritic cells and the induction of protective T cell responses upon immunisation with VLPs

Inducing T cell responses by therapeutic vaccination requires appropriate activation of antigen presenting cells. The use of virus-like particles (VLPs) containing TLR ligands has demonstrated remarkable potential in modulating the immune response for prophylactic vaccines as well as immunotherapy or prophylactic vaccines. Here, we aimed to employ VLPs associated to TLR ligands as tools to modulate cytotoxic response by CD8⁺ T cells and provide further insight in the development of T cell-based immunotherapy. We have investigated the transcriptomic profiles in dendritic cells (DCs) from mice immunised with VLPs containing distinct classes of nucleic acid and correlate the expression patterns with the efficiency of T cell responses induced. We identified key pathways activated in DCs that are involved in the appropriated induction of T cell responses and show evidence for the modulatory effect of CCL2 on the CD8⁺ T cells mediated response. We have also provided evidence for the regulation of IFN- α responses induced by STING upon immunisation with Q β but not Q β (CpG) and shown evidence that reduction of expression of CD101 may be a potential mechanism of tumour control by cancer vaccines based on Q β (CpG). These insights shed light on immune networks that are pivotal for the induction of potent cytotoxic T cell

responses and identify key genes for appropriate DC activation and subsequent modulation of the adaptive immune response.

Chapter IV - Innate pattern recognition receptors and IgG class switching – Implications for vaccine design

An universal influenza vaccine has been a long sought goal in the field of vaccines. Here, using an heterologous VLP presenting the highly conserved extracellular domain of the M2 protein from the influenza viruses as an antigen, we demonstrated that the type of the RNA packaged into VLPs can alter the quality of the induced humoral response, namely by modulating isotype switching. We have also provided evidence that the process is predominantly TLR7 dependent, and ruled out a role for MAVS and possibly its upstream sensor RIG-I. Finally, we have evaluated the impact of the quality of humoral responses (i.e. the ratio of different IgG subclasses induced by the vaccine) in the efficacy of the vaccination to protect against influenza-related morbidity in a murine influenza model.

Our findings provide further considerations for the rational design of VLP-based vaccines and the immunomodulation exerted by TLR7 ligands packaged within the particles. Based on this work, scientists can be better informed with respect to the impact of manufacturing choices for vaccine production on the induced immune responses and additional variables that can influence the immune response and vaccination outcome.

Chapter I – Introduction

1.1 Literature Review

Literature review was performed by searching the term “vaccines” in the database Pubmed. The revision process was initially focused on reviews analysing the current state of development of therapeutic and prophylactic vaccines. In addition, the latest recommendations and information available through the World Health Organisation (WHO) website were also used as a start-point. Manuscripts on the topic of innate immunity and immunisations and specific articles within the topic were also selected in order to narrow down the topic to the following subjects: virus-like particles, PRR ligands as adjuvants, Influenza vaccines, therapeutic vaccines against tumours.

This literature review aims to summarize the current landscape of the vaccine development field and to layout the rationale behind the proposed study, which employs VLP-based vaccines to selectively engage and modulate the innate immune response for therapeutic and prophylactic vaccines.

1.2 Vaccines – an Overview of the past and the way forward

The first reports of active immunisation dates back to 1100 with the description of a technique called variolation, consisting of inoculating healthy subjects with dried scab material from patients that recovered from smallpox⁵. Later, Edward Jenner

in the 1790s formally demonstrated that immunisation of healthy subjects with fluids of patients previously exposed to cowpox would confer increased protection against smallpox⁶, by doing so, Jenner revolutionised human populational health and strategies to intervene and prevent diseases. Since then, according to the WHO there have been several licensed vaccines against at least 26 infectious diseases (table I.1) available for human use. It has been estimated that vaccines alone have contributed significantly to an overall life-span increase of 30 years with the prevention and eradication of diseases⁷.

Among the vaccines approved for humans, there are four main categories: live, inactivated, sub-unit, and bacterial toxoid⁸. (i) Live vaccines may either employ the attenuated pathogen or a heterologous virus usually belonging to the same family or quasi-species of the target virus, and that are able to confer cross-protection. Live vaccines based on the live-attenuated pathogen, contains the pathogen that underwent successive passages in cell lines or non-natural hosts, resulting in strains with reduced pathogenicity. (ii) Inactivated vaccines consist of the killed pathogen, the inactivation process is usually achieved by formaldehyde treatment or irradiation of the etiologic agent of the disease. Both live and inactivated vaccines require special storage conditions to avoid reversion to the pathogenic phenotype, which poses a logistic challenge in remote areas of developing countries, leaving room for further improvement in the vaccines within this category. Live-attenuated vaccines although often highly immunogenic and efficacious, carry the associated risk of reversion to the virulent form of the pathogen. Whilst inactivated vaccines may be safer, they are commonly accompanied by the problem of reduced immunogenicity and low efficacy. (iii) The third type, subunit vaccines, encompasses any formulation that utilises parts

of the antigens in the creating of the vaccine. In this category it is included vaccines generated through recombinantly expressed proteins, VLPs, glycoconjugate, and polysaccharide based vaccines. (iv) Lastly, the toxoid-based vaccines consist of the toxin derived from the pathogen that has undergone processes of chemical inactivation resulting in the loss of capacity to cause disease while conserving neutralising antigenic targets⁹. Although it can be argued that toxoids are also a type of subunit vaccine, they are classified within their own group owing to the fact that those vaccines do not aim to neutralise the pathogen, but the toxin itself.

Early immunisation strategies predominantly employed live attenuated or chemically inactivated pathogens as seen in figure 1, which illustrates the vaccines produced to date according to the technology employed. As the threat posed by infectious diseases became greatly reduced due to the successful immunisation programs around the world and eradication of certain pathogens, the associated risks accepted for any vaccine or medication was likewise reduced¹⁰. Currently, the immunogenicity versus safety is the main paradigm guiding vaccine development, where it is necessary to produce vaccines safe to the individual however virulent enough to elicit a protective immune response¹¹. The success of previous vaccines has imposed further pressure on the matter, stimulating the transition from live attenuated vaccines to safer subunit vaccines or vaccines based on safe vectors, such as Adenoviruses and MVA.

Table I.1: Vaccines licensed for humans

Disease	Type	Year	Pathogen
Cholera	Inactivated	1896	Bacteria
Dengue	Subunit	2015	Virus
Diphtheria	Toxoid	1923	Bacteria
	Subunit	1988	
Haemophilus influenzae type B (HiB)	Subunit	1993	Bacteria
	Subunit	1987	
	Inactivated	1985	
Hepatitis A	Live	2001	Virus
	Inactivated	1995	
	Subunit	2001	
Hepatitis B	VLP	1989	Virus
	VLP	1986	
	VLP	1981	
Hepatitis E	VLP	2011	Virus
	VLP	2006	
HPV	VLP	2007	Virus
	VLP	2009	
	Inactivated	2015	
	Inactivated	2012	
Influenza	Inactivated	2005	Virus
	Live	2003	
	Inactivated	1945	
Japanese Encephalitis	Inactivated	1992	Virus
Malaria	Subunit	2015	Protozoan
	Live	1973	
Measle	Live	1968	Virus
	Live	1963	
	<i>Inactivated</i>	<i>1963</i>	
	Subunit	2005	
Meningococcal meningitis	Subunit	1981	Bacteria
	Subunit	1978	
	Live	1967	
Mumps	Live	1973	Virus
	Live	1967	
	Subunit	2000	
Pneumococcal disease	Subunit	1983	Bacteria
	Subunit	1977	
	Inactivated	1915	
Pertussis	Subunit	1991	Bacteria
	Inactivated	1949	
Pneumococcal disease	Subunit	1977	Bacteria
	Inactivated	1990	
Poliomyelitis	Live	1961	Virus
	Inactivated	1955	
	Live	1980	
Rabies	Live	1885	Virus
	Inactivated	1914	
	<i>Live</i>	<i>1998</i>	
Rotavirus	Live	2006	Virus
	Live	2006	
	Live	1979	
Rubella	Live	1971	Virus
Tetanus	Toxoid	1937	Bacteria
	Inactivated	1976	
Tick-borne encephalitis	Live	1961	virus
	Live	1941	
Tuberculosis	Live	1921	Bacteria
	Subunit	1989	
Typhoid	Inactivated	1896	Bacteria
	Live	1952	
	Live	1798	
Varicella	Live	1995	Virus
	Live	1974	
	Sub-unit	2005	
	Live	1978	
Yellow Fever	Live	1953	Virus
	Live	1930	

*Vaccines written in italic have been withdrawn from the market.

Source:
<http://www.immunize.org/timeline/> accessed on 19th of July of 2018.

Historically, the choices available during the design phase of a vaccine are constrained by the three following variables: the technologies available at the time of vaccine development, immunogenicity, and tolerability. Until the development of *in vitro* techniques and recombinant DNA in the decade of 1970's¹¹, the most common type of vaccine were live and inactivated. After the 1970s however, recombinant techniques had expanded the choices of strategies available for vaccines development, resulting in several new recombinant vaccines including VLPs. This has been to the detriment of advances in live and inactivated vaccines (figure I.1), and it is expected that this trend may likely continue in the field.

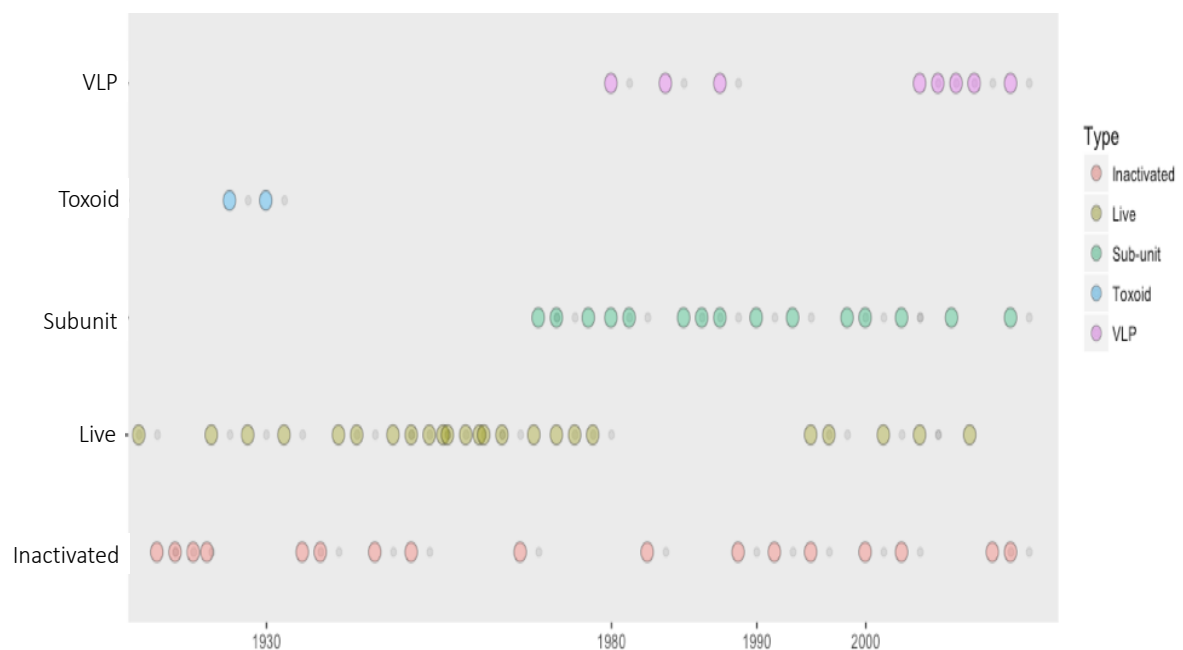


Figure I.1 - Type of vaccines licensed for humans.

Vaccines licensed for humans classified according to the type of vaccine: inactivated, live pathogen, live-attenuated, subunit, toxoid or VLP and approximate year of licensing. Source: Immunize.org¹². In addition to the four categories, VLPs are highlighted for being the focus of this thesis.

Considering the trend-shift towards safer subunit vaccines and that formulations of this kind often induce low immunogenicity, it is of great importance to refine the techniques available for vaccine development and understand the interactions of vaccines with the immune system. Basic research in the field of recombinant and subunit vaccines can lead to important insights that will support the development of new vaccines and could also help improve the immunogenicity and safety of the existing ones. Moving towards safer recombinant vaccines has also become of major importance as a response to address the growing and sometimes unfounded public concerns towards vaccine safety¹⁰.

The rationale for subunit vaccine development includes the selection of an immune-dominant antigenic target, usually a surface protein of the pathogen recognised by neutralising antibodies. Proteins alone however, are often unable to elicit an immune response once removed from the context of the pathogen their so called pathogen associated molecular patterns (PAMPs), as postulated by Janeway *et al* on his work on pathogen recognition¹³. The solution to this problem was discovered empirically with the use of adjuvants such as Alum, showing that in order to overcome the low immunogenicity of subunit vaccines, it becomes necessary to combine the chosen antigen with immunogenic substances that are able to induce a non-specific inflammatory response in parallel to the presentation of the antigen¹⁴.

1.3. Lessons from the immune system to the vaccine development field

The immune system of vertebrates starting as far back in the evolutionary tree as the agnates, is traditionally divided in two modular branches, the innate and the adaptive immune system¹⁵. There are two major defining characteristics of the aforementioned branches of immunity, the first is the general mechanism of recognition of the invader, and the second is the level of the specificity of the response mounted against the antigen. Starting with the innate immune system, the general mechanism of action can be described as a first line of defence, mounting a fast, non-specific response through the identification of general pathogen-associate or danger/ damage patterns or by simply creating mechanical or physiological barriers against infection¹³. Regarding the level of specificity of this response, the pathogen recognition by the cellular branch of the immune system is mostly mediated by germline encoded pattern recognition receptors (PRRs) expressed by immune cells, that do not recognise specific antigens but their conserved associated patterns¹³. For instance, certain PRRs are able to recognise the level of secondary modification in the nucleic acid, which is different between prokaryotes and eukaryotes, leading to different type and magnitude of responses¹⁶. Although the initial immune response elicited will not be specific to the etiological agent, the innate system will trigger general responses against that class of pathogen (*e.g.* virus, bacteria or helminth). The general innate response will attempt to control the pathogen whilst also recruiting, activating, and orchestrating the establishment of the adaptive immune response. The adaptive response on the other hand, can be classified as a second line response as it develops in a later stage of infection or pathology and may result in immunological memory against that specific etiological agent. The general mechanism of action is pathogen-specific as it relies on antigen and receptor interaction followed by clonal

expansion of the cognate cells. The classification in these two branches indeed facilitates the study of this complex system. However, the separation of innate and adaptive may fail to address the intricacies of the blurred boundaries of the two systems. Whenever possible, it is pivotal to analyse the findings of immunological and vaccine research in the light of these two connected branches that are constantly interacting and cross-modulating the immune responses.

In the prophylactic vaccine development field, the main goal is to induce a robust adaptive response that will lead to immunological memory against a given pathogen¹⁷. For years, some would claim that the field was biased towards the study and activation of the adaptive immunity. The case to favour adaptive immunity over innate relies on the fact that both B and T cells – two main representative cell types of the adaptive immune response - have antigen-specific and receptor-based interactions and establish memory, while innate immunity is unable to directly perform either. Contrary to B cells, which as APCs are also able to obtain and process their own antigens, T cells do not respond unless they undergo antigen presentation and priming events¹⁸. These functions are performed primarily by the innate immune system, namely by DCs. For T cell-based vaccines, considering the imperative need of innate immunity activation, it becomes paramount to include the innate immune system into the deliberations for vaccine design. For B cells, although not essential, innate and T cell help may be necessary in cases where the antigen alone fails to induce B cell immunity. The distinct nature of interactions of DCs, B and T cells and the indispensable role for DCs at inducing T cell responses is highlighted in figure I.2.

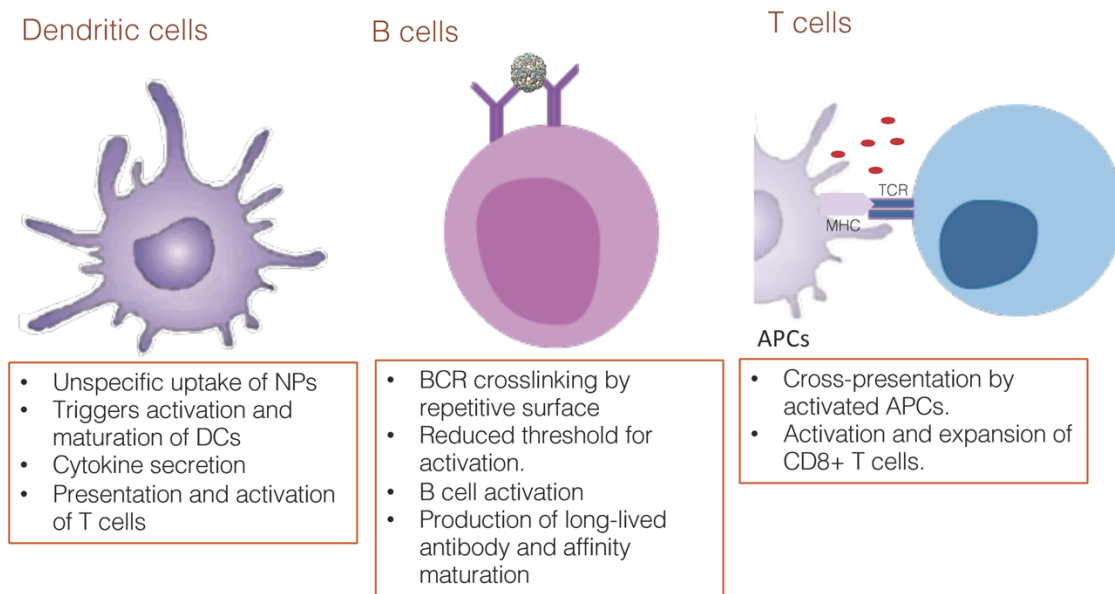


Figure 1.2 - Interaction of immune cells and viral particles

Interaction of viral particles and relevant immune cells. APCs such as dendritic cells are the main cells recognizing and driving the immune response against nanoparticles. The unspecific uptake guarantees that those cells will internalize and process most of the pathogens and molecules. The uptake of NPs triggers maturation of DCs and secretion of relevant cytokines that will stimulate other cells, such as T lymphocytes, and modulate the immune response. Humoral immune response is favoured by the repetitive surface which promotes BCR crosslinking and activation of B cells, leading to activation and production of long-lived antibody responses. Cellular and cytotoxic responses are driven by the APCs that internalized the NPs and cross-present the antigens to T cells. *Adapted from: Harnessing Nanoparticles for Immune-modulation and Vaccines, Gomes et al⁴.*

1.4 Induction of B and T cell responses by vaccination

In light of the proven success of vaccines against infectious diseases, scientists started to develop similar approaches to prevent and/or treat non-infectious diseases following the same strategy of educating the immune system to control the offense. Most of the vaccines developed until the 1900s were the result of

empirical work since very little was known about the immune system and how to modulate its responses. The scenario however, had dramatically changed after the development of technologies and advances in the field of immunology and vaccinology¹¹ with an especial mention to the brilliant work of Metchnikoff, considered the father of cellular immunology¹⁹. Nowadays, vaccines are developed using the so called rational design that applies the lessons learnt about the immune system and its relevant cells, thus contributing to the reduction in the role of empiricism and supporting the development of safer and stronger vaccines.

With the exception of two vaccines including the Herpes Zoster vaccine, where T lymphocytes have been shown to play an important role in immunogenicity²⁰, all the vaccines in use to date rely on activation of antigen-specific B cells that will in turn produce neutralising antibodies. Humoral immunity alone however, may not be enough to control diseases with a more complex life-cycle such as malaria. In addition to that, other diseases caused by intracellular pathogens or pathogen-free conditions, such as cancer may also not be restrained by vaccines that only induce antibodies. For situations of this kind or for therapeutic vaccines, the consensus is that it is necessary to develop vaccines that are also able to induce T lymphocytes in addition to humoral responses.

B and T lymphocytes recognise antigens through interaction of the antigen binding variable regions of the BCR or TCR (B cell receptor and T cell receptor, respectively) with the antigen. B and T cells receptors share a common evolutionary origin and had later diverged in structure and signalling cascades. Nonetheless, TCRs and BCRs follow the same basic rules; the diversity in the repertoire is produced through somatic recombination and the effector activity is

achieved by activation and clonal expansion of the relevant clones. The major differences between BCRs and TCRs is the threshold for activation, the means of antigen recognition (native antigen versus processed by APCs, respectively), and the number of distinct activation signals that each cell needs to undergo the genetic and epigenetic reprogramming program to become effector cells. Such differences in the activation threshold are deemed as one of the reasons why it remains a challenge to properly activate and induce T cells as opposed to B cells based therapies. Even with an incomplete understanding of their biology, scientists have nonetheless been able to induce effective humoral responses with vaccines.

The capacity of B and T lymphocytes to respond to stimuli and the differences in the activation and establishment of B and T cell responses in the context of vaccines will be further explored in the following sections.

1.4.1 B cell biology – considerations for vaccine development

Vaccine-mediated induction of neutralising humoral responses have contributed to the near eradication of pathogens and contributed to the reduction of threats imposed by infectious diseases. B cell activating vaccines are usually prophylactic and aim to produce antibodies against antigens that are expressed on the surface of pathogens. Non-empirical approaches to develop B cell activating vaccines had only become possible with the work of scientists such as Paul Ehrlich and Von Behring who identified antibodies and contextualised the concept of the complementarity of antigen and antibody interactions. Ehrlich had also identified antibodies as the active compound in serum therapy²¹ laying the foundations of the study of humoral responses and therapeutics. Nowadays, significantly more is known about the biology of B cells and the process that leads to formation of

memory B cells. However, several gaps still remain in how to translate such findings into therapeutic solutions or prophylactic vaccines.

B lymphocytes are central to the humoral responses, being the precursor cell type that, upon activation in a finely regulated process, gives rise to plasma cells (PCs), the professional antibody secreting cells²². The first event in B cell activation is the BCR mediated antigen recognition. The strength of this signal and the presence of other activation cues will affect the fate of the cell and the quality of the humoral response²³. Given that the antigen is able to overcome the activation threshold of the cell, there is a mobilization of Ca^{2+} from the endoplasmic reticulum in the intracellular compartment supported by a second wave of influx of extracellular Ca^{2+} . The aforementioned changes in the cationic balance of the cells leads to phosphorylation and activation of signalling mediators, which in turn results in the expression of several surface receptors, intracellular signalling molecules, and mobilisation of transcriptional factors to the nucleus. The final stage of this process is the commitment of the activated cells to the PC fate, producing and secreting large amounts of antibodies in some cases for the entire life span of the individual.

There are two means of providing sufficient activation stimuli to B cells. The first is dependent on the characteristics of the antigen and the second is through T helper cells (T_H). When the antigen alone is able to activate B cells without any further help of T_H cells, such antigens are called T cell independent (TI). On the other hand, soluble antigens that do not sufficiently activate B cells, can induce humoral responses if secondary signals such as PAMPs and danger associated molecular patterns (DAMPs) accompanying the antigen for induction of Th cells, leading to Th dependent response, such antigens are likewise called T_H dependent (TD)¹⁴.

Upon antigen recognition there are two main forces that govern the strength of the interaction, BCR oligomerization and mechanical forces. The first being governed by the number of BCRs crosslinked or oligomerised by the antigen, which largely depends on the level of multimerization and repetitiveness of antigenic targets in the surface of the antigen²⁴. A consequence of that is that the more repetitive the structure of the antigen, the more BCRs in the same cell will be cross-linked by the antigen leading to a stronger signalling wave. The second signal is a result of mechanical traction forces, that are governed by the B cell immunological synapses with the antigen presenting cell or the antigen itself, meaning that antigens that have higher stiffness will be better at stimulating B cell responses²⁵. Following activation, the BCR-antigen complex will be internalised, during this process, antigens reach endosomal compartments where TLR7 and TLR9 reside²⁶. In the case of pathogens such as viruses and bacteria, the internalisation will expose the nucleic acid to those receptors. This will in turn initiate in parallel a signalling and transcriptional reprogramming that has been shown to impact the quality of the humoral response through promotion of class switching of IgG to subtypes with higher effector capacities and somatic hypermutation that promotes affinity maturation^{27,28}.

The aforementioned considerations are of extreme importance for antigen design in vaccines. By observing such characteristics, the amount of adjuvants employed in the formulations can be reduced or even eliminated through the design of antigens with an optimal structure for B cell activation and antibody production.

1.4.1.1 B cell inducing vaccines - Challenges and unmet needs

Although the field of vaccines has consistently delivered solutions to many of the major threats to public health, there are still several challenges remaining. Considerable worldwide efforts by the scientific community are directed towards the development of vaccines against malaria, HIV, dengue, TB, and influenza to name a few. Such studies have greatly contributed to the understanding of the aforementioned diseases. However, further work is still required to guarantee the development of effective vaccines or therapies.

There are several possible explanations or reasons as to why the B cell-based vaccines used to date have not worked for the aforementioned diseases: (i) first due to the complexity of the pathogen, which may not be solely controlled by antibodies; (ii) second due to the antigenic targets chosen, which may not always be appropriate to confer protection and neutralisation; and (iii) finally the possibility that the developed vaccines may be inducing inappropriate humoral responses, either regarding the magnitude or the polarisation of the response. The options to tackle such problems lies in improving the design of the vaccine, including both the antigenic target and the vehicle or adjuvant used, or even moving to other strategies, such as vaccines which induce T cells.

1.4.2 T cell biology – considerations for vaccine development

T lymphocytes are a potent effector cell type of the adaptive immune system that is further subdivided in two subtypes based on the expression of the molecules CD4 or CD8, having a major role in controlling chronic infectious diseases and

cancer. CD4⁺ and CD8⁺ lymphocytes exert effector function that include cytokine production in response to activation, and in addition to that CD8⁺ T cells can directly kill infected cells²⁹. CD4⁺ T cells also modulate and provide help to B cells responses and the development of CD8⁺ T cells³⁰, hence the widespread interest in developing immunotherapies capable of selectively modulating and inducing T cell responses³¹.

Owing to the potentially deleterious capacity of T cells for the host, it is not surprising that there are several signals that need to be given to T cells in order to activate the effector program, as this tightly regulated process avoids excessive damage to the host and onset of auto-immune diseases³². The first layer of protection against deleterious T cell response is the tiered activation program that requires at least three signals: (i) MHC-restricted antigen and TCR interaction, (ii) co-stimulation, (iii) and signal three cytokines¹⁸. The first signal can be either given by APCs or virtually any cell type expressing MHC-I, the remaining two signals are given exclusively by immune cells. In particular by activated DCs that create a specific milieu of chemokines and other active molecules that will shape the T cell responses¹⁸. The second protective layer is the low frequency of a given antigen-specific T cell clone in the steady-state. A given T cell clone needs to undergo activation and cell division that leads to an increase of several orders of magnitude in the clone frequency³¹, which is not the case for B cells. In addition to that, the identification of the inhibitory molecules PD-1 and CTLA4 have shown an additional mechanism of T cell control, and T cell based immunotherapy had only become a feasible endeavour following the development of the checkpoint inhibitors. In parallel, these novel immunotherapies have helped in to revealing important clues about the biology and the mechanisms to fine-tune CTL

responses³³. This complex and tiered activation program that needs to be in place in order to activate T cells on one side guarantees a well-controlled cytotoxic response, but on the other hand it makes the endeavour of developing T cell based vaccines into a cumbersome process.

Considering that scientists in the vaccine field have been unable to induce long-term T cell responses empirically, the study of the immune system and T cell biology has become a topic of great interest to vaccinologists. For T cell vaccine development, there are two manners of giving the activation signal 1 – *i.e.* antigen presentation - to T cells. The first is through endogenous MHC-I restricted presentation, which can be attained by the use of viral vectors expressing the antigen of interest. The second strategy relies on targeted delivery of the vaccine to APCs, such as DCs that are able to cross-present particulate exogenous antigens to T cells; this is the case for non-replicative VLP-based vaccines. For the remaining two activation signals necessary for T cell responses, appropriate activation of DCs is required.

The first hurdle to be overcome in T cell activation is to promote sufficient expansion of the antigen-specific T cell population. It is estimated that the frequency of a given T cell clone is in the order of 200-2000 cells³⁴. Infections caused by certain viruses such as the extreme case of LCMV, brings the frequency of the virus specific clones up to 50% of the total T cell pool³⁵. Although 50% frequency may be a rather extreme case, it is estimated that a substantial frequency must be attained in order to provide therapeutic value. An illustration of this is the CAR-T cells therapy against leukaemia. The few successful procedures were found in patients that had circulating frequencies of about 40% of the CAR-T cells post

infusion, and a correlation of frequency of the T cells clone and protection was proposed³⁶. To date, there are very few adjuvants that are consistently able to induce substantial T cell clonal expansion in different models. One strategy had used a combination of TLR-ligand with CD40-ligand³⁷ and a second used CpG, the TLR9 ligand; another one employs viral vectors^{38,39}. However, these successful cases were demonstrated in murine models, and had reduced effect in humans^{40,41}. Nonetheless, (i) viral vectors and (ii) PRR ligands are currently considered the best choice as adjuvant candidates for T cell-based immunotherapy and several studies are employing one of the two systems in order to achieve efficient adaptive immune responses^{42,43}. Moving forward with immunotherapy and learning more about other inhibitory molecules can provide additional targets for modulating the immune system and finally bring T cell based immunotherapy to the clinics.

1.4.2.1 T cell inducing vaccines - Challenges and unmet needs

Currently, there are several vaccines in pre-clinical or phase I studies aimed at evoking T cells to establish protection and immunity. The most common disease targets are different types of cancers, where the role for T cells in tumour elimination is well established. Progress has been made with the combination of T cell based vaccines and checkpoint inhibitors (trial: NCT01176474) or cytokines that improve T cell activity such as IL-2 (trial: NCT00005949). For other diseases such as HIV and malaria there is still a debate on whether a vaccine should aim to induce T cell responses or not. In the HIV field, a promising T cell inducing vaccine candidate, the MRKAd5, a viral vectored based vaccine produced by Merck that was shown to induce great immunogenicity profiles with CD8+ and CD4+ T cells producing high levels of IFN- γ and IL-2. However, it had to be withdrawn from a phase IIb trial after showing no protection in comparison to the placebo group.

The disappointing results of this trial shows that our correlates of efficacy for T cell vaccines, at least against HIV, may need to be revisited and further studies may be necessary in order to finally produce efficient subunit or vectored T cell inducing vaccines⁴⁰.

The need for T cell based therapeutic vaccines in addition to the traditional prophylactic vaccines is well illustrated by malignancies induced by HPV. Whilst there are currently three highly effective prophylactic vaccines against HPV approved for humans since 2007, the prevalence of HPV-related cancers is high worldwide and is predicted to remain so until a critical percentage of the population is immunised against the virus⁴⁵. More urgently however, there is a large percentage of previously HPV infected people at risk of developing malignancies, and for whom prophylactic vaccines had been shown to be ineffective⁴⁶. This is likely a limitation of the mechanism of protection of these three vaccines relying mostly on the induction of neutralising antibodies, that are able to prevent the initial infection but not to control the tumours associated to the virus. The alternative is to develop therapeutic vaccines able to induce cell-mediated control of the tumour. In HPV-related cancers, immunotherapy stands as an attractive alternative considering that the transformed cells will constitutively express some of the oncoprotein from the HPV at high levels⁴⁶. An example of that is the E7 protein from the HPV virus, marking such cells with a clear antigenic target for T cell mediated killing. With respect to the above, although there are already several prophylactic vaccines against HPV, clearly there is still an unmet need for therapeutic alternatives against HPV that are able to engage T cells. In addition to that, the development of a therapeutic vaccine against HPV will strengthen the case for T cell-based vaccines in general. They represent

an excellent model to study therapeutic vaccines, since HPV-associated tumours express a virus-related antigen instead of the self-proteins expressed in tumours that are not directly caused by oncoviruses.

In summary, the major challenges faced when developing T cell immunotherapies are: (i) promote clonal expansion of T cells, (ii) activate T cells in the appropriate polarisation and effector programme, (iii) find the right pre-clinical correlates of the immune response to assess vaccine efficacy.

1.5 Overcoming challenges for T cell-based vaccines

Tumours and other pathogens have co-evolved to evade the immune responses and thus have to produce several immune modulators that halt T cell mediated protection. Although the pitfalls and challenges in the field may sound discouraging, good news from current evidence has demonstrated that T cells can be an effective immunotherapy against treatment-resistant cancers. Juxtaposed with the failures of antibody-based vaccine candidates, this highlights the need for a better understanding of the immune system and T cells in particular. In the following sections, the methods and directions taken in the attempt to overcome the challenges in the T-cell vaccine field will be discussed.

1.5.1 Viral vectored vaccines

Recombinant viral vectored (VV) vaccines are classified as a subunit vaccine and are based on modified viruses that can promote transient infection and expression of the antigenic target encoded and delivered by the vector⁴⁷. The immunogenicity

is a result of both the (i) expression of the foreign protein by the infected cells, that can activate the adaptive immune system and the (ii) general inflammatory responses elicited by the virus, mimicking a real viral infection⁴⁸.

There are different classes of VV based on different viruses, Vaccinia and adenoviruses being two of the most widely used vectors. VV had shown encouraging results, especially for CTL responses upon heterologous prime-boost. This immunisation scheme employs a prime with a DNA vaccine, such as MVA, followed by a boost with a viral vector such as adeno-based vectors, resulting in transgene-specific CD4⁺ and CD8⁺ T cell responses in different disease models including HIV⁴⁹ and hepatitis C virus (HCV)⁵⁰.

Although VV do show enormous potential, the high level of modification that such vectors undergo in order to be rendered safe are shown to impact the immunogenicity of such particles⁴⁸. In addition to that, viral vectors are not devoid of other problems that need to be circumvented in order to facilitate the use of such platforms. The major problems with vaccines of this kind are either related to the logistics of vaccine production or to resultant efficacy. For the former, VV-based vaccines require constant revision of immunogenicity and genetic stability of the vaccines; and the production process is still inefficient, which can be a problem to guarantee supply of a given vaccine, which directly impacts the price per unit of a vaccine⁴³. Regarding the efficacy, VV must be screened for pre-existing immunity, which has been shown to reduce efficacy. In addition to that, the optimal dose must be carefully assessed as viral vectors at high dose have been shown to induce considerable side-effects. To date, vaccines that were licensed using such platforms have not achieved satisfactory efficacy as for the case of the RTS,S Malaria vaccine and further improvements are necessary⁵¹. Nonetheless, the development of viral

vectored vaccines continues at a remarkably fast pace, and it is hoped that efforts currently employed in this field will overcome the aforementioned difficulties, allowing them to be translated in to highly efficient therapies and vaccines in the future.

1.5.2 Dendritic cells and T cell-based vaccine development

As previously mentioned, selectively engaging the innate immune system is important to achieve efficient immune responses through vaccination. DCs being the main antigen presenting cell in the immune system and the bridge between the innate and the adaptive responses have a central role in shaping immune responses, raising interest for both prophylactic and therapeutic strategies for disease control. DCs are a heterogeneous cluster of phagocytic cells descending from an unique hematopoietic lineage that further differentiate into functionally distinct subtypes classified according to the ontogeny, surface marker expression, and transcription factors³². For the scope of this thesis, only the traditional classification of DCs into conventional DCs (cDCs) and plasmacytic DCs (pDCs) will be considered. Considering the functional classification, cDCs encompasses DCs mostly dedicated to antigen presentation and priming of naïve T cells relying on their diverse mechanism of antigen uptake and presentation for T cells. pDCs on the other hand, although still fully capable of antigen uptake and presentation, are classified as a cell type specialised into producing type I IFN, thus having a central role in shaping the immune response to control viral infections.

Depending on the activation programme DCs undergo and the immune mediators produced, the adaptive response can be biased either towards tolerogenic or

inflammatory effector responses by T cells⁵³. This phenomenon is a consequence of the manner DCs are activated prior to antigen presentation, which can be either due to DC-intrinsic maturation programs or pathogen induced⁵⁴. The role of DCs in the orchestration of the immune response is well established in the literature^{18,55,56}, in addition to that, their importance becomes more prominent when studying T cells. Mouse models lacking DCs have further established their essential role in the initial activation of naïve T cells, priming, and the recalling of memory responses⁵⁷. DCs are the only cell type capable of not only initiating the activation program of T cells through antigen presentation of MHC-I and II restricted peptides to T cells, but it is also able to provide the two additional activation signals. They accomplish this by up-regulating co-stimulatory molecules that help to modulate the quality of the activation programme of T cells. Also, DCs contribute to T cell maturation and maintenance of that state in a paracrine and autocrine manner.

When aiming to develop T cell-based vaccines, it is expected that the relevant antigen-specific T cell clone will expand, having been primed to have an effector phenotype, and remain as a pool of memory T cells. DCs are important to cross-present the antigen to the naïve cells and more importantly, to provide additional signals that will dictate the fate and reprogramming of T cells⁵⁵. Vaccines that fail to activate the appropriate inflammatory reprogramming of DCs, although antigen presentation may still happen, the clonally expanded T cells may not exert the protective effector response necessary for efficacy. Thus, it is reasonable to say that based on the available evidences that in order to induce effector T cell responses vaccines one should aim firstly to target and properly activate DCs.

1.5.3 PRRs and vaccines

During the 20th century, Alum was the only adjuvant licensed for human use and still remains as an essential part of most of the vaccines developed thus far, providing protection and near eradication of certain pathogens. Alum however, has its limitations, for instance Alum is unable of inducing any measurable CTL responses and mostly induces Th2-biased immune responses, propelling the vaccine field to search for new adjuvants⁴². The identification and study of PRRs started with the discovery of the Toll-like receptors (TLRs) in the early 90s based on the work lead by Akira⁴⁸, Janeway¹³, and Beutler⁵⁹ *et al.* This opened up an enormous field that explored diverse themes, from how organisms develop, to how cells interact and respond to their surroundings. The activation of both DCs and T cells is largely controlled by the PRRs engaged by different stimuli⁶⁰ and further studies on PRRs and the orchestration of immune responses led to the acknowledgement of the major role of such sensors in re-programming of gene expression, metabolism and overall biology of cells⁶¹. As a result, a growing number of PRR ligands are being developed and studied aiming to provide tools to modulate the immune system and the development of therapies and adjuvants. In addition to TLRs which can recognise compounds such as nucleic acid and lipoproteins, three additional classes of PRRs have been described including the (i) retinoic acid-inducible gene I-like receptors (RLRs) that recognise intermediary species of cytoplasmic RNA; the (ii) nucleotide oligomerization domain-like receptors (NLRs, also known as LRR, NACHT, and PYD domain proteins) recognizing molecules such as ATP, peptidoglycans, and toxins; and (iii) cytosolic DNA sensors such as the cyclic-GMP-AMP synthase (cGAS) that recognises dsDNA^{13,62,63}. As for TLRs, the aforementioned classes of PRRs are likewise being

explored as therapeutic targets and have been producing encouraging results. Table I.2 summarises the receptors expressed by DCs and the agonists that have been licensed or are in advanced pre-clinical studies.

Through the engagement of PRRs by their cognate ligands, DCs undergo stereotypical responses and physiological adaptations that include profound changes in the metabolism of those cells. The genetic and metabolic reprogramming of activated cells will support the production of immune mediators such as chemokines, that will orchestrate and amplify the immune response⁶¹. Selective engagement of PRRs has shown an enormous potential in modulating the immune response to specific ends and fine tuning the immune system^{28,64}.

As previously discussed in section 1.2, subunit vaccines often require adjuvants. Proteins alone, even of foreign or “non-self” origin are often non-immunogenic; this is a result of the several mechanisms in place, selected to avoid excessive and deleterious auto-immune responses. However, if administered with PAMPs or perhaps danger signals, as postulated by Polly Matzinger with the danger model⁶⁵, even short peptides can be rendered immunogenic. Both PAMPs and DAMPs are recognised by PRRs expressed in virtually every cell type but specially in DCs, that are not only able to sense but also to respond to such signals. These two models explain the growing interest within the vaccine field in understanding the biology of those receptors and how their modulation could be exploited for vaccine development.

Table I.2: PRR ligands as vaccine adjuvants

Receptor	Adjuvant	Class of compound	Vaccines ¹	Immune responses	Natural Ligand
cGAS	di-cyclic DNA Chitosan	Nucleic acid Polyssacharide		Th1, CD4+ CD8+ T cells	dsDNA
TLR2/6	Pam2CSK4	synthetic diacylated lipopeptide		Th1, CD4+ T cells	Microbial cell wall components
TLR3, RLRs	Poly-(I:C) and derivatives	synthetic dsRNA		Ab, Th1, CD8+ T cells	pppRNA, dsRNA
TLR4	AS01*	Liposome containing TLR4 agonists	Mosquirix (Malaria)	Ab, Th1	LPS
	AS02	Liposome containing TLR4 agonists		Ab, Th1	
	AS04*	Liposome containing TLR4 agonists	Fendrix (HBV), Cervarix (HPV)	Ab, Th1	
	Lipid A*	7-acyl lipid A (LPS analog)		Ab, Th1	
	MPLA*	Monophosphoryl lipid A (LPS analog)	Pollinex Quattro (Allergy)	Ab, Th1	
	RC529*		Supervax (RBV)	Ab, Th1	
TLR5	Flagellin	Flagellar protein		Ab, Th1+Th2	Flagelin
TLR7	Imiquimode*	RNA		Ab, Th1, CD8+ T cells	ssRNA
TLR9	<i>CpG ODN</i>	synthetic DNA rich in C and G		Ab, Th1, CD8+ T cells	CpG rich DNA
	<i>QbG10</i>	VLP containing A type CpG		Ab, Th1	
	IC31	antibacterial peptide and synthetic ODN		Ab, Th1, CD8+ T cells	

Pattern recognition receptors and ligands harnessed for immunomodulation in vaccines. Adjuvants followed by (*) had been licensed for clinical use. HBV: Hepatitis B Virus, HPV: Human Papilloma virus. 1 Licensed vaccines.

LPS-based adjuvants

Currently, there are several PRR ligands in use both in advanced pre-clinical stage and some already licensed for human use (table I.2). The most common type are adjuvants based on the bacterial lipopolysaccharide (LPS) sensed by TLR4. Adjuvants based on LPS retain TLR4 agonistic capabilities with reduced pyrogenic side-effects common to the native form of LPS. Two highly effective vaccines in use today, HBV and HPV include MPLA, an adjuvant derived from LPS, into their

formulations showing good efficacy and tolerability in vaccines that induce protective antibody responses⁶⁶. Of note however, the LPS-based adjuvants have limited capacity to induce CD8⁺ T cell responses.

RNA-based adjuvants

Research on viral infections has contributed to the recognition of both dsRNA and ssRNA as potent immune-stimulators. Soon after, synthetic analogues of RNA, such as Poly IC and Poly ICU were developed and employed as adjuvants for vaccines and revealed an excellent approach to induce potent adaptive immunity⁶⁷. dsRNA is recognised by TLR3 and MDA5 receptors; Such receptors are similarly the target of the synthetic RNA analogues. ssRNA on the other hand, is recognised by endosomal TLR7 and 8 and it is more labile to degradation by temperature and/or the almost ubiquitous RNase enzyme in comparison to dsRNA. Therefore, in an attempt to improve the pharmacokinetics of compounds of this kind the RNA is commonly associated with carriers such as liposome, viral vectors, and VLPs that naturally pack nucleic acid⁶⁸. Adjuvants targeting either TLR3, TLR7/8, or MDA5 produce distinct outcomes in terms of protection and immune responses, however the efficacy of such compounds are often measured in terms of *in vitro* production of IL-12 and type I IFN secretion by DCs⁶⁹. Such observations suggests that future studies needs to go beyond the measurements of type I IFNs and IL-12 in order to understand the differences in outcome of the responses orchestrated by such ligands and their cognate receptors. Another TLR7 ligand in use as an adjuvant is the molecule R837 (Imiquimode). The addition of Imiquimode to vaccine formulation has been reported to improve cellular responses against viruses and tumours and stands as an attractive choice for adjuvants of CD8⁺ T cell responses⁷⁰. In general, RNA-based adjuvants have been shown to improve

overall immunogenicity, albeit not to the same extent, especially by promoting Th1-skewed responses and improving the quality and magnitude of humoral responses^{68,71,72}.

CpG-rich DNA based adjuvants

CpG rich ODNs (synthetic oligodeoxynucleotides containing unmethylated CpG) are the main TLR9 ligand and have consistently shown encouraging results as adjuvants in vaccines against a range of diseases. Moreover, CpGs are deemed to be one of the more promising candidates for CTL induction and immune modulation considering their ability of (i) to activate a range of immune cells such as NK cells, DCs, B cells and macrophages, (ii) to activate type I IFN responses, and (iii) in controlling the Th1 / Th2 balance⁷³. There are three main classes of CpG are named A (or D), B (or K), and C. The classification is based on the biological effect, length, and structure of the sequences (figure I.3). Although CpGs belonging to different classes signals through the same receptor (*i.g.* TLR9), the activation program of the cells exposed to CpG is highly distinct and so is the therapeutic outcomes induced by each CpG type^{74,75}. In addition to distinct outcomes of immune responses raised by different types of CpG, the TLR9 recognition and activation is sequence dependent and is different across species⁷³.

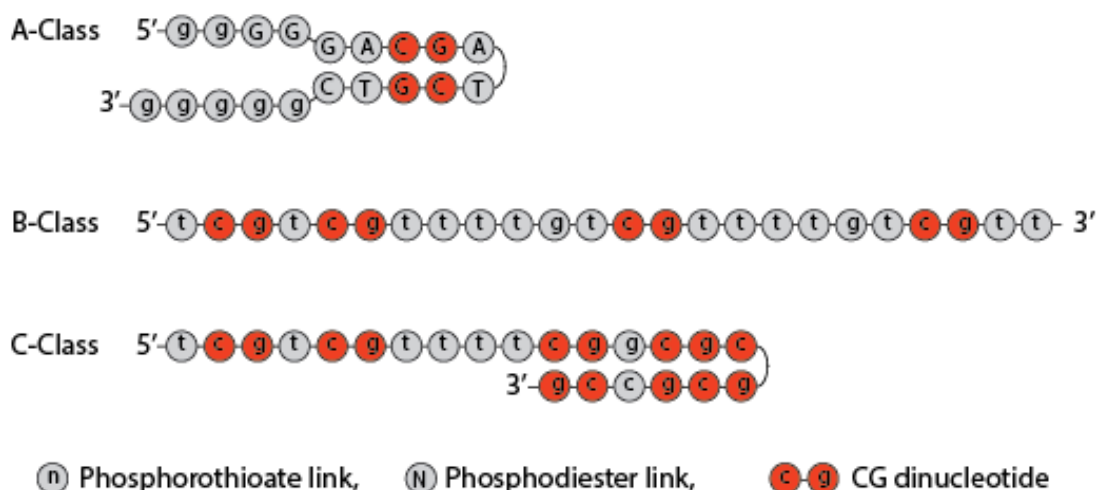
Type A CpGs are CpG-containing ODN sequences connected by phosphodiester bonds and includes a palindromic motif with a poly-G string at their 3' end comprising of phosphorothioate bonds. CpGs of this class lead to strong type I IFN responses from pDCs with reduced activation of NF- κ B-dependent pathways and reduced production of pro-inflammatory cytokines⁷⁶. One example of clinical use of A type CpGs was demonstrated in a treatment against allergies employing a A

type CpG named G10 associated to a VLP, named CYT003-QbG10. This formulation when administered either in absence or concomitantly to the allergen displayed encouraging results against certain types of severe allergy such as allergic rhino conjunctivitis in a phase IIb clinical study⁷⁷. The proposed mechanism of action is a strong Th1 polarisation in opposition to the Th2-skewed allergic response and regulatory T cells, thus ameliorating the symptoms even in the absence of the allergen. Type B CpG on the other hand, is a poorer inducer of type I IFN production by pDCs but it is one of the best adjuvants in inducing CTL responses in pre-clinical studies. B type CpG are shown to induce strong antigen-specific clonal expansion of multifunctional CD8⁺ T cells that produce key cytokines such as IFN- γ and TNF- α ⁷⁸. In terms of structure, B type CpGs are usually shorter in length, containing only phosphorothioate bonds and do not form secondary structure as opposed to type A (figure I.3)⁶⁴. Based on the discrepancies of A and B type CpG, a different class of CpG-rich ODN was developed called C type. C type CpGs were designed as a hybrid of the structure of A and B types, containing a phosphodiester backbone (as A type) and the palindromic motif containing CpGs. C type CpGs are able to induce IFN- α production by pDCs in similar levels compared to A type and are also good B cell stimulators similarly to the B type⁷⁶.

In addition to the therapeutic applications, CpGs of different class can also provide important clues on TLR signalling and type I IFN responses. An example of that was elegantly demonstrated by Honda *et al* that elucidated the differential IRF7 signalling and endosomal trafficking on pDCs and cDCs that leads to the distinct capacities of each subtype to produce IFN- α in response to certain PRR stimulation⁷⁹. Those findings were a result of the investigation of the distinct

endosomal trafficking and signalling of different CpG type A and B that lead to a better understanding of TLR signalling machinery and methods to modulate the response raised by each CpG⁷⁹.

CpG ODN Classes



Source: <https://www.invivogen.com/cpg-odns-classes>

Figure I.3 – CpG ODN classes

Primary and secondary structural differences of CpG ODN class A, B and C. Adapted from: Invivogen (<https://www.invivogen.com/cpg-odns-classes> accessed on July 14th 2018).

Agonists of cytosolic nucleic acid receptors

In the recent years, agonists of the cGAS/STING pathway have also been employed as adjuvants against viral diseases such as influenza and cancer models. The major cGAS agonist is dsDNA, that upon binding to cGAS promotes conformational changes that activates the enzymatic activity of the molecule resulting in the production of cyclic-GMP-AMP (cGAMP), a cyclic dinucleotide (CDNs) second messenger⁸⁰. Such CDNs are secondary signalling molecules present in bacteria and mammalian cells that trigger a type I IFN response. Pre-

clinical studies employing CDNs as adjuvants in a vaccine against tuberculosis had shown a Th1 and Th17-biased response with protection similar to the attenuated bacillus⁸¹. Another adjuvant that activates cGAS/STING pathway is Chitosan, a polysaccharide that has been widely used as an adjuvant. Chitosan was recently shown to exert its immune enhancing activity through mitochondrial stress and activation of the cGAS/STING pathways leading to strong CD4⁺ T cell responses and improved humoral responses in different disease models^{82,83}. These encouraging results are fuelling further research on this novel class of adjuvants targeting the cytosolic sensors and increase the breadth of applications of such compounds.

In general, PRR ligands included as adjuvants have consistently been shown to render vaccines more immunogenic and improve protection. Moreover, many PRR ligands have the advantage of being derived from naturally occurring compounds, such as nucleic acids or prokaryotic proteins, which makes the process of development and production easier compared to the development of new chemical compounds, for instance. Thus, PRR ligands currently stand as attractive alternative for the development of new therapies.

1.5.4 Vaccines in the “omics” era

Finding the appropriate immune markers that correlate with the efficacy of a vaccine or treatment during the pre-clinical phase of development is not a straight forward process. Several vaccine candidates with excellent immune responses in the pre-clinical stage resulted in disappointing efficacy and protection after moving to clinical phases. With the dawning of transcriptomic techniques and other systems biology approaches several publications emerged on the topic of

investigating early predictive signatures that would correlate with protection and efficacy of the vaccine. The transcriptional profile of peripheral blood mononuclear cells (PBMCs) of immunised subjects in the early time-points post yellow fever YF-17D immunisations revealed an early gene transcriptional signature in DCs associated with the later protective immune response of the highly efficacious yellow fever vaccine⁸⁴. The same approach had been used to compare the transcriptional reprogramming that DCs undergo prior to induce T cell clonal expansion and effector activity in response to several popular recombinant vaccine platforms. The aforementioned study lead by Dérian *et al* had revealed a set of genes named by the authors Sig1 that were differently regulated and are correlated to the development of different types of CTL responses⁸⁵. Studies of this kind are extremely useful for two reasons, firstly, they provide reliable tools to the cumbersome and expensive endeavour of evaluating efficacy of vaccines. Secondly, such studies provide insights and useful lessons regarding the immune system, not only offering information on how this complex system functions but also elucidating genes and pathways that can be targeted by therapeutic modulators in a plethora of pathologies, from infectious to auto-immune diseases.

As previously discussed, B cell based vaccines have historically been developed even in times when only less sophisticated techniques were available; T cell vaccines on the other hand had shown slower progress. Validating and understanding the impact of genes and pathways differently regulated in DCs and T cells will support the vaccine field and allow this promising type of vaccines to finally reach the clinics.

1.6 Virus-like Particles and the future of vaccine development

Virus-like particles, as the name suggests are supra-molecular particles derived from the capsid or sometimes envelope of real viruses, which retains most of virus-associated characteristic such as structure and size⁴. VLPs were first described following the observation of viral particles devoid of infectious material that naturally occur in certain viral infections. A notable case is hepatitis B virus (HBV), where non-infectious particles were purified from the fluids of infected individuals among infectious viral particles⁵⁶. The purified particles were used to immunise healthy subjects against the HBV virus, at the time being then the first case of immunisations employing VLPs. Later, the HBV VLPs were produced *in vitro* by recombinant expression of the hepatitis B surface antigen (HBsAg), which had rendered the vaccines safer and made the production scalable to larger commercial scale manufacture.

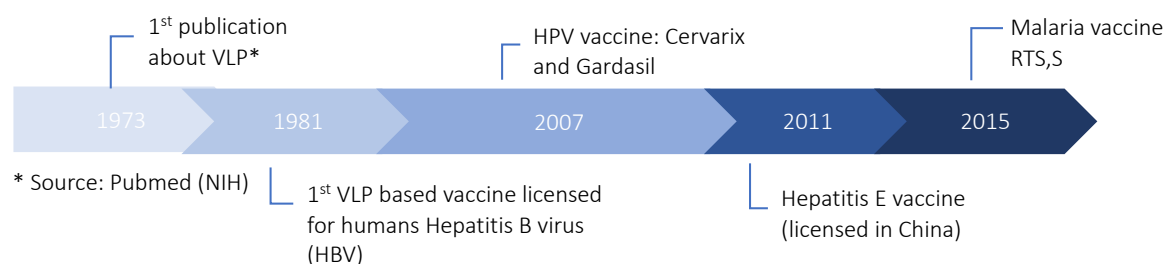


Figure I.4 - VLP and viral vectored based vaccines licensed for humans.

The successful HBV vaccine laid the foundations of VLP-based vaccines. Since then, two vaccines against two different sets of serotypes of HPV⁴⁵, one against the Hepatitis E virus (HBE)⁵⁷ and a HBsAg based vaccine against malaria⁵¹ had also been approved for clinical use (figure I.4).

One characteristic in common between the VLP-based vaccines developed against HBV, HPV, and HBE is that the VLPs were derived from the homologous virus that are the etiological agent of the disease. This approach has the benefit of preserving all the antigenic characteristics of the original virus with the exception of their infectious capacity. However, this strategy limits the development of VLP-based vaccines to not only viral diseases, but also only to viruses from which stable VLPs can be derived from. An additional caveat to developing VLPs from several individual viruses is that the process of producing a new and stable VLP, that is also economically viable, requires production that adheres with good manufacturing practice (GMP) settings. A rather time-consuming, technically demanding and cumbersome exercise that would need to be repeated for each virus VLP. An attractive alternative would be to produce a single well characterised VLP platform that can be used for different diseases⁸⁸. This latter approach has been widely employed in the past decades and proven highly efficacious. Yielding several vaccine candidates to different types of disease, ranging from the traditional prophylactic vaccines targeting infectious agents to vaccines against non-infectious diseases such as hypertension⁸⁹ and nicotine addiction⁹⁰.

The high immunogenicity of VLPs is correlated to the retention of the viral fingerprint that is widely recognised as a PAMP and pathogen associated structural pattern (PASP) by the immune system. The rationale behind producing a VLP presenting heterologous antigens is to associate the otherwise non-immunogenic antigens to this platform, effectively extending the viral fingerprint

of the VLP to the antigen of interest. The immunogenicity of VLPs will be further discussed below in the following sections.

1.6.1 Viral fingerprint

Vertebrates co-evolved with a plethora of pathogens such as viruses and bacteria and a great part of the biology of vertebrates is a direct result of this close relationship. The innate immune system is no exception⁹¹ being well equipped to identify and respond against pathogens. Doing so by recognizing patterns that are associated with viruses and prokaryotic pathogens, the so called PAMPs and DAMPs¹³. The co-evolution of immune system and pathogens becomes blatantly evident on the theory of clonal selection postulated by Burnet in the 1950s as a result of his work with influenza and antigenic shift and drift⁹². With an evolutionary perspective in mind, the high immunogenicity of VLPs can be summarized in the three following characteristics: 1) size, 2) geometry, and 3) presence of packed nucleic acids, characteristics that most VLPs retain, with the main difference being the that VLPs are replication incompetent. The viral fingerprint will be discussed in the following sections within the scope of vaccine development.

Size – The size of particles is now recognised as a PASP for two reasons: The first reason is due to the apparent absence of host proteins that fall within the same size range of viruses, and second as a consequence of the draining properties of particles within the lymphatic system, that due to the fenestrated nature and size of vessels favours the cell-free drainage of particles smaller than 200nm¹⁷. Interestingly, it is recognised that there are three categories of size that impact the

immunogenicity of an antigen. The first category is for proteins smaller than 10nm. Virtually all the vertebrates host protein in the extracellular space are encompassed within this size range, moreover as previously discussed, proteins smaller than 10nm are seldom immunogenic when administered without the addition of immunogenic signal. The second class includes particles within 20-200 nm, particles within this size range are virtually absent in the extracellular space of vertebrates and this size range is characteristic of most viruses, hence the pathogen-associated pattern. Particles smaller than 200 nm, can freely drain to the subcapsular region of lymph nodes from the periphery, where several immune cells reside and initiate the immune response by reaching the B-cell follicles²³. The third category encompasses particles larger than 200 nm, which are unable to enter the pores of the lymphatic vessels and can only reach lymph nodes if associated and processed by an APC²³. This concept has been empirically demonstrated in an elegant paper showing that lymph node resident dendritic cells preferentially uptake particles that fall within the second category and are more efficient at reaching lymph nodes, which was correlated to higher immunogenicity³. This is a very important consideration for the vaccine development field specially at an age of subunit vaccines where it is necessary to improve immunogenicity².

Geometry – The consequence of viruses having a small genome is that they are limited to encode very few structural proteins to form the capsid. The capsid shell is commonly formed by one or two monomeric protein subunits repeated symmetrically and arranged in a quasi-equivalent conformation in either rod-like or spherical structures²⁴. Rod-like capsids have a helical symmetry and the number of monomers varies according to the genome length packed into the particle. Icosahedral capsids on the other hand form polyhedrons with at least 60

monomers or multiples of 60, depending of the length of the genome. As a result of the polyhedron symmetry, spherical capsids are classified according to the triangulation (T) – or multiples - of the 60 monomeric subunits forming the capsid, as proposed by Caspar and Kull⁹⁴. The great majority of icosahedral capsids follows the T3⁹⁵ symmetry, although sheets, tubes and multi-layered shells can also be formed *in vitro* depending of the conditions of the solution where such viruses are assembled⁹⁶. A consequence of that is that spherical capsids only pack a fixed amount of nucleic acid due to the constraints of the capsid. The multimeric nature of the structure and geometry of viruses and VLPs results in a stiff, highly repetitive surface with geometrically organised antigenic arrays as schematically represented in figure I.5⁹⁷.

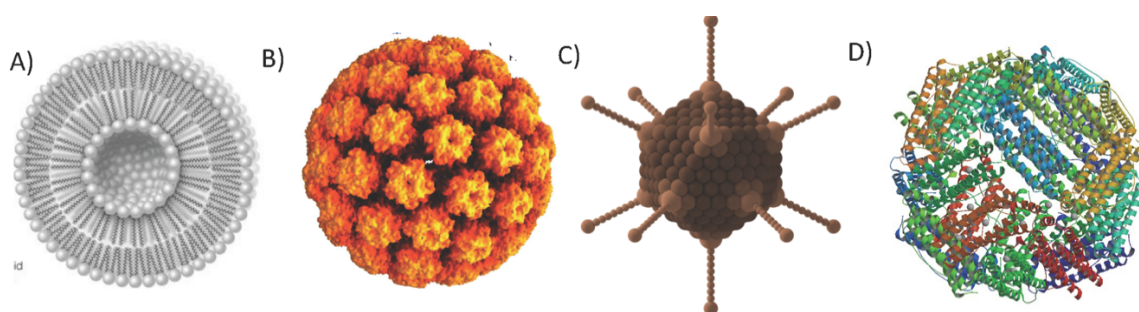


Figure I.5 - Nanoparticles repetitive structure.

(A) Liposome: schematic representation of a phospholipidic liposome; (B) VLP derived from HPV virus, PDBID: 1DZL; (C) Viral vector: schematic representation of viral adenovirus.; (D) Self-assembled proteins: X-ray structure of ferritin PDBID: 2X17. Protein Data Bank Identification (PDBID). *Adapted from: Harnessing Nanoparticles for Immunomodulation and Vaccines, Gomes et al⁴.*

Multi-protein structures with a repetitive conformation such as viruses are almost absent in vertebrates, in particular in the extracellular space and under non-

pathological conditions in contrast to other domains of life where they are common. These geometrically organised structures are categorised as a PASP and it plays an important role in triggering immune responses⁴. Repetitive structures are more likely to have deposition of components of the fluid-phase innate immune such as pentraxins, natural antibodies, and fixation and activation of components of the complement system. Triggering the cascade of the complement facilitates the opsonization of antigens for several immune cells such as macrophages and DCs, reduces the BCR activation threshold through the co-stimulatory role of C3 degradation products engaging CD21 on B cells, and promotes antigen trapping in the germinal centre via CD35. Those elements contribute to the generation of memory B cells and long-lived plasma cells (PCs) and the overall immune response⁸. In addition to that, the multimeric antigenic surface of VLPs itself promotes the crosslinking and simultaneous activation of the BCR, which promotes the activation of B cells. In summary, several components of the immune system have evolved to recognise and trigger an immune response against agents with repetitive structure and this can be harnesses to render sub-unit antigens into highly immunogenic just by mimicking the repetitive surface of viruses.

Nucleic acids – In order to guarantee success of infection and spread of their genome, the tertiary structure of viruses' capsid is selected to assemble around and pack their own genome based on protein-RNA/DNA interaction that usually favour packing their own genome over other nucleic acid⁹. Nucleic acids are polyelectrolyte sequences that have negative net charge and fold into secondary structure depending of their length and charge. The charge and conformation of the polyanionic sequences facilitates the capsid assembly process by contributing

to the thermodynamic forces and stabilising protein-protein interactions. An interesting consequence of that is that the re-assembly process of VLPs can be performed *in vitro* and the type of nucleic acid packaged can be selected in order to engage different PRRs.

The inherent capacity of VLPs to assemble around nucleic acids contributes to the immunogenicity of such particles as immune cells are multiply equipped to detect foreign nucleic acids. Using VLPs packing nucleic acid such as RNA and CpG also improves the pharmacokinetics of such compounds as VLPs are readily phagocytosed by immune cells and delivered to their endosomal compartment of cells. The endosomal compartment of cells is precisely where some of the relevant receptors for nucleic acids are located, as for TLRs. A consequence of this targeted delivery is a reduction on the dose necessary to achieve activation and a reduction of side-effects as VLPs are phagocytosed only by immune cells such as DCs, macrophages, and B cells.

A remarkable advantage of packing nucleic acid into nanoparticles, in addition to the immune modulation is to improve the economic viability of the vaccine. The case of CpG illustrates this advantage very clearly. CpG has been widely used in pre-clinical studies as a vaccine adjuvant and more than 600 clinical trials up to now and has recently obtained regulatory approval for clinical testing of CpGs. The suggested dose in the phase I-II clinical trial of the compound D35, for instance is 20mg/Kg⁹⁰, which could make treatment unfeasible from an economic perspective. As a mean of comparison, the Q β VLP for instance, packs 120nM of CpG per mg protein¹⁰⁰, considering that the average dose of VLPs used for humans in clinical trials was of 1 mg of VLP in the MelQbG10 trial¹⁰¹, the per dose amount

of CpG is reduced to less than 1µg/dose as opposed to 120mg/dose considering an adult of 60Kg. This argument alone represents a compelling reason to include packaged versions of the CpG as comparison in pre-clinical and clinical studies as a default.

1.6.2 Q β VLP and vaccine development

As previously discussed, heterologous antigens may have its immunogenicity greatly increased if associated to a VLP. This is a rather important finding for two reasons. First, it eliminates the need of developing a VLP from each virus to whom a vaccine is being developed against. Second, it allows the development of vaccines against diseases or pathogens that are not caused by viruses. To take advantage of that, it is desired to have a robust, stable and well characterised model VLP. Several VLPs have been developed throughout the years with this purpose, examples of that are AP205, Q β ¹⁰² and MS2¹⁰³ derived from a *E. coli* bacteriophages, and the Hepatitis B core antigen (HBcAg)¹⁰⁴, the most widely studied VLP to date¹⁰⁵.

An optimal model VLP is highly immunogenic, should be produced in high yields, easy to purify with high purity, is stable and does not require special storage conditions. In addition to that, such VLP would preferably be free to operate. A good example of a VLP that has all of such characteristics is the Q β VLP. Q β VLP was first cloned and described in 1993 by Pumpens *et al.* Q β is a *E. coli* RNA phage that had been extensively employed in different vaccine formulations in the past assisting in pre-clinical development of several vaccines against viral diseases¹⁰⁶, nicotine addiction¹⁰⁷, melanoma¹⁰⁸, and allergy⁷⁷ to name a few.

As most of RNA phages, 180 copies of the capsid monomer are organised in quasi-equivalent conformation forming an icosahedral structure that assembles around RNA⁹. The packed RNA interacts with arginine-rich repeats through electrostatic forces and orients particle assembly⁹⁵. The nucleic acid is essential for assembly of the VLP, however once the VLP is correctly assembled the nucleic acid can be removed by different methods for RNA degradation without affecting the stability of the particles¹⁰⁹. Moreover, the empty Q β capsid can be re-packed or re-assembled with different types of nucleic acids, such as CpGs⁷⁸. The superior stability of Q β as a VLP and the several well characterised methods for manipulating the particle resulted in a versatile platform that can be modified and adjusted according to the needs of a given vaccine under development.

1.7 Preface

As pointed out by Stanley and Susan Plotkin in a very concise and elegant review about the development of vaccines published in 2011, every vaccine developed after the *M. bovis* bacille Calmette–Guérin vaccine, was developed firstly relying on theoretical and immunological hypothesis¹¹. They claim that by applying the lessons learnt in immunology and the new techniques in system biology will lead scientists to have better decision-making algorithms in place reducing the time and cost necessary to bring a vaccine from the lab bench to the clinics. More theoretical knowledge in the adjuvant field will allow scientists to choose and develop adjuvants based on an understanding of the mechanism and the optimal circumstances in which each adjuvant should be used. Moreover, it is paramount to increase our knowledge and options of adjuvants able to engage T cells considering the great potential for immunotherapy that such cells represent. The questions addressed on this thesis is a response to the aforementioned unmet needs in the vaccine field, aiming to offer valuable insights in the interactions of vaccines and the immune system and how this knowledge can be directly translated to the development of safer and more immunogenic vaccines. The specific aims of each chapter will be specified on each chapter.

Chapter II: VLPs as a platform for T cell based vaccine – Implications of size and mechanism of antigen presentation for vaccine design

2.1 Introduction

Since the discovery of the first virus-like particle (VLP) derived from hepatitis B virus (HBV) in 1970³⁶, the field of VLP-based vaccines has expanded from autologous virus-targeting vaccines – *i.e.* HBV vaccines and the recent HPV Gardasil and Cervarix⁴⁵ - to powerful vaccine platforms that can be used to present heterologous and even self-antigens, in addition to applications as delivery systems for controlled release of additional adjuvants (reviewed⁴).

The rationale for vaccine development had traditionally relied on prophylactic vaccines, however the concept of educating the immune system to target and control diseases have also be expanded to therapies against established diseases. HPV is the main cause of cervical cancer worldwide and since 2007⁴⁵, prophylactic vaccines had been licensed and are currently being used across the world. However, to those previously exposed to the virus, the prophylactic vaccine has been shown inefficient. HPV-related tumours express certain HPV proteins at high levels, that contrary to non-virus related cancers, expressing an easy foreign antigenic target, rendering HPV-related tumours a perfect target for therapeutic vaccines.

The development of VLPs presenting heterologous antigens usually involves the selection of a suitable VLP and the antigen of choice that should be physically

associated to the particle either by genetic fusion or biochemical crosslinking. The genetic conjugation of VLP and antigen offers two main benefits: 1) To guarantee uniform decoration of the particle with the antigen and 2) reduce the number of steps in manufacturing the VLP, which renders the process cheaper and faster. However, the addition of a heterologous antigen may disrupt the tertiary structure of the VLP, alter the biophysical properties of the particle or even fail to expose the antigen on the surface, restricting the possibilities of vaccine formulation to antigens that do not interfere with the assembly of the VLP. Biochemical conjugation is often the choice for antigens that disrupt the VLP or whenever the goal is to develop vaccines for fast response as in the case of epidemics. However, the additional manufacturing step and the resulting efficiency of conjugation can vary greatly between batches, which could be a major problem for GMP production and commercialisation. Therefore, to provide alternatives and understand the requirements for appropriate induction of the immune response against heterologous antigens displayed on VLPs is of major importance in the field of vaccines.

It is considered common sense that physical association of adjuvants and VLPs displaying heterologous antigen ensures simultaneous delivery of all vaccine components to the same antigen-presenting cells (APCs). Concomitant delivery of antigen and adjuvant promotes cellular activation and potent antigen-specific immune responses, which it is expected to result in production of antibodies and T cell activation⁸. However, when considering the differential modes of antigen acquisition of different APCs, for instance DCs and B cells, physical conjugation may not be necessarily required to achieve the same level of activation. DCs are considered the major APC due to its remarkable capacity of antigen uptake

throughout the body via unspecific micropinocytosis, receptor mediated endocytosis and phagocytosis¹¹⁰. This diverse uptake machinery enables DCs to uptake virtually any type of antigen. B cells on the other hand, acquired antigens via receptor-mediated endocytosis, the consequence of that is that B cells are limited to the uptake of antigens that are recognised by their cognate BCR¹¹¹. This is an essential feature of humoral responses that depend on Th cells, as the receptor-mediated interaction restricts B cells to only respond to Th cells that recognize the same antigen.

In a scenario where antigen and VLP are physically associated, DCs via non-specific endocytosis will process and present the antigen displayed on VLPs whilst receiving costimulatory signals by the VLP-incorporated adjuvant¹¹⁰. Similarly, antigen-specific B cells recognizing the antigen via BCR will also be exposed to VLPs linked to the antigen. On the other hand, in formulations of vaccines where antigens are not physically associated with the VLP, both components should be equally taken up by DCs, but not by B cells that will either uptake the antigen, the VLP or nothing, depending of the specificity of the BCR. Recent literature has shown this empirically by demonstrating that the adjuvant CpG linked to an antigen enhances B cell responses promoting isotype switching and affinity maturation by activating B cells directly through TLR9 recognition¹¹², which required simultaneous internalization of antigen and CpG²⁷. By contrast, CpGs mixed with antigen primarily enhance B cell responses by facilitating Th cell activation, thereby increasing antibody responses indirectly¹¹³. In summary, it seems important that the immunological target cells - DCs or B cells - are simultaneously activated by the adjuvant and exposed to the antigen⁵³, however the means to achieve this end may be different for the DCs and B cells.

Here, we demonstrate in mice that physical association of particulate antigen and VLPs loaded with the adjuvant B type CpG 1668 is critical for B but not T cell responses. As a model system, we used the E7 protein from human papilloma virus (HPV), which spontaneously forms oligomers with molecular weight ranging from 158 kDa to 10 MDa and an average size of 50 nm¹¹⁴. E7 oligomers were either chemically linked or simply mixed with VLPs loaded with DNA rich in non-methylated CG motifs (CpGs), a ligand for toll-like receptor 9. E7-specific IgG responses were strongly enhanced if the antigen was linked to the VLPs. In contrast, both CD4⁺ and CD8⁺ T cell responses as well as T cell-mediated protection against tumour growth were comparable for linked and mixed antigen formulations. Here, we demonstrated that a VLP-based vaccine containing CpG protects against morbidity and mortality related to tumour growth. In addition to that, our data shows that B cell but not T cell responses require antigen-linkage to the carrier and adjuvant for optimal vaccination outcome, elucidating another characteristic of the immune system that can be directly translated and applied to the development of immunotherapies.

2.2 Experimental Approach and Research Questions

2.2.1 Experimental Approach

This chapter aims to explore the differential modules of interaction of VLPs and the main immune cells following therapeutic immunisation in a tumour model. Draining properties of VLPs and interaction with DCs is characterised. Tumour

protection mediated by immunisation is assessed. T and B cell response against VLP and antigen of different sizes is measured.

2.2.2 Research Questions

1. Can a heterologous VLP presenting an HPV-derived antigen confer protection and tumour control as a therapeutic vaccine?
2. How does antigen size impacts the immune response of B and T lymphocytes against a tumour antigen presented in a VLP-based vaccine?
3. Is linkage of antigen and VLP necessary for improved immunogenicity even when antigen and VLP have the same size?

2.3 Materials & Methods

A complete list of materials and reagents, suppliers and catalogue codes utilised in this thesis can be found in the supplementary materials (Table S.3 to S.7). Experiments were designed, performed, and analysed by the writer of this thesis unless stated otherwise. In the case of experiments performed by collaborators, the name of the author is indicated alongside the data, but design and analysis of every experiment were performed by the writer of this thesis.

2.3.1 VLP and E7 Production

The 98 amino acids oncoprotein HPV-E7 containing a short C-terminal GGC linker was expressed in *Escherichia coli*, solubilized from the inclusion body fraction by 8M urea and purified using affinity and size-exclusion chromatography. The denatured protein migrated as a 14-kDa single band in reducing SDS-PAGE (data

not shown). Following refolding by dilution and dialysis against NaCl-MES-containing buffer, E7 protein spontaneously formed oligomers. E7 protein was cloned and purified by Ana Flace.

Q β Plasmids were transformed into *E. coli* JM109. A seed culture was prepared by inoculating a single colony into LB medium containing Ampicillin and growing the static culture overnight at 37°C. For expression, the overnight culture was diluted in M9 medium supplemented with casamino acids (Fisher Tech), glucose, MgCl₂, Vitamin B, and Ampicillin followed by incubation at 37°C with vigorous aeration for 14–20 hours. Cells were re-suspended in NaP buffer (20 NaHPO₄, 150 mM NaCl, pH 7.2) containing 1 mg/ml lysozyme, 0.1% Triton X-100, 125U of Benzonase per grams of cell, and lysed by three freeze thaw cycles followed by ultrasonication. Clarified lysates were purified by anion exchange with Fractogel® EMD TMAE (S) (Merck), equilibrated with NaP buffer, protein was eluted with a gradient of NaP/NaCl buffer (20mM Na HPO₄, 1M NaCl, pH 7.2). The VLP-containing fractions were further purified by size exclusion chromatography using the Sephacryl S500 column (GE healthcare) equilibrated with PBS. The absorbance was monitored at 214 nm, 260 nm and, 280 nm and the selected fractions were analysed by SDS-PAGE followed by Coomassie staining and agarose gel stained with SYBR safe and Coomassie.

2.3.2 CpG ODN and Antigen Sequence

CpGs 1668 with phosphorothioate backbone were purchased from Invivogen (sequence: 5' tccatgacgttctctgatgct 3'). The protein and peptides E7₄₉₋₅₇

were produced in a modified version with additional 3 aa (GGC) added to the C terminus (Proimmune) to allow coupling to VLPs. E7 protein sequence UniProt database: P03129. E7₄₉₋₅₇ peptide sequence: RAHYNIVTFGGC.

2.3.3 Measurement of Anti-E7 and Anti-VLP total IgGs

Antigen-specific total IgG were measured by ELISA. 96-well plates were coated overnight with 5 µg/mL of unmodified VLP or 5 µg/mL of E7 oligomers. Plates were blocked for 2 h with 2% bovine serum albumin phosphate-buffered saline (PBS), serum obtained from vaccinated or control mice (12 dilutions starting at 1:100 to 1:12,500) was added and plates were incubated for 2 h at room temperature. Total IgGs was detected by horseradish peroxidase-labelled goat anti-mouse immunoglobulin G (IgG-HRP) (Sigma) followed by the addition of 3,3',5,5'-tetra- methylbenzidine (TMB, Sigma). Reactions were halted with 0.1M H₂SO₄ and absorbance read at 450 nm (OD450). Titres are expressed as serum dilutions reaching the half-maximal OD (OD50).

2.3.4 Association ELISA

Plates (Nunc-Immuno MaxiSorp) were coated with anti-E7 antibody, each of the vaccine preparations were added at a concentration corresponding to 60 ng/mL of E7 protein. Following washing and incubations, anti-Qβ monoclonal antibody was added followed by secondary detection antibody goat anti-mouse IgG-HRP and TMB. Data is expressed in OD450.

2.3.5 Vaccine Preparation

Vaccines were prepared by chemical coupling using the heterobifunctional cross-linker succinimidyl-6-(β -maleimidopropionamido)hexanoate (SMPH, Pierce). Purified Q β VLPs (2 mg/mL in PBS) were derivatised by a 1-h incubation at room temperature with a 10-fold molar excess of SMPH. Free cross-linker was removed by diafiltration with Amicon Ultra Centrifugal Filters, 100 kDa MWCO. Derivatised Q β VLPs and E7₄₉₋₅₇-GGC (peptide at five-fold molar excess) or E7 protein were then incubated for 3 h at room temperature to allow cross-linking. Unbound material was removed by diafiltration with Amicon Ultra centrifugal filters, 100 kDa MWCO for the peptide and size exclusion for the E7 protein. Efficiency of cross-linking was analysed by SDS-PAGE.

Packaging of CpG into Q β VLPs was performed by digestion of bacterial RNA packaged into VLPs during recombinant expression with RNase A (Merck) for 5 h at 37° C in low-ionic strength buffer. RNase-treated VLPs were combined with 120 nM/mL of CpG and incubated for further 3 h at 37°C. Excess of CpG and RNase was removed by dialysis against PBS.

2.3.6 Immunisation and Sample harvesting

This study was carried out in accordance with the recommendations of the Animals (Scientific Procedures) Act 1986 (ASPA) and European Directive 2010/63/EU on the protection of animals used for scientific purposes.

C57BL/6 mice (female 9–12 weeks old; Envigo) were injected s.c. with 80 μ g of either vaccine formulation. Spleen or blood was collected from the tail vein and serum and PBMCs separated for further analysis at indicated time points.

Spleens were isolated and a single-cell suspension was prepared by incubating organs with 1 mg/mL collagenase D (Roche) and 0.04 mg/mL DNase I (Boehringer) in 5% FSC containing DMEM, for 30 min at 37°C. Organ pieces were passed through a 70-µm cell strainer and stained for cell-specific markers. PBMCs were collected from the tail vein in PBS containing 0.25mM of EDTA. Red blood cells were lysed using ACK buffer (Lonza). Freshly isolated splenocytes were treated with FcBlock (eBioscience) and stained with the following fluorochrome-labelled antibodies: anti-CD3, anti-CD8a, Live-Dead dye, anti-IFN-γ, anti-TNF-α, and anti-IL-17 (all from eBioscience). E7-specific tetramer were obtained from NIH tetramer facility. Cells were incubated with Anti-CD8 and 2.5ug/mL of E7-tetramer for 30 min.

2.3.7 In vivo Trafficking experiment and In vitro uptake of VLP and peptide

E7 protein or E7₄₉₋₅₇ were labelled with AlexaFluor 488 C5-maleamide as instructed by the manufacturer. Qβ VLPs were labelled with AlexaFluor 647 or PE containing a NHS reactive group as instructed by the manufacturer (all from Thermo Fischer). C57BL/6 mice (9–12 weeks old; Envigo) were injected s.c. with 30 µg of fluorescent VLP and peptide into the hind leg.

Popliteal and inguinal LNs were isolated and a single-cell suspension was prepared by incubating LN with 1 mg/mL collagenase D (Roche) and 0.04 mg/mL DNase I (Boehringer) in 5% FSC containing DMEM, for 30 min at 37°C. Organ pieces were passed through a 70-µm cell strainer and stained for cell-specific markers. The following fluorochrome-labelled antibodies were used: anti-CD11c, anti-CD8a (all from eBioscience), anti-F4/80, Live/Dead Aqua cell stain (all from Life Technologies).

Primary culture of DCs from LNs of a naïve mouse was prepared as described previously and incubated for 24h with 10nM of E7₄₉₋₅₁ conjugated with Alexa488 and 1 µg/mL of Qβ conjugated with Alexa647. CD11cF4/80⁺ DCs were subsequently analysed by flow cytometry for uptake of peptide and Qβ.

Flow cytometry analysis were performed using a BD FACSCanto II. Data analysis was performed in the FlowJo software (TreeStar).

2.3.8 Tumour Model and vaccinations

Female C57BL/6 mice at age of 10–11 weeks were injected with 1.5×10^5 TC-1 cells expressing HPV16 E7 oncoprotein. Eight days post injection of tumour cells, mice developed palpable tumours and were subjected to a weekly immunisation schedule with E7 coupled or mixed to Qβ(1668) for 49 days. Tumour size was recorded daily and was calculated as follows: $W \times W \times L / 2$, where W is tumour width (centimetres) and L is tumour length (centimetres). Mice showing signs of excessive suffering or reaching tumour size larger than 1,000 cm³ were euthanized. Survival rate was analysed by Log-rank (Mantel-Cox) test. Tumour challenges and monitoring of mice were performed by Ana Flace.

2.4 Results

2.4.1 Characterization of Qβ-VLPs and E7

Upon expression and purification of VLPs and antigens, we have characterised the size of Qβ and E7. Qβ particles upon expression assembles within the prokaryotic

host without requiring further folding. E7 protein however, requires refolding upon expression and purification. Size-exclusion chromatography (SEC) analysis of the refolded E7 showed a wide distribution of the elution peaks corresponding to molecular weights between 17 kDa and 10 MDa (void volume of the column, figure II.1.A, upper panel). To reduce the heterogeneity of the supramolecular forms of E7, the refolded protein was treated with reducing agent DTT (1,4-dithiothreitol), which resulted in a narrow molecular weight distribution to a single peak with calculated average size of 600-800 kDa (figure II.1.A, bottom panel) confirming the role of disulphide bonds in E7 multimerization. The oncoprotein spontaneously forms spherical oligomers with a calculated diameter of 50nm as estimated by electron microscopy (EM) and described elsewhere¹¹⁴.

The monomeric capsid protein of the Q β bacteriophage with a molecular weight of 14 kDa was expressed in *E. coli*. Upon expression, the monomers spontaneously dimerizes and self-assembles into a supramolecular icosahedral VLP with molecular weight of approximately 3 MDa¹⁰². The size and integrity of VLP was assessed by EM and dynamic light scattering (DLS) demonstrating that Q β VLPs are single particles with average diameter of 30 nm (figure II.1.D). The SEC analysis of the purified VLP indicated a narrow size distribution on a S500 column (GE) (figure II.1.B). The above characterization supports the claim that Q β VLPs and non-reduced E7 oligomers exhibit roughly comparable sizes of 30–50 nm.

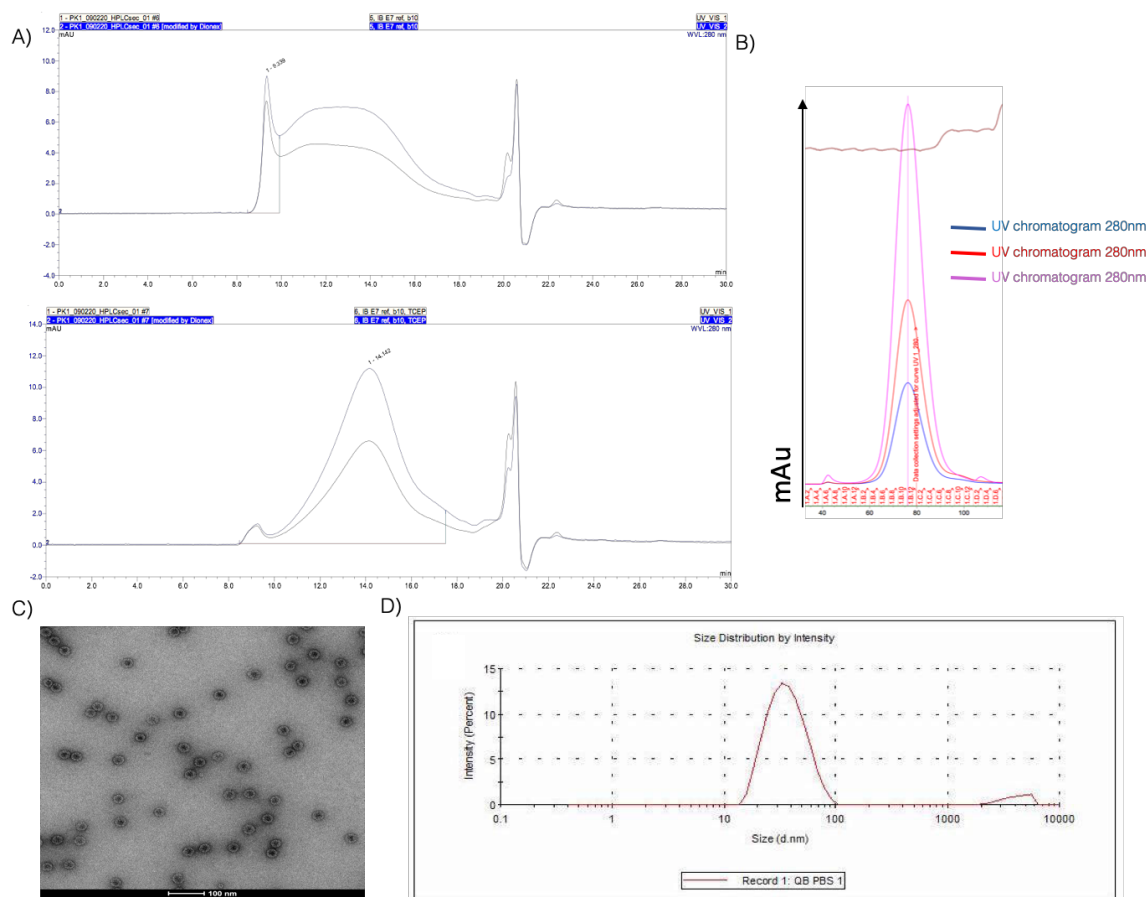


Figure II.1 - Size characterisation of Q β VLPs and E7 protein.

A) Size-exclusion chromatography of refolded E7 in buffer without reducing agent (upper panel) using TSKgel G4000SW column (resolution between 20,000-7x10⁶ Da). Refolded E7 (bottom panel) forms oligomers with elution time between 9.3 and 15 min. **B)** Size-exclusion of Q β VLPs in a S500 column eluted with PBS. Narrow peak show narrow size distribution. **C)** EM of Q β VLPs **D)** Dynamic light scattering of purified VLPs. Data is representative of 28 independent purifications. Purification of E7 was performed by Ana Flace.

2.4.2 Size dependent drainage of particles to lymph nodes and uptake by resident DCs

In order for antigens in the periphery to initiate an immune response, it is necessary for such antigens to reach secondary lymphoid organs, such as LNs. Upon s.c. injection, we assessed the drainage of VLP and antigen to LNs. VLP and the E7 protein were labelled with different fluorescent tags and injected in the hind

leg of C57BL/6 mice. Popliteal LNs were harvested 24h later and DCs analysed for uptake of Q β and E7. A subpopulation of resident DCs (CD11c^{high}F4/80⁻) in the draining LNs was found associated to E7 and Q β VLPs (figure II.2.C). A total of 23% of DCs from the popliteal LN were double positives for Q β and the antigen E7, 46.9% positive for the VLP only and 24% positive to E7 only. Thus, antigen and adjuvants, i.e., E7 and Q β VLPs, were simultaneously taken up *in vivo* by a large percentage of DCs even though there was no physical association of the two particles. To guarantee the absence of interaction or association of Q β and E7 upon mixing, an association ELISA test was performed. The ELISA plate was coated with E7-specific antibodies and incubated with the mixed or coupled formulation of vaccines. Following washing steps that removed unbound vaccine, Q β was only detected by Q β specific antibodies on the coupled formulations (figure II.2.B) ruling out possible non-covalent interactions between Q β and E7.

To corroborate that the size of particle was influencing the distribution and subsequent immune response following s.c. injection, we tested the trafficking to LNs of the E7-derived immunodominant peptide H2-Db E7₄₉₋₅₇ mixed to the VLP in similar conditions. Injection of free peptide E7₄₉₋₅₇ labelled likewise with the same fluorophore and labelled Q β VLPs did not result in simultaneous uptake by DCs in draining LNs (figure II.2.D). As a matter of fact, the peptide could not be found in LNs in the same period of time. To exclude potential methodological limitations in visualization of peptides by flow cytometry, DCs were pulsed with the peptide and VLP *in vitro*. Primary cultures of LNs resident cells of a naïve mouse were incubated for 24 h with the fluorescent peptide E7₄₉₋₅₁ and Q β and CD11c⁺F4/80⁻ DCs were subsequently analysed by flow cytometry for uptake of peptide and VLP. As shown in figure II.2.E, almost 50% of the DCs were double positive for the peptide and Q β , confirming the capacity of small peptides to be efficiently taken up by DCs

in vitro and detected by flow cytometry. This supports the hypothesis that peptides injected s.c. do not efficiently traffic to the LN due to the size, protease stability and propensity to bind to MHC class I molecules on any cell.

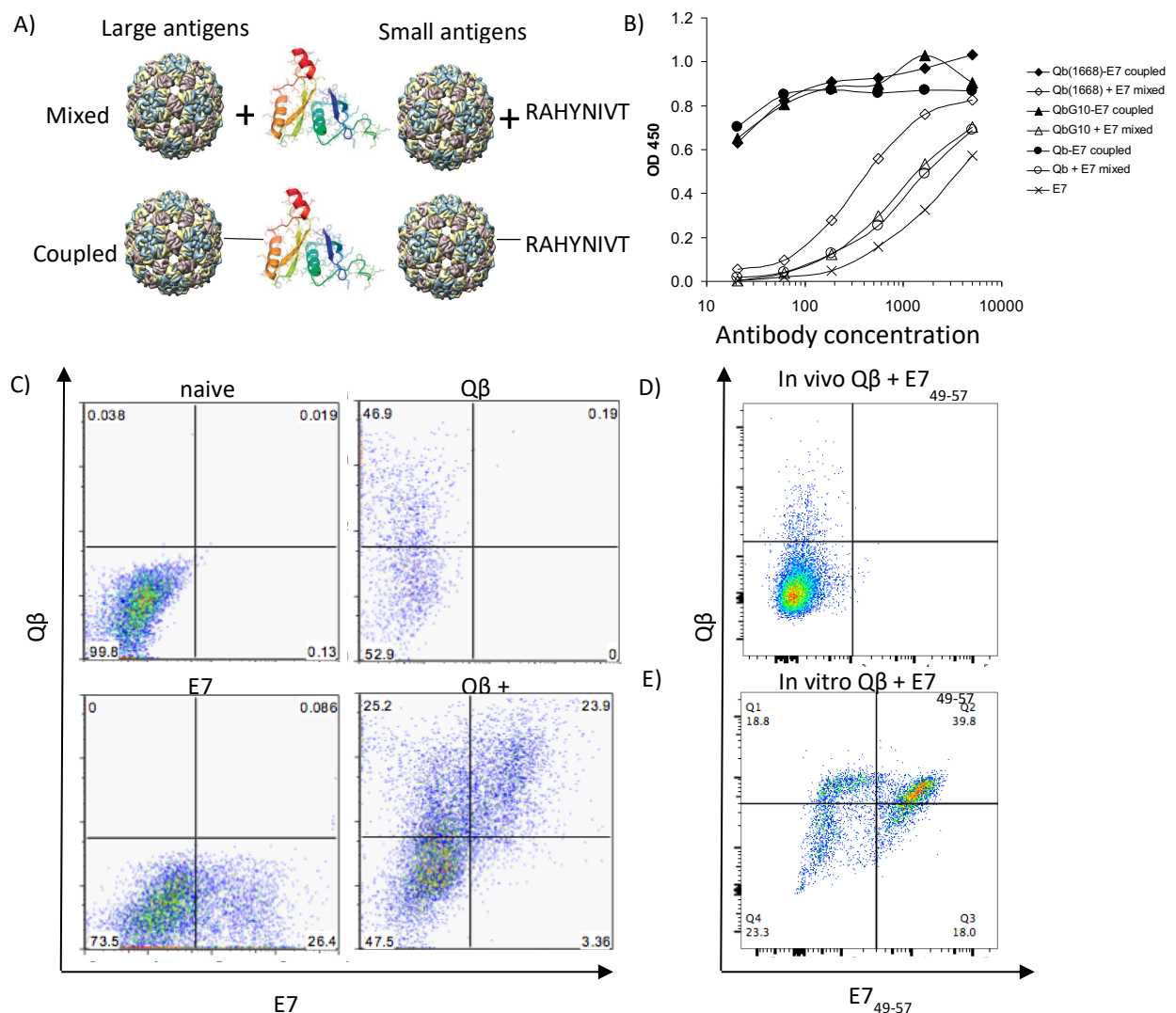


Figure II.2 - Draining of Qβ VLP and antigens to LNs.

A) Schematic representation of the system of antigens. VLPs have an average size of 30nm, E7 protein of 50nm and the peptide has a size of 1.7 kDa. The plus signal (+) indicates the mixed formulation of VLP and antigen, the dash (---) indicates chemical coupling of antigen and VLP. **B)** Immunoplate assay demonstrating the absence of physical association between VLP and E7 protein in the mixed formulation. Plates were coated with anti-E7 antibody, each of the vaccines preparation were added at a concentration corresponding

to 60 ng/ml E7 protein. Following washing and incubations, anti-Q β monoclonal antibody was added followed by detection antibody. Data expressed in optical density (OD). **C)** Q β -VLP mixed with E7 oligomers are taken up by the same population of DCs in the draining lymph nodes. Mice were injected either with Q β -VLP-Alexa488 or E7-phycoerythrin (PE) or a mixture of both particles (100 μ g of each). The uptake of the fluorescent proteins into DCs of the draining lymph node (popliteal) was analysed by flow cytometry. Frequency plot is gated on DCs (CD11c⁺ F4/80⁻). **D)** Frequency plot of cells from draining LNs 24h post injection with 100 μ g of Q β -Alexa647 and 100 μ g of E7₄₉₋₅₇ peptide labelled with Alexa Fluor 488. **E)** In vitro uptake of Q β -Alexa647 and E7₄₉₋₅₇ Alexa488. Single cell preparation from draining LN of naïve C57BL/6 mice was prepared and pulsed for 24h with 1 μ g/mL VLP and 10 nM of peptide. Frequency plot is gated on DCs (CD11c⁺ F4/80⁻). Plots are representative of results obtained from 3 mice. Trafficking results are representative of 2 independent experiments. ELISA was performed only once.

2.4.3 Protective T cell responses are dependent on packaged B-type CpG packaged into VLPs

We next investigated the immune response elicited by the vaccine candidates. To this end, C57BL/6 mice were immunised either with E7 protein alone, E7 mixed with CpG, Q β (CpG) coupled with E7, and Q β (CpG) mixed with E7. The production of the cytokine IFN- γ by CD8⁺ and CD4⁺ T cells upon in vitro re-stimulation was evaluated on day 8. E7 alone or mixed with free CpG induced low frequency of cytokine-producing T cells, with levels similar to the background (figure II.3.A and II.3.B). E7 protein mixed or coupled with Q β (CpG), on the other hand, efficiently induced CD4⁺ and CD8⁺ T cells producing IFN- γ demonstrating the superior immunogenicity of a vaccine based on Q β packaging CpGs (figures II.4.A and II.4.B). Of note, levels of cytokines produced by CD4⁺ and CD8⁺ T cells from mice immunised with E7 protein either mixed or coupled to Q β (CpG) were similar indicating that coupling does not affect the outcome of immunisation.

In order to study antigen-specific production of cytokines by T cells in more detail, we focused on the two most potent vaccine candidates , i.e. Q β (CpG) and E7, either mixed or coupled

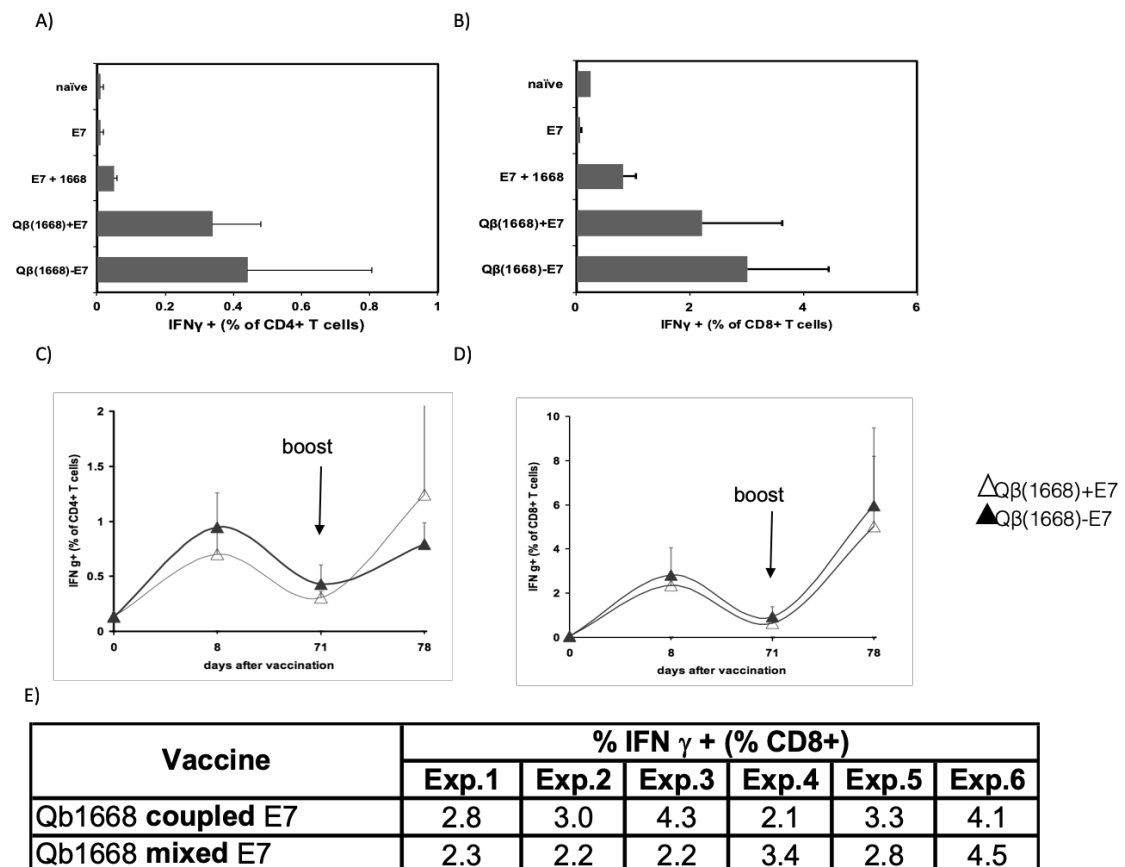


Figure II.3 - Coupling of antigens with similar size to VLPs is not required for T cells responses.

C57BL/6 mice were immunised and T cell responses were assessed 8 days later. **A)** IFN- γ production by CD4+T cells after immunisation with E7 protein, E7 mixed with free CpG 1668, Q β (CpG) mixed (+) with E7 and Q β (CpG) coupled (-) with E7. **B)** IFN- γ production by CD8+T cells after immunisation with mixed or coupled vaccines. Boost was administered 70 days after first dose, blood was collected on day 0, 8, 71 and 78 for IFN- γ measurements. **C)** Kinetics of IFN γ production by CD4+ T cells from blood. **D)** Kinetics of IFN- γ production by CD4+ T cells from blood. Data represented as mean + standard deviation n = 5 mice per group. Results are representative of 6 individual experiments, summarised in **E)**.

Mice were primed on day 0 and boosted on day 71. Cytokine production was assessed 8 days after priming and on day 71 and 7 days post boosting (day 78). The production of cytokines by CD4⁺ and CD8⁺ T cells (figure II.3.C and II.3.D, respectively) was similar for the mixed and coupled formulations suggesting that physical association of the antigen and adjuvant of same size is dispensable for antigen specific T cell responses. 70 days after immunisation, cytokine production was reduced before boost but recovered to previous levels in the blood upon boosting by day 78, indicating that memory T cells could have been induced by the vaccines.

2.4.4 Same size antigens and adjuvant do not require coupling for protective T cell responses and eradication of established tumours.

Upon establishing the (i) superior capacity of formulations including Q β (CpG) at inducing IFN- γ production by CD8⁺ and CD4⁺ T cells in response to re-stimulation with E7 and that (ii) DCs would simultaneously take up antigen and VLP even in the absence of physical crosslinking leading to strong T cell responses, we investigated whether the immune response observed was protective. To assess the therapeutic capacity of the vaccine-induced T cells, mice were injected with TC-1 cells expressing HPV16 E7 oncoprotein. Palpable tumours were observed around 8 days post injection of tumour cells, when mice were subjected to a weekly immunisation schedule with E7 coupled or mixed to Q β (CpG) for 49 days.

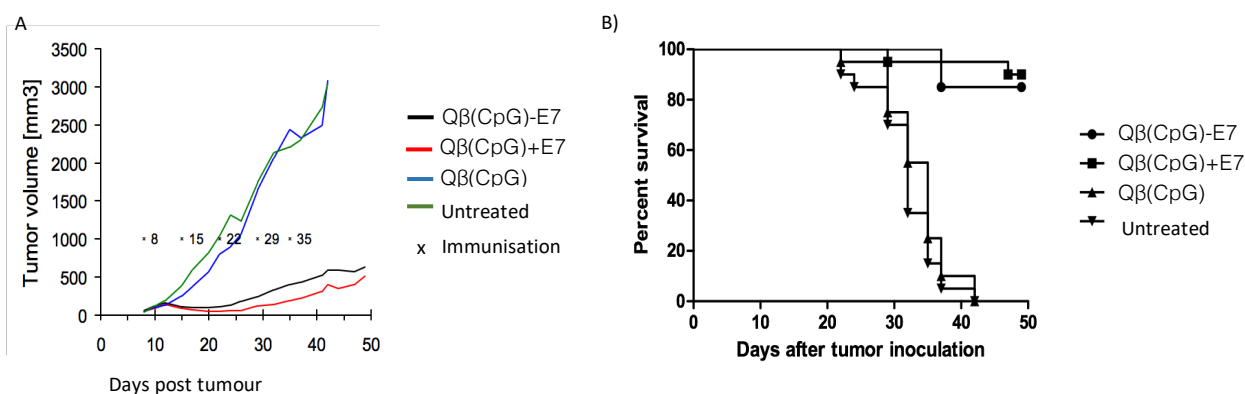


Figure II.4 - Tumour protection induced by immunotherapy.

A) Tumour growth in mice receiving vaccines or untreated controls over 50 days post tumour inoculation. C57BL/6 female mice (n=5 per group) were injected with 1.5×10^5 TC-1 cells expressing HPV-16 E7 oncoprotein. Crosses indicate the weekly immunisation schedule. **B)** Percent of survival and tumour growth in immunised groups was monitored over time and represented in a Kaplan–Meier survival curve and analysed using the Log-rank test; *P<0.05. Tumour implantation and mice monitoring were performed by Ana Flace. Results are representative of two independent experiments.

T cell responses induced by vaccination were correlated with the protection against tumour growth and survival. In untreated and control group immunised with Qβ(CpG) only, tumours grew unrestrained (figure II.4.A) and 50% of mice reached severity scores requiring euthanasia by day 32 (figure II.4.B). In contrast, coupled and mixed formulation of Qβ(CpG) and E7 protein vaccines protected against tumour growth resulting in survival rate of more than 80% until the end of the study. Thus, covalent linkage of the E7 to Qβ(CpG) VLPs is not required for induction of protective T cell responses against solid tumours if both antigen and adjuvants are particulate.

CTL responses from challenge was assessed following the end of challenge for immunised mice that had survived and non-challenged controls. Both vaccination regimens (coupled and mixed) induced CD4⁺ T cell responses with multifunctional lymphocytes producing IFN- γ , TNF- α , and IL-17 (figure II.5.A-C) and a high frequency of E7-specific CD8⁺ T cells producing TNF α and IFN- γ (figure II.5.E-F). Likewise, as observed in the blood, E7 protein crosslinked to Q β induced similar frequencies of cytokine-producing T cells compared to the mixed formulation in splenic T cells.

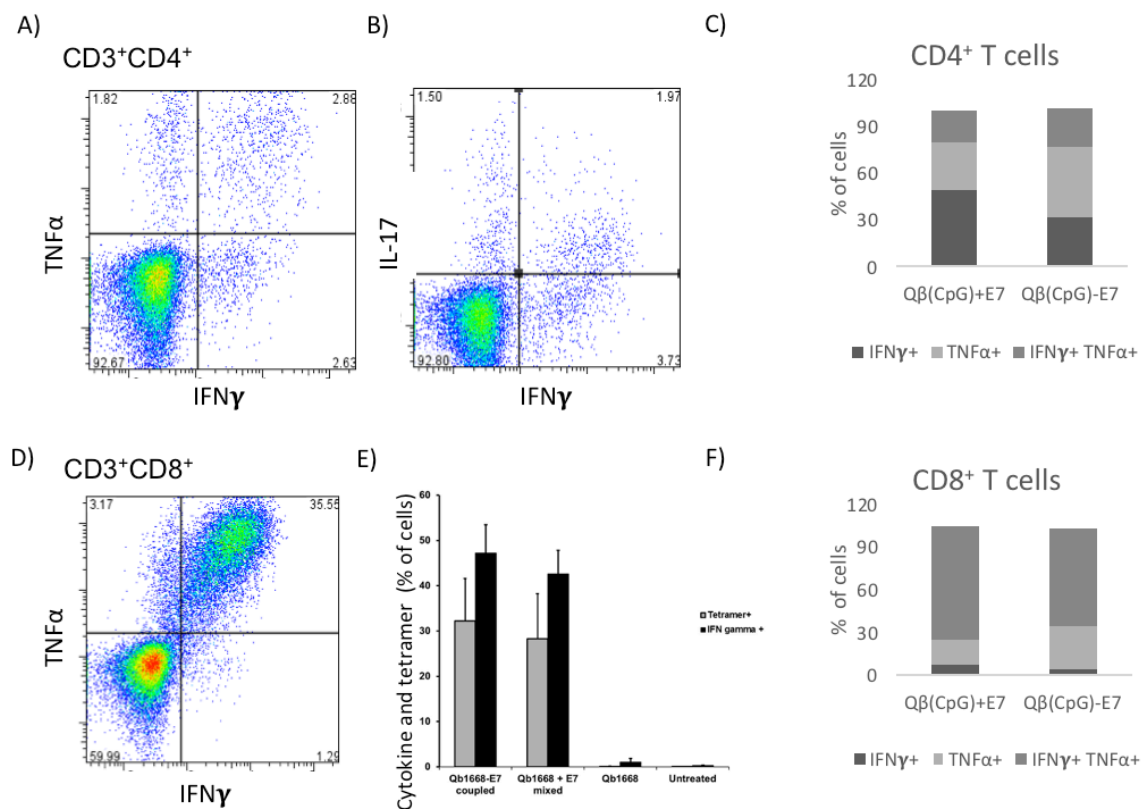


Figure II.5: Coupling of similar size antigens to VLPs is not required for cytokine production by T cells.

A) TNF- α and IFN- γ production by CD4⁺ T cells, **B)** IL-17 and IFN- γ production by CD4⁺ T, **C)** cytokine production by CD4⁺ T cells from immunised mice upon in vitro stimulation **D)** Frequency of cytokine producing cells among CD8⁺ T cells from immunised mice upon in vitro stimulation. **E)** Cytokine production by E7-tetramer specific CD8⁺ T cells. **F)** Frequency

of cytokine producing cells among CD8⁺ T cells upon in vitro stimulation. Data is representative of two independent experiments.

In marked contrast to the results obtained with particulate E7 proteins, for small peptides, linkage to the VLP was essential. Specifically, mice immunised with the peptide E7₄₉₋₅₇ coupled to Q β (CpG) induced antigen-specific CD8⁺ T cell producing high levels of IFN- γ and TNF- α in marked contrast to CD8⁺ T cells from mice immunised with E7₄₉₋₅₇ mixed to Q β (CpG), that produced 3 times less IFN- γ and TNF- α (figure II.6).

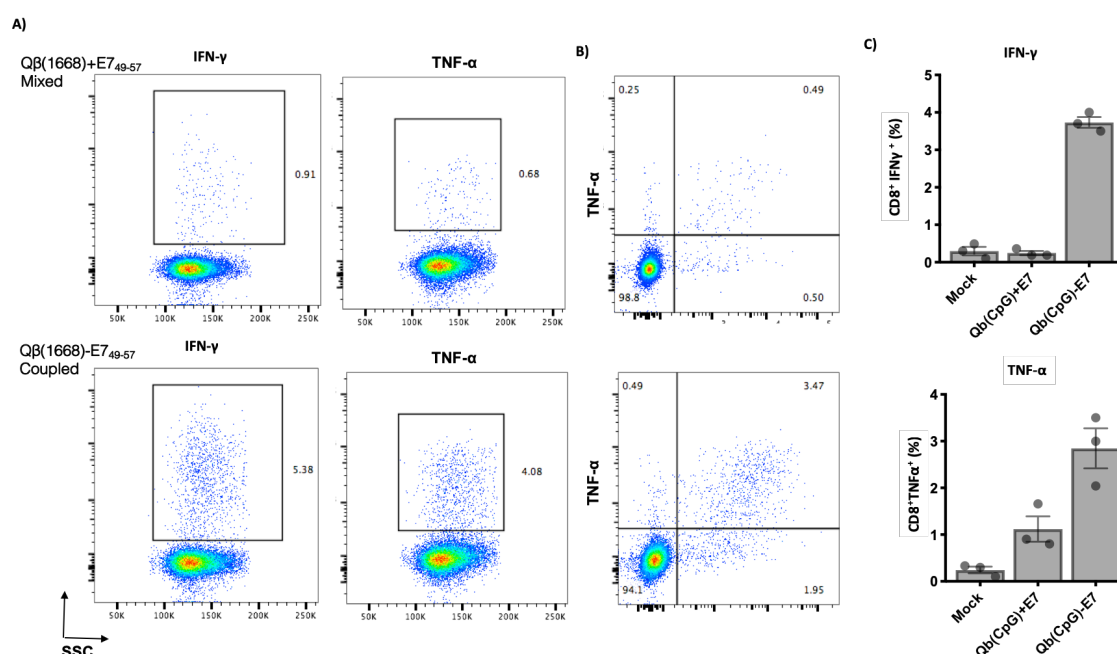


Figure II.6 - Coupling of small peptides and VLP is required for activation of CD8⁺ T cells.

C57BL/6 female mice were immunised in a prime-boost regimen (day 0 and day 7) s.c. with 50 μ g of coupled or mixed Q β (CpG) and E7₄₉₋₅₇. Spleens were harvested on day 14 and cells analysed for cytokine production. Cells were stimulated for 6h with peptide and analysed by flow cytometry. **A)** IFN- γ and TNF- α production by CD8⁺ T cells upon in vitro re-stimulation. **B)** Coupled vaccine (bottom) induces multifunctional CD8⁺ T cells but not mixed (upper). **C)** Percentage of CD8⁺ T cell producing IFN- γ and TNF- α post in vitro

stimulation. Data represented as mean plus SEM, n = 5 mice per group. Experiment is representative of 2 independent experiments. Cells analysed by FCM, 1×10^7 cells acquired per sample. Values are plotted after subtraction of background values from non-stimulated samples.

2.4.5 Optimal B cell responses are dependent of organization and repetitiveness of antigens

Considering that VLPs are well known for their remarkable capacity to induce high levels of antibodies, we next analysed E7 and Q β specific humoral responses. Serum from immunised mice was collected and analysed by ELISA. Vaccines with antigen covalently coupled to the VLP induced 10 times more E7-specific antibodies compared to the mixed formulations and 100 times more than the E7 protein alone (figure II.7.A). Conversely, IgG levels produced against Q β were higher in the mixed formulation when compared to the coupled counterpart since covalent coupling of antigens in the surface of the VLP hinders antibody production against the VLP as it reduces the surface area of the VLP being exposed (figure II.7.B).

Those observations were even more pronounced when measuring humoral response directed against small antigens as peptides. IgG levels against the E7₄₉₋₅₇ peptide in mice immunised with the mixed formulation were comparable to the background (figure II.7.C). But coupling the peptide to VLP promoted high levels of antigen-specific IgG. Hence, in contrast to T cell responses, optimal B cell responses requires physical linkage of all forms of antigen and adjuvants in order to induce appropriate levels of antibody responses irrespectively of the size of the antigen.

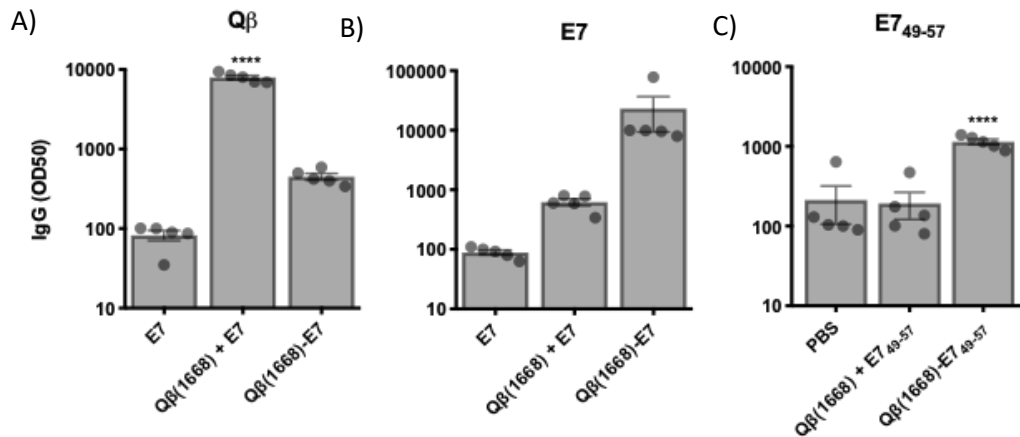


Figure II.7 - Covalent linkage is required for optimal responses of B cells.

A) Total IgG against E7 expressed in log scale of OD50 on day 71. **B)** Total IgG titres expressed in log transformed OD50 against Qβ at day 71 measured by ELISA. Mean with SEM in log transformed titres. **C)** Antibody titres expressed in OD50 against the peptide E7₄₉₋₅₇ on day 14. Values as mean (n=5 mice per group) plus SEM in log transformed titres. Antibody titre is representative of two independent experiments. Statistical differences were assessed by unpaired t test with Welch's correction. **** P>0.0001

2.5 Discussion

In the current study, we demonstrated in a tumour model expressing the HPV derived antigen E7 that a particulate VLP-based nanoparticle packaging CpG is effective as a therapeutic vaccine against established tumours. We have demonstrated that coupling of antigens and VLP of similar size is essential for improved humoral responses but entirely dispensable for protective CTL responses.

We speculate that the differential requirement of coupling for optimal B and T cells responses is a result of the distinct activation mechanism of B and T lymphocytes. For T cell activation, APCs, specifically DCs, need to be exposed and activated by

antigen and VLP. Following activation of APCs, priming and antigen presentation to T cells occur⁵⁵. The adjuvant effect of VLP for B cell responses on the other hand, is the result of direct recognition and BCR-mediated uptake of the VLP by antigen-specific B cells, thus requiring coupling of antigen to the surface of VLP.

The manner by which each cell type is exposed to the antigen and adjuvant impact greatly the activation and subsequent response of a given immune cell. We demonstrated that antigen and VLP of similar size (~30-50nm) reach secondary lymphatic organs at the same time and are simultaneously taken up by DCs. Simultaneous uptake of antigen and VLP by DCs guarantees that this important class of APCs will be activated by the adjuvant to prime T cells whilst processing and presenting the antigen to the primed effector cells. We correlated appropriate DC activation to the orchestration of protective T cell responses.

On the other hand, in order to benefit from the superior capacity of VLPs to activate B cells, antigens need to be physically associated to the VLP to be rendered immunogenic by being exposed on the surface in a repetitive and organised manner⁹⁷. Moreover, by having antigens crosslinked to VLPs, BCR-mediated endocytosis of the antigen will result in endocytosis of the VLP that will then deliver its cargo of TLR ligands packaged inside the VLP, further activating B cells that will in turn produce high affinity antibodies against the antigen^{26,112}. Conversely, in mixed formulations, B cells with BCRs recognizing E7 will fail to promote endocytosis of VLPs loaded with CpGs unless E7 is linked to the VLPs as summarised in figure II.8. The B cell-intrinsic activation is shown to be of major importance in this system as not even with the efficient activation of CD4⁺ T cells, humoral response was still reduced in absence of coupling.

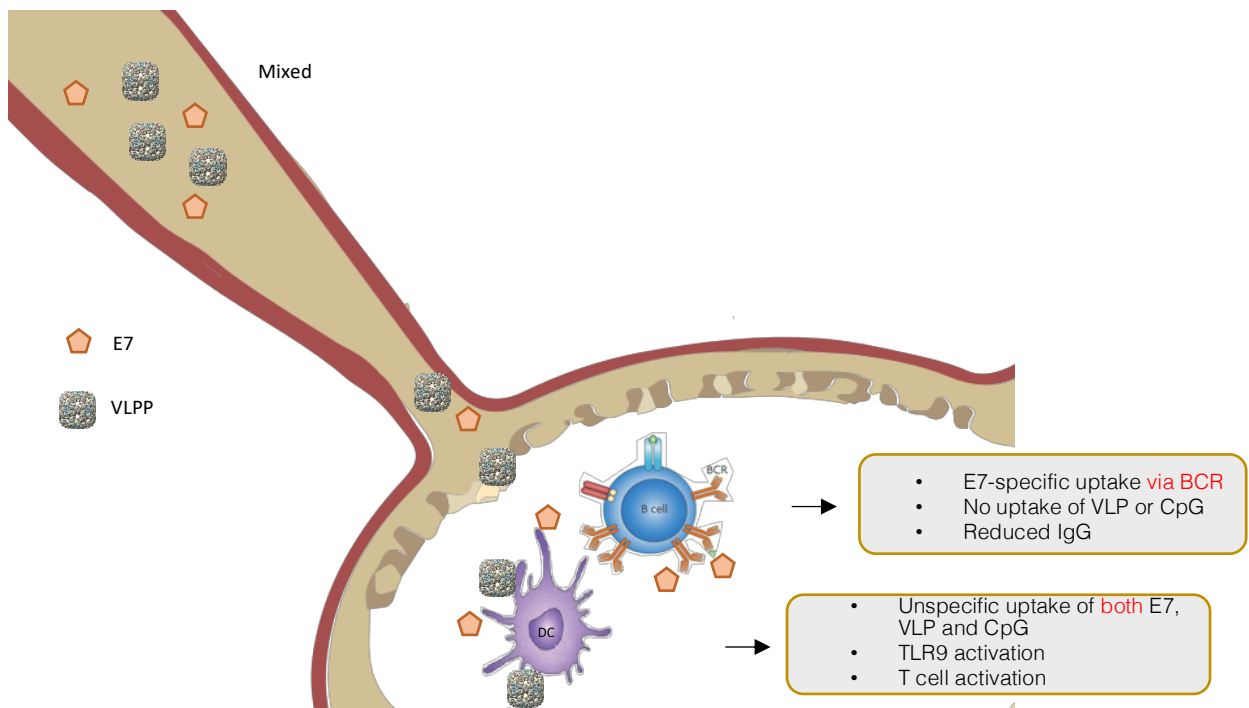


Figure II.8 - Similar size antigens interaction with DCs and B cells.

Of particular note, the conclusions from this study offers further insight to a growing body of evidence suggesting that size of antigens conveys major influence on factors driving immunity^{3,115-117}. Peptides are well known to be unable of inducing relevant immune responses; this is likely a result of inefficient drainage to LNs, degradation, or uptake and presentation by resident MHC class I positive cells *in vivo* that does not results in activation and fail to migrate to the LNs and activate T cells. We provide further evidence to this by showing that administered peptides failed to charge LN resident DCs unless coupled to VLPs.

Collectively, these observations are important for vaccine development, as they elucidate distinct requirements for induction of optimal B versus T cell responses and indicate that the use of both particulate antigens and adjuvants with similar size dispenses the necessity of conjugating the two components of the vaccine

without affecting the protective immune response mediated by T cells. This may be especially important for the development of therapeutic vaccines and fast response vaccines for epidemics, for this knowledge can be used to design antigens that would not require coupling, streamlining the manufacturing process, reducing costs and in particular timelines.

Finally, based on the findings of this work, a new advantage is attributed to nanoparticles. By manipulating the size of antigens, vaccines that do not induce high titres of antibodies while inducing potent T cell responses can also be developed, standing as an interesting tool to investigate the immune system and the interplay between humoral and cellular immunity induced by vaccination.

Chapter III: Early transcriptional reprogramming in Dendritic cells and the induction of protective T cell responses upon immunisation with VLPs

3.1 Introduction

In spite of all the prophylactic vaccines in use to date relying on antibody mediated protection there is a big interest in developing vaccines that induce protection mediated by T cells. Therapeutic vaccines against chronic viral infections and cancer seem to benefit from the induction of T cell responses, in particular cytotoxic responses mediated by CD8⁺ T cells (CTL)^{101,118,119}. Induction of effective antigen-specific T cell responses has proved itself a complex endeavour that will require major developmental strides before reaching the clinics. The complexity of cytotoxic T cell responses and the difficulty in activating such cells by immunisation in comparison to B cells is likely a direct consequence of the higher level of damage that T cells can inflict to the host in case of uncontrolled deleterious immune response⁵³. The discovery of T cell checkpoint inhibitors is a clear indicator of the several molecular restraints that had evolved in our immune system in order to control and modulate the activity of these cells.

Considering the distinct regulation and complexity of activation events for B and T lymphocytes as previously discussed in section 1.4 (chapter I), it is reasonable to assume that unlikely B-cell based vaccines, attempts to develop T-cell inducing vaccines will not be successful until we have a better understanding of the

regulatory networks of T cell responses, thereby providing a rationale to develop tools able to modulate such networks.

Dendritic cells and the orchestration of CTL responses

T cell responses are tailored by the innate immune system through events of cross-presentation and cross-priming promoted by antigen presenting cells (APCs), especially DCs³⁹. Appropriate activation of APCs is critical for successful vaccine development strategies since APCs are responsible not only for initiating but also for modulating the adaptive immune response. This is a consequence of APCs being the only cell types capable of take up antigens in the periphery, process, and migrate to sites of T cell activation, where such APCs will express co-stimulatory molecules and cytokines that activate and recruit other cell types to mount the immune response. Therefore, understanding the factors that drive appropriate activation of APCs and correlated to the adaptive response becomes paramount for vaccine development, especially where aiming for T cell mediated protection.

Subunit vaccines and adjuvants for T cells

The process of developing safer vaccines has in some cases also rendered antigenic candidates less immunogenic. Thus, the search for an appropriate adjuvant - *i.e.* an adjuvant that will modulate the response towards the desired outcome - can be considered almost as important as the choice of the antigenic target during vaccine development. TLR ligands have emerged as a promising adjuvant for vaccines due to their safety and remarkable ability to enhance immunogenicity with efficacy already proved in the clinics^{73,101,120}. However, unlike traditional antibody-inducing vaccines, the choice of adjuvants is dramatically reduced when developing T cell-based vaccines. TLR7 and TLR9 ligands, especially in combination with delivery

systems such as nanoparticles and VLPs, have been showing promising results in the induction of cytotoxic T cells and have been successfully employed in a plethora of pre-clinical vaccines for different applications. Understanding the initial events induced by TLR-ligands, in particular in DCs, can be a valuable tool for the development of effective adjuvants and therapeutic vaccines. To gain insight into these early events, we have employed systematic and integrative analysis of *in vivo* gene expression in DCs preceding three types of CD8⁺ T cell responses after vaccination: (i) absent versus (ii) inefficient versus (iii) protective T cell responses. We have previously demonstrated that VLPs containing B type CpG are able to induce protective T cell responses in tumour models caused by HPV¹²¹ and viral infections¹²² while its VLP counterparts containing RNA or no nuclei acid were unable to provide protection against the tumour growth and associated mortality. Using the same vaccines model, we have investigated the transcriptional reprogramming that DCs undergo in the first day following immunisation and the initial events and pathways correlated to cross-priming of T cells by DCs.

3.2 Experimental Approach and Research Questions

3.2.1 Experimental Approach

This chapter aims to explore the initial events following immunisation that precede protective T cell responses. By using formulations of VLPs containing different immunostimulatory sequences we investigated the impact of such vaccines in the response mediated by dendritic cells. Our aim was to find early correlates of the immune response orchestrated by DCs and the subsequent CTL response with a direct impact in the *in vivo* responses triggered by vaccines. Some

of the immune-correlates of efficient T cell responses used to measure vaccine efficacy are (i) clonal expansion of antigen-specific T cells, (ii) production of key cytokines. In our case, IFN- γ and TNF- α were chosen considering the major role of these two cytokines in mediating CTL responses and the demonstrated correlation of polyfunctional T cells and viral control¹²³.

3.2.2 Research Questions

1. Does packaging of different classes of CpGs into VLPs impacts the activation of DCs and the overall immune response?
2. What are the immune-correlates in the immune response triggered by DCs that influence T cell responses?
3. What is the impact of a TLR7 and a TLR9 ligand to the global transcriptomics of DCs immunised with VLPs?
4. What are the main immune pathways activated in response to immunisation with VLP and TLR ligands and how do they correlate to the magnitude and quality of the resultant CTL response?
5. How does the transcriptional reprogramming of DCs translate to the production and secretion of cytokines?
6. Which cytokines produced in the early time frames following immunisation are associated to later development of CTL responses?

3.3 Material & Methods

A complete list of materials and reagents, suppliers and catalogue codes utilised on this thesis can be found on the supplementary materials (Table S.3 to S.7).

Experiments were designed, performed, and analysed by the writer of this thesis unless stated otherwise. In the case of experiments performed by collaborators, the

name of the author is indicated alongside the data, but design and analysis of every experiment were performed by the writer of this thesis.

3.3.1 VLPs, vaccine preparation and immunisations

Q β VLPs were prepared as previously described in section 2.3.1.

The A type CpG G10 was custom made by Eurogentec. Sequence: 5'gggggggggggacgatcgtcgggggggggg 3'. CpGs 1668 with phosphorothioate backbone were purchased from Invivogen (sequence: 5' tccatgacgttcctgatgct 3'). The immunodominant peptides p33 was produced in a modified version with additional 3 aa (GGC) added to the C terminus (Proimmune, UK) to allow chemical crosslinking of peptide to VLPs. P33 peptide sequence: KAVYNFATMGGC.

Packing of CpG into Q β VLPs was performed by digestion of bacterial RNA packaged into VLPs during recombinant expression with RNase A (Merck) for 5 h at 37°C in low-ionic strength buffer. RNase-treated VLPs were either further purified to remove RNase and degrade RNA or combined with 120 nM/mL of CpG and incubated for further 3 h at 37°C. Excess of CpG and RNase was removed by dialysis against PBS. Re-packing and integrity of VLPs were assessed by running samples on a native agarose gel (1%) in TAE buffer (Tris, Acetic-acid and EDTA). Gel was stained with SYBR safe (Invitrogen) to detect nucleic acid, followed by Coomassie blue stain (Expedon).

Vaccines were prepared by chemical coupling using the heterobifunctional cross-linker succinimidyl-6-(β -maleimidopropionamido)hexanoate (SMPH, Pierce,

Rockford, IL, USA). Purified Q β VLPs (2 mg/mL in PBS) were derivatised by a 1-h incubation at room temperature with a 10-fold molar excess of SMPH. Free cross-linker was removed by diafiltration with Amicon Ultra Centrifugal Filters, 100 kDa MWCO. Derivatised VLPs and P33-GGC (peptide at five-fold molar excess) were then incubated for 3 h at room temperature to allow cross-linking. Unbound material was removed by diafiltration with Amicon Ultra centrifugal filters, 100 kDa MWCO for the Efficiency of cross-linking was analysed by SDS-PAGE stained with Instant Blue Protein Stain (Expedon).

3.3.2 Mice

Wild-type C57BL/6 mice were purchased from Envigo (UK). CCL2 knockout mice were described previously¹²⁴ and purchased from Jacksons lab, USA. STING and 9. Mavs-/- mice were provided by Jan Rehwinkel and were initially obtained from J. Tschopp (Lausanne)¹²⁵.

All mice used in this study were bred in specific pathogen-free (SPF) conditions. This study was carried out in accordance with the recommendations of the Animals (Scientific Procedures) Act 1986 (ASPA) and European Directive 2010/63/EU on the protection of animals used for scientific purposes.

3.3.3 Tetramer staining and cytokine measurement

Total splenocytes of immunized mice were isolated 24h following immunisation and a single-cell suspension was prepared as described on section 2.3.4 and cultured for further 12h. Secreted cytokines (G-CSF, GM-CSF, IFN- γ , IL-1 α , IL-1 β ,

IL-2, IL-4, IL-5, IL-6, IL-7, IL-9 IL-10, IL-12p40, IL-12p70, IL-13, IL-17, CCL2, CCL3, CCL5, CXCL1, CXCL2, CXCL10) were measured with a Milliplex kit (Merk-Millipore, France) following the manufacturer's instructions. Luminex MAGPIX system with xPONENT software was used for data acquisition and data analysis. IFN- α , IL-12 and IL-1 were measured by ELISA following manufacturer's instruction (Thermo Fisher, R&D, and R&D, respectively).

The T cell response was assessed 7 days following immunisation. Single cell suspension from spleen was prepared and described in the section 2.3.4. For tetramer staining freshly isolated splenocytes were stained and immediately analysed with anti-CD8 and p33 Tetramer. For Intracellular staining, cells were re-stimulated with 1 μ g/mL of p33 peptide for 1h, followed by the addition of secretion inhibitors Monensine and Brefeldina (eBioscience) and further incubation for 5h.

3.3.4 Antibodies and flow cytometry

Flow cytometry was performed using a BD FACSCanto II and the following antibodies:

APC-Cyanine7 anti-CD11c (N418, Invitrogen), PercyPCy5.5 anti-CD3, AlexaFluor700 anti-CD8, FITC anti-TNF α (eBioscience), APC anti-IFN γ (Life Tech), PE anti-CD101 (Thermo), PE anti-CD86 (eBioscience), Acqua Live-Dead dye (Thermo), FITC anti-CD40, APC conjugated Tetramer-p33₃₃₋₄₁ (H-2D^b KAVYNFATC, NIH core tetramer facility) were used.

For intracellular staining of IFN- γ , IL-12, and TNF- α , splenocytes were fixed and permeabilized with BD Cytotfix buffer. Antibody staining was performed subsequently to Fc receptor blockade (monoclonal antibody 2.4G2 to mouse CD16-CD32, 10 μ g/ml) in PBS supplemented with 0.1% FBS.

Data analysis was performed in the FlowJo software (TreeStar).

3.3.5 Cell preparation and sorting

Spleens were isolated and a single-cell suspension was prepared by incubating spleens with 1 mg/mL collagenase D (Roche) and 0.04 mg/mL DNase I (Boehringer) in 5% FCS containing DMEM, for 30 min at 37°C. Organ pieces were passed through a 70- μ m cell strainer, red blood cells were lysed with ACK solution (Lonza).

Cell sorting - Sorting of splenic DCs was performed with immunomagnetic positive selection of mouse CD11c⁺ cells (EasySep Mouse CD11c, StemCell Tech) according to the manufacture's instruction. Purity was not assessed for this experiment.

BMDC and BMpDC generation - bone marrow cells were cultured in RPMI supplemented with 10% FCS, glutamine, sodium pyruvate, penicillin, and streptomycin for 6–8 d in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) containing X-63 cell supernatant for generation of conventional BMDCs or FLT3 (Insight Biotech) for generation of BMpDCs.

DCs immunisation – fully differentiated WT BMDCs were pulsed for 1h with Q β (CpG)-p33, washed and injected i.v. in the tail vein of WT and CCL2 KO

recipients. CTL responses were assessed 7 days later as described in the section 3.3.3 of this thesis.

3.3.6 Immunofluorescence and endosomal trafficking of VLPs and free CpG

Cells were cultured on glass-bottom, 0.17mm tissue culture dishes (MatTek GLASS) pre-coated overnight with poly-L-Lysine. BMDCs were generated as described in section 3.3.4. and seeded overnight. Cells were transfected with the LysoTrack dye following the manufacturer's instructions (Life Tech). Q β VLP were packed with a B type CpG 1668 coupled to a fluorescent dye (custom made, Eurogentec). Confocal microscopy studies were performed with an oil immersion objective (63x oil immersion, numerical aperture 1.4) on Zeiss LSM 710 Multiphoton Confocal Microscope.

B type CpG sequence and modifications: 5' tccatgacgttcctgatgct 3' coupled to AlexaFluor 547 dye. Packaging of CpG-AF547 was performed as described on section 3.3.1.

3.3.7 RNA preparation, RNA-seq and primary data analysis

Sample preparation

Total RNA was extracted from sorted splenic DCs (as described on section 3.3.5) from immunized mice following the manufacturer's protocol (RNeasy Midi Kit, Qiagen), using an on column DNase-I digest step.

The High Throughput Genomics Facility, Wellcome Trust Centre for Human Genomics (Oxford, UK), executed the Library preparation and sequencing.

Material was quantified using RiboGreen (Invitrogen) on the FLUOstar OPTIMA plate reader (BMG Labtech) and the size profile and integrity analysed on the 2200 or 4200 TapeStation (Agilent, RNA ScreenTape). Library preparation was completed using TruSeq Stranded Total RNA kit (Illumina) following manufacturer's instructions. Libraries were amplified on a Tetrad (Bio-Rad) using in-house unique dual indexing primers (based on DOI: 10.1186/1472-6750-13-104). Individual libraries were normalised using Qubit, and the size profile was analysed on the 2200 or 4200 TapeStation. Individual libraries were normalised and pooled together accordingly. The pooled library was diluted to ~10 nM for storage. The 10 nM library was denatured and further diluted prior to loading on the sequencer. Paired end sequencing was performed using a HiSeq4000 75bp platform (Illumina, HiSeq 3000/4000 PE Cluster Kit and 150 cycle SBS Kit).

Data analysis

All statistical and bioinformatics analysis was performed using R language (<https://www.r-project.org>) and Bioconductor packages.

Trimmomatic 0.35 package was used in order to quality filter reads and remove adapter contamination¹²⁶. The first 10 and the last base were removed after visual inspection of the read quality distribution. After trimming, read length was 64BP.

For read mapping, Star aligner v2.5.2b¹²⁷ was used on the Gencode M12 transcriptome (mm10) with settings "--outSAMmultNmax 20". Reads were assigned to genes using featureCounts v1.5.1¹²⁸ with settings "-C -B -M -s 2 -p". To calculate log2 fold change estimates between groups, a negative binomial general linear model was fit using DESeq2¹²⁹ and only genes with a Benjamini-Hochberg¹³⁰ corrected Wald test FDR of less than 5% were labelled significant. The script was developed with the assistance of Dr Julius Müller.

The overrepresentation of pathways within groups of DE genes was computed by applying a one-tailed Fisher's exact test. Only top-level pathways with $P < 0.0001$ for overrepresentation were considered. Plots were generated in R language with the package ggplot2.

Heatmaps were generated using counts per million multi mapped (CPM-MM) values as input. CPM values are mean centred, row scaled and standardized. Hierarchical clustering was performed by using the distance based Euclidean distances using complete linkage clustering.

3.3.8 Tumour model

1×10^6 B16F10p33 melanoma cells were injected into one flank of RAG^{-/-} mice (6-8 weeks old; Envigo). Twelve days later the growing tumours were collected and processed for transplantation into C57BL/6 mice (6-8 weeks old) into one flank. The test group of transplanted C57BL/6 mice was treated s.c. with 120 μ g Q β (CpGs)-p33, while the control groups was left untreated. Tumours were collected and tumour volume was measured using a caliper according to the formula $V = (W \times W \times L) / 2$, where V is tumour volume, L is tumour length and W is tumour width.

Data is represented as mean $\pm$ SEM. Comparison between the groups was performed by Student's unpaired *t* test (two-tailed or one-tailed). Survival rate was analysed by Log-rank (Mantel-Cox) test. This experiment was performed in collaboration with Mona O. Mohsen (University of Oxford/University of Bern).

3.3.9 TILs isolation

Tumours were digested by DNaseI (Boehringer) and collagenase D (Roche) in 5% FSC containing DMEM for 1 h at 37°C. Tumours were then smashed in 100µm cell strainer (Sigma-Aldrich) and washed 2x with PBS. Tumour-infiltrating lymphocytes (TILs) were collected by Ficoll-paque premium (GE Healthcare) and centrifuged for 30 min at RT at 1800 rpm. TILs were collected and kept on ice for ICS experiment.

TILs were stained with live/dead, Fc block and then anti-CD8α, IFN-γ, CD11b, Ly6C, MHC-II, CD45 mAb (eBioscience), and analyses were carried out by flow cytometry. Survival rates were evaluated and compared using the same designated vaccines per group. Transplanted C57BL/6 mice (6-8 weeks old; Harlan) were immunised twice a week and tumour sizes were monitored daily and measured using a calliper as explained previously (section 3.3.8). Mice were culled according to the humane end-point as per the score-sheet following ethical regulations in place at University of Oxford and University of Bern. This experiment was performed with the assistance and collaboration of Mona O. Mohsen (University of Oxford/University of Bern).

3.4 Results

3.4.1 Packaging of CpGs into VLPs alters endosomal trafficking and immune responses

Most VLPs derived from RNA phages rely on interactions between protein and nucleic acid to assist in the assembly and stability of the particle. After expression and purification, the content of VLPs can be manipulated with the removal of the nucleic acid followed by *in vitro* re-packing with different poly-anionic sequences such as nucleic acid of different kinds (figure III.1.B). A schematic representation of the re-packing of VLPs is shown in figure III.1.A and methods described in section 3.3.1.

CpGs belonging to different classes trigger distinctive immune activation programs when associated with liposomes compared to the free molecule. Such differences have been thoroughly investigated in the past decades. The phenomena are in part a result of altered endosomal trafficking that CpGs undergo when associated to nanoparticles¹⁷. The consensus is that depending of the secondary structure and sequence of the CpGs, the molecules will traffic through distinct endosomal routes. For instance, it has been demonstrated that B type CpGs when associated with the liposome DOTAP, gets retained for longer in the endosomal compartment before reaching lysosomes¹⁷. This spatial-temporal distribution dictates the interaction with components of the signalling cascade of TLRs. To investigate whether the same phenomena would be influencing our system, a time-lapse experiment of CpG uptake by BMDCs was performed by pulsing cells with the B type CpG 1668 containing a fluorescent dye. We have found that free CpG co-localises with the lysosomal marker LysoTrack as fast as 6 minutes after addition of CpG to the cells (figure III.1.C). Next, the spatiotemporal

distribution of Q β (1668) in BMDCs was tested in a similar setting. To that end, BMDCs were pulsed with the VLP containing CpG with a fluorescent tag, washed and fixed at the indicated time points (figure III.1.D). Co-localisation of packed CpG and LysoTrack dye was delayed in comparison to the free CpG. At the time-point 10 min (figure III.1.D upper panel), no co-localisation was observed of the CpG and the LysoTrack dye. At the 20 minutes time point, high level of co-localisation could then be detected, showing that the CpG takes longer to reach the lysosomal compartment. In accordance to literature, packing CpG into VLP alters the endosomal trafficking of those molecules¹⁷, the relevance of this observation to the immune response was assessed next.

Considering the superior pharmacokinetics of CpGs packaged into VLPs (discussed in chapter I, section 1.6.1) and previous data demonstrating the adjuvant effect of B type CpG on a therapeutic vaccine against HPV (chapter II of this thesis, ref: ¹²¹) we wanted to evaluate the impact of packaging different classes of CpG, type A and type B into VLPs in the context of T cell-inducing vaccines. To this end, the A type CpG G10 and the B type CpG 1668 were packed into Q β VLPs and immune responses triggered on pDCs and cDCs and T cells were assessed. Hereafter, VLPs packaging CpG will be represented by Q β (G10) for type A CpG G10, and Q β (1668) for B type CpG 1668.

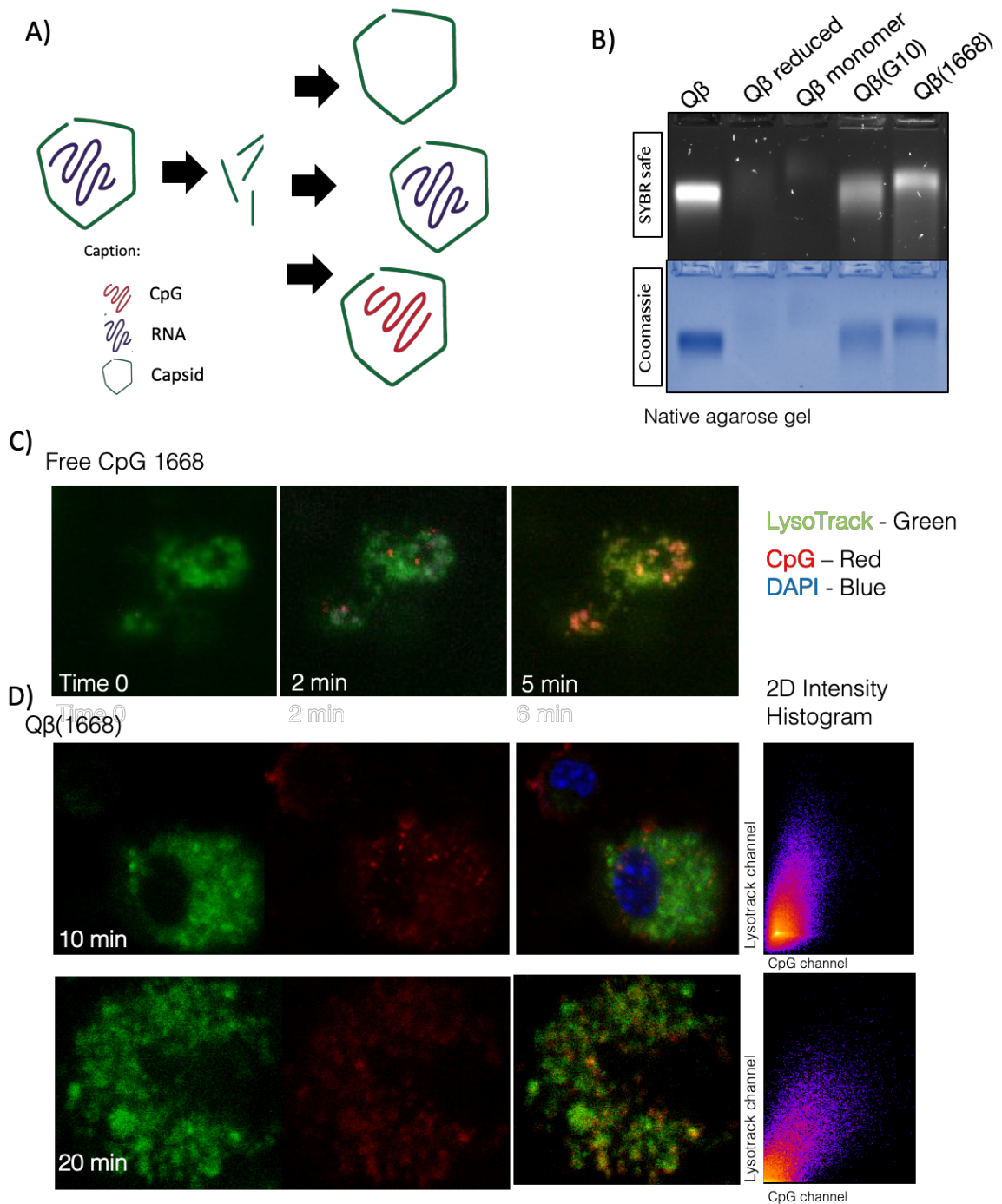


Figure III.1: Packing of CpGs on VLP alters the immune response induced by VLPs

A) Schematic representation of re-packing of VLPs with polyanionic molecules. **B)** Re-packing of VLPs analysed by co-localization of protein and nucleic acid in a native agarose gel stained with SYBR safe for nucleic acid (upper panel) and Coomassie for proteins (lower panel). First lane shows the migration pattern of a purified and intact VLP packing RNA. Lane 2 and 3 with samples from the reduced and disassembled VLP. 4 and 5 shows the re-assembled VLPs with A type CpG and B type CpG. **C)** Time-lapse of *in vitro* uptake of free CpG 1668 by BMDCs. Lysosomes is represented in green (GFP), CpG in red (Alexa Fluor 567). **D)** Endosomal trafficking of B type CpG packed into Q β VLP. BMDCs were pulsed with the VLP for 10 min (upper panel) or 20 min (bottom panel), washed and immediately fixed.

First image in green shows the endosomal compartment, followed by the CpG channel. Third image contains the superposition of both channels (plus DAPI stain for the nuclei). 2D intensity histogram showing pixel colocalization of the two channels. Data is representative of 3 independent experiments.

Firstly, we investigated the T cell responses induced by Q β (G10) and Q β (1668). To this end, VLPs re-packed with either G10 or 1668 CpGs were crosslinked with the immunodominant model peptide p33, derived from the Lymphocytic Choriomeningitis virus (LCMV). Mice were then immunised s.c. and CTL response were assessed by tetramer staining 7 days later (figure III.2.A). Q β VLP containing G10 induced relevant T cell responses, with the p33-specific population comprising 2% of the CD8⁺ population. Mice receiving Q β (1668)-p33 had at least 3-fold increase on the p33-specific T cells compared to Q β (G10) (figure III.2.B), confirming that B type CpG is superior to G10 at inducing clonal expansion of antigen-specific T cells.

In order to confirm whether packing CpGs into Q β would influence the activation state of DCs freshly isolated splenocytes were incubated with VLPs containing CpGs of either type A or B. 24h later, activation markers and cytokines were measured. Total splenocytes, pDCs, and cDCs responded in a distinct manner to free CpGs and their packed counterparts. The activation molecule CD86 was up-regulated in DCs in response to all preparations. Free B type CpG was found to be superior at inducing CD86 up-regulation especially in pDCs, where a more striking difference was observed (figure III.2.C). Type A CpG performed better when associated to the VLP. TNF- α was produced mostly by pDCs and at higher levels by the cells treated with free B type CpG or the VLPs containing either CpG (figure III.2.D). IL-12, a key cytokine associated to Th1 response and known to be induced at high levels by B type CpG was produced in different levels depending

not only on the stimuli but also the cell type being investigated (figure 2.E-F). cDCs produced higher levels of IL-12 in comparison to pDCs in response to free A type CpG and Q β (1668). When measuring IL-12 produced by total splenocytes (figure III.2.F), a different trend was observed with higher levels of IL-12 being produced in response to free CpGs. We can speculate that other cells types such as B cells that are also equipped with TLR9 would be contributing to the production of IL-12, as previously described¹⁸, however this was not assessed in this experiment.

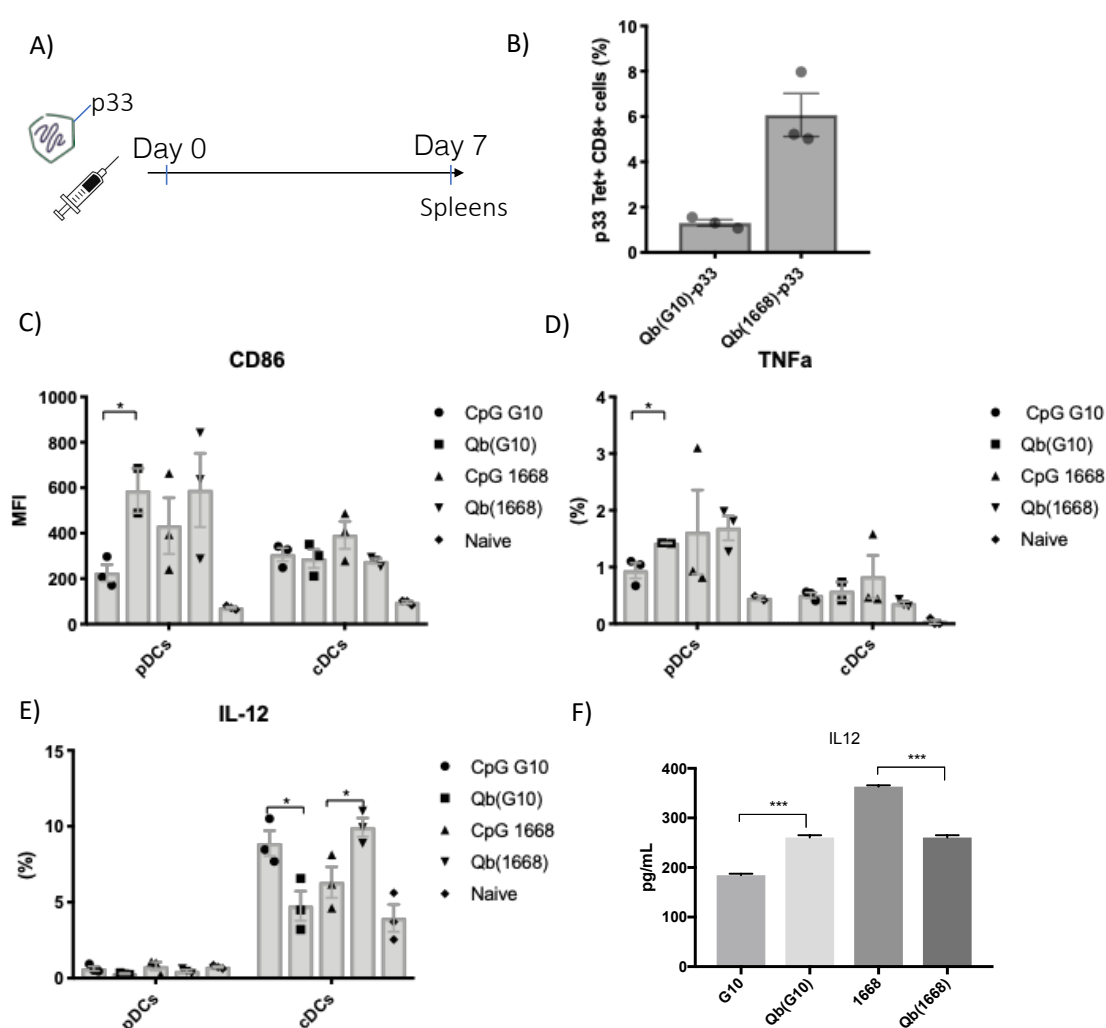


Figure III.2 – Packing CpGs into VLPs alters endosomal trafficking of CpG.

A) Immunisation schedule. Mice (n=3) were immunised s.c. with 50 μ g of Q β containing either G10 type A CpG (Qb(G10)-p33), or 1668 B type CpG Q β (1668)-p33. Spleens were collected 7 days later and p33 antigen-specific CD8+ T cells were analysed by **B)** p33-

specific Tetramer staining. Data represented as mean + SEM. **C)** CD86 expression and **D)** TNF- α production by splenic pDCs and cDCs. **E)** IL-12 production by splenic cDCs (CD11c+CD317-) and pDCs (CD11c+CD317+) treated with 1 μ M of the respective CpG or 100 μ g/mL of VLP for 24h. **F)** IL-12 production by murine splenocytes incubated with either 1 μ M of the free CpG or 100 μ g/mL of the respective VLP for 24h. Data represented as Mean + SEM. Each observation represents cells from one individual mouse, n = 3 mice per experiment. Statistical significance was measured by ANOVA followed by multiple *t*-tests followed by Bonferroni and Dunn's correction for multiple testing ****p*<0.001, **p*<0.01.

3.4.2 Q β packing B type CpG induces superior antigen-specific CD8+ T cell responses compared to Q β packing RNA

It has been consistently shown in the literature that VLP-based vaccines containing CpG are able to induce strong immune responses and enhance protection in different challenge models. For instance, Q β (CpG) coupled to a LCMV-derived peptide had shown excellent protection in a modified vaccinia virus challenge model⁹⁹ expressing the p33 antigen and tumour cells expressing the E7 protein from HPV¹²¹. The consistent and superior ability of Q β (CpG) to induce protection in different models in a CTL-dependent manner propelled us to further investigate the initial events in the immune response that were leading to appropriate activation of T cells.

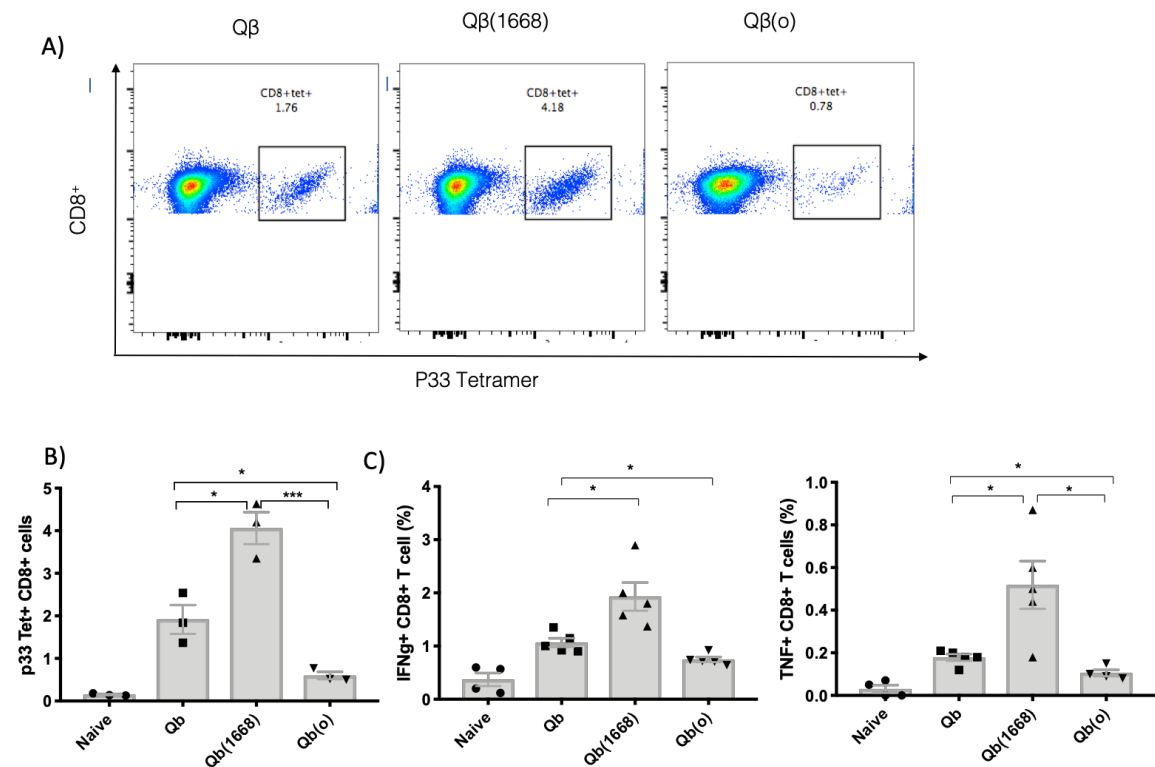


Figure III.3: The type of TLR ligand impacts the CTL responses elicited by Qβ-p33 vaccines

C57BL/6 mice were vaccinated s.c. with 50ug of VLP-p33 containing RNA (*E. coli*), CpG class B or the empty capsid. **A** and **B**) p33-specific tetramer staining of CD8⁺ T cells from 3 immunised mice and **C**) ICS staining of CD3⁺CD8⁺ T cells 7 days post vaccination. 6h stimulation with p33 peptide from 5 immunised mice. Data represented as Mean + SEM. Statistical significance was measured by unpaired two tailed *t*-tests followed by Bonferroni-Dunn's correction for multiple testing. **p*<0.01, ****p*<0.0009. Data is representative of 3 independent experiments.

To demonstrate the differential induction of T cell responses promoted by different TLR ligands, CpG, RNA or absence of TLR ligand *in vivo*, VLPs containing B type CpG 1668 (hereafter represented as Qβ(CpG)), prokaryotic RNA (represented as Qβ) and the VLP without nucleic acid (as Qβ(o)) were crosslinked to the immunodominant LCMV-derived peptide gp33₃₃₋₄₁ and mice were immunised via s.c. route. The antigen specific CTL response was evaluated 7 days later after immunisation. Addition of CpG to VLPs had not only increased the expansion of p33-specific T CD8⁺ cells as previously demonstrated by tetramer staining (figure III.2.B and figure III.3.A and B), but had also boosted their capacity for producing

key cytokines such as IFN- γ and TNF- α (figure III.3.C). Formulations containing RNA although still able to induce antigen specific T cell responses and cytokine production, had a less pronounced response compared to B type CpG counterparts. VLPs devoid of nucleic acid on the other hand, had responses comparable to the samples harvested from naïve mice.

Having established and validated the biological effect to investigate CTL responses, we then moved to interrogate the reprogramming of the innate immune system focusing on DCs hoping to find correlations between the activation of DCs and CTL responses.

3.4.3 Transcriptional reprogramming in splenic DCs 24h post immunisation with VLPs containing TLR9 or TLR7 ligands as adjuvants

To evaluate the impact of the nucleic acid packaged within the VLP in the innate immune response, mice were immunized with 50 μ g of the respective VLP s.c. and 24h later spleens were collected and processed for analysis. Total RNA was extracted from sorted CD11c⁺ murine splenic cells. Following rigorous quality control, RNA samples were analysed by genome-wide RNA-sequencing.

Mice immunised with VLP devoid of TLR ligands were used as control for the groups receiving VLPs associated with TLR ligands. The baseline normalised log2 gene expression values for genes with a significant change in expression between groups (adjusted P value < 0.05 FDR) were classified as differentially expressed genes (DEGs). The differential expression of these genes over time was analysed for statistical significance by one-way analysis of variance (ANOVA);

TLR engagement by either CpG or RNA led to differential expression of more than 3800 genes in comparison to the formulation devoid of TLR (3855 genes between Q β (CpG) versus Q β (o) and 4504 genes between Q β versus Q β (o)), showing the major transcriptional changes that TLR engagement promotes on the cell, while Q β vs Q β (CpG) had far fewer genes differently expressed, with 279 genes in total (figure III.4.A and B).

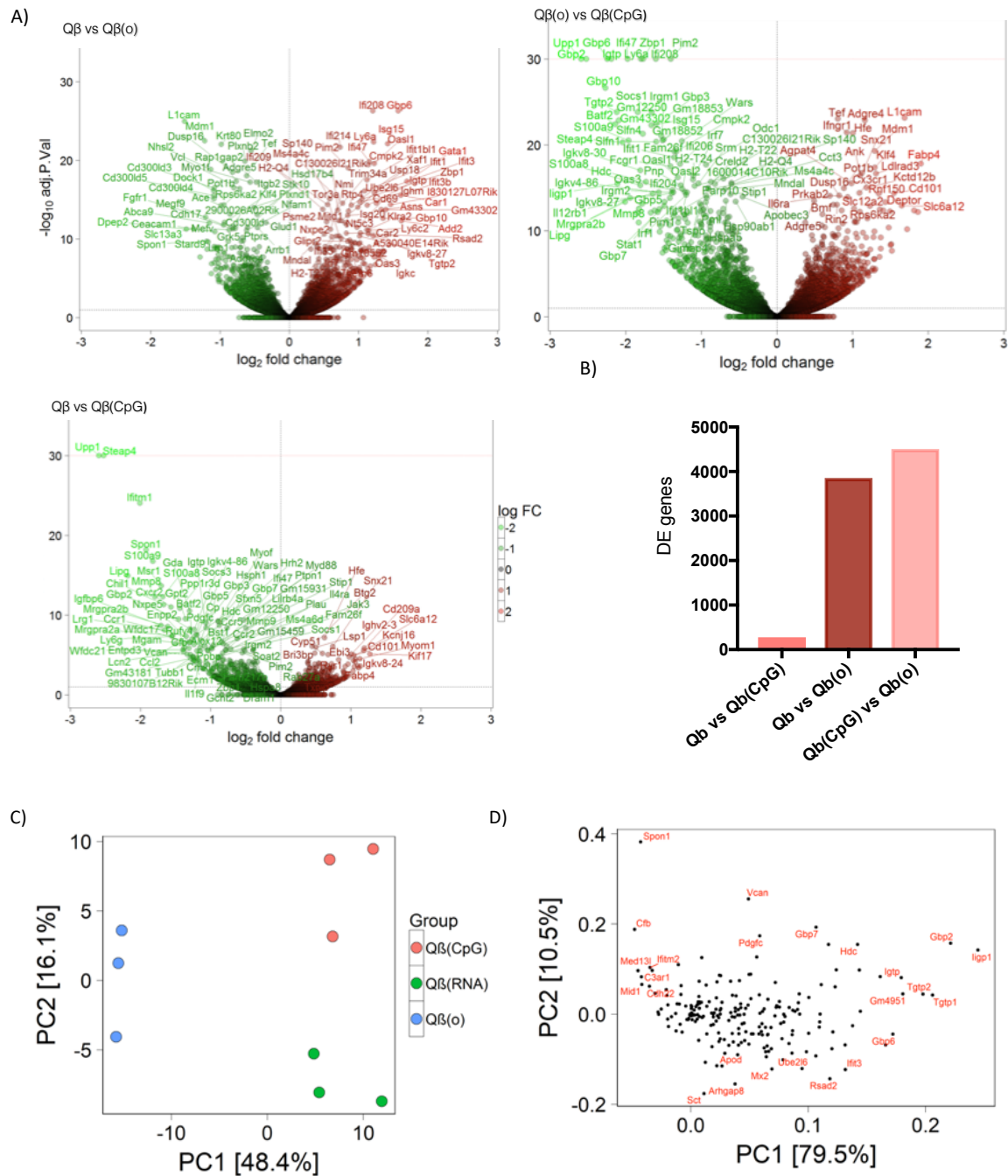


Figure III.4: Transcriptional reprogramming of DCs 24h after immunisation.

A) Volcano plot of genes differently expressed between Q β versus Q β (o), Q β (o) versus CpG and Q β versus Q β (CpG), respectively. Log₂ fold change on the x-axis and the -Log₁₀ p values on y-axis. Up-regulated genes are represented in red and down-regulated gene in green. **B)** Number of genes differently expressed between the groups with a FDR of 10%. **C)** Principal component analysis of genes differently expressed among the groups. The effects of the principal component 1 (PC1) is shown on x-axis and principal component 2 (PC2) shown in the y-axis. **D)** Genes with major contribution to sample clustering. RNA sequencing was performed only once.

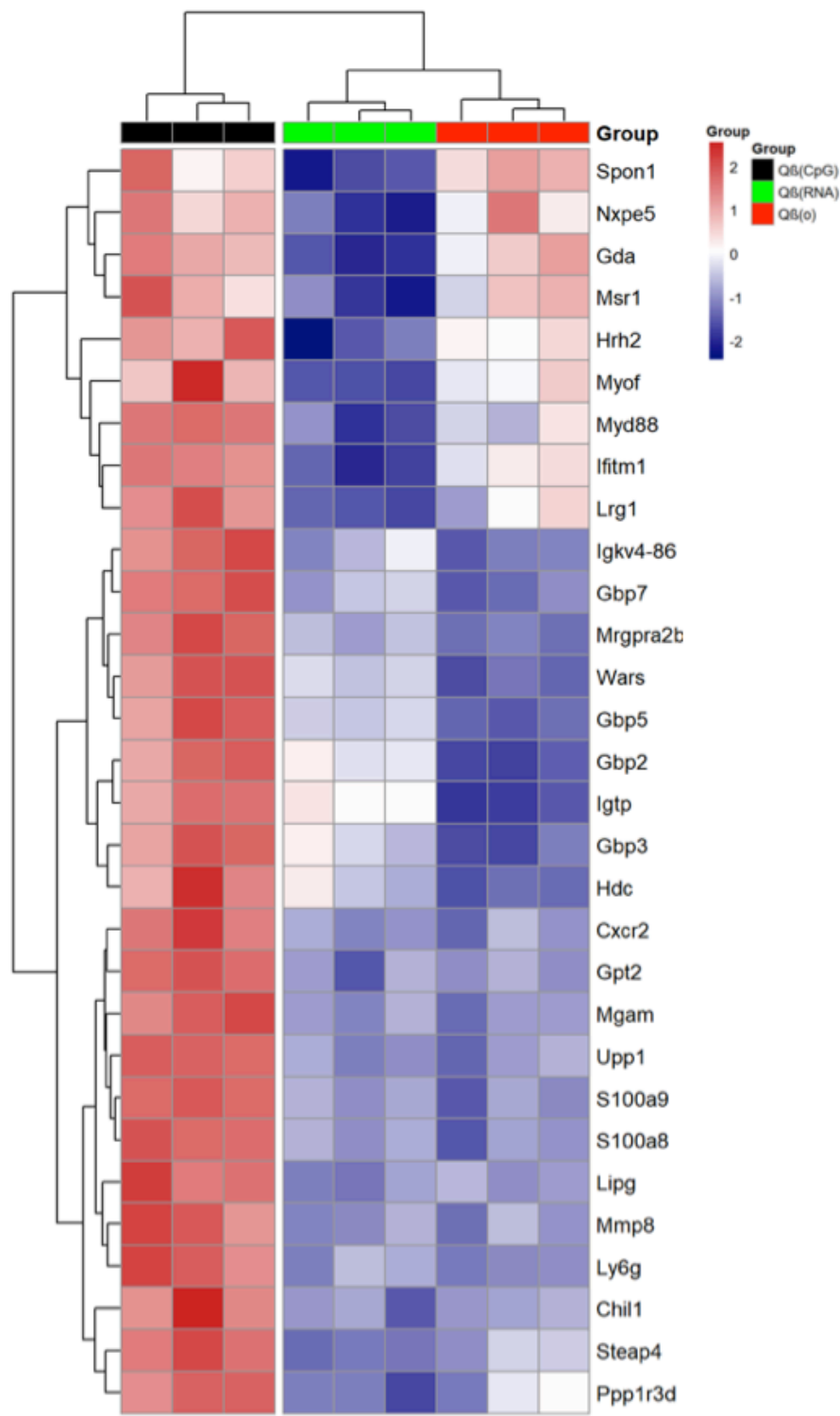


Figure III.5 – top 30 differentially expressed genes

Hierarchical clustering of top 30 differentially expressed genes. Regularized log transformed count data were row scaled and hierarchical clustering was applied to Euclidean distances using complete linkage clustering.

Later, an exploratory analysis on the data was performed aiming to find patterns and biological relevance in the data. The aim of this analysis was to assist with the characterisation and understanding of the regulation of genes controlling the immune response. An unsupervised principal component analysis (PCA) was performed on the data set and revealed the expected clustering of the samples according to the type of PRR ligand received alongside the VLP (figure III.4.C and D). Considering that the three groups had received the same VLP packing different immunogens, the genes most significantly contributing to the clustering of the samples (figure III.4.C and D) can be attributed to the distinct transcriptional reprogramming triggered by the immunogens and not the VLP itself. With such evidences indicating that the differences seen among the groups were a result of the TLR ligands present in the vaccine, further analysis were carried out to assist the identification of genes and processes induced on DCs in response to the vaccines.

The top 30 differently expressed genes between the groups were analysed for hierarchical clustering performed by calculating the Euclidean distances using complete linkage clustering method (figure III.5.E showing the top 30 genes differently expressed between Q β (CpG) versus Q β (o)). Clustering analysis showed a distinct expression profile between the three groups with genes mostly involved in immune responses and TLR signalling such as the gene coding for the adaptor molecule Myd88, the alarmins S100a8/9 and members of the family guanylate binding proteins (GBPs). In addition to genes involved in immune responses, several genes responsible for controlling cell homeostasis, cell signalling, and metabolism were also differently expressed. Examples of note were the Steap4 and MMP8 genes, two metalloredutases that have been shown to

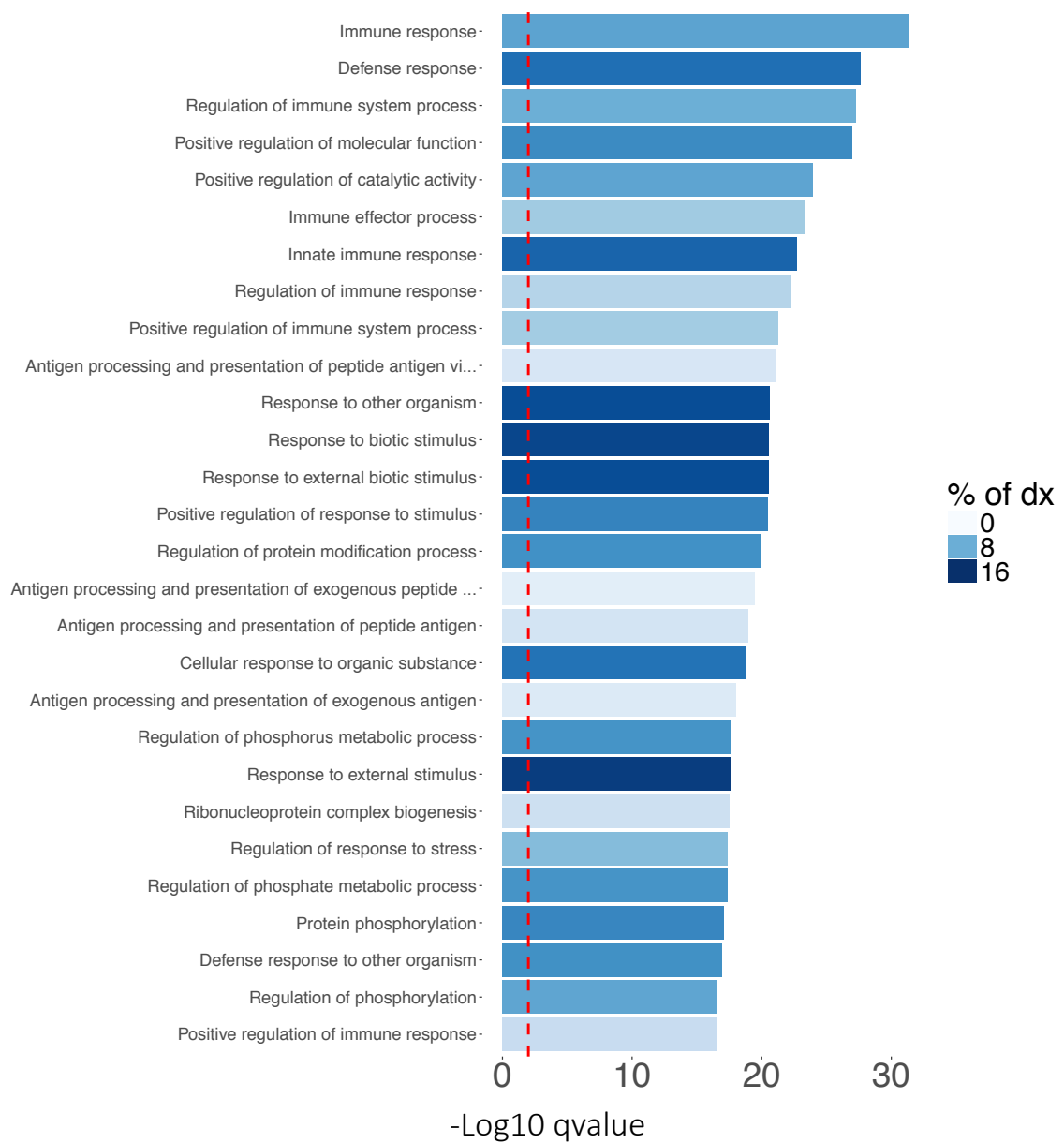
integrate metabolism and inflammatory responses¹³², and Upp1¹³³ an enzyme that catalyses events related to transcription and transduction of proteins. It is noteworthy that the major transcriptional changes that DCs undergo upon TLR engagement are related to either metabolism or immune responses. This a direct consequence of the metabolic reprogramming that DCs needs to undergo in order to support the immune response, gene transcription and translation, protein and cytokine production, and cell migration, which represent major energetic demands for the cell⁶¹. Regardless of the presence of TLR ligands however, Q β clustered closer to Q β (o) compared to Q β (CpG), showing the top 30 genes differently regulated in response to CpG is quite distinctive to RNA-induced responses.

Interestingly, when comparing the entire list of genes differently expressed among the three groups, Q β and Q β (CpG) were remarkably similar compared to Q β (o). This indicates that albeit of the differences seen on the clustering analysis of the top 30 genes differently expressed between Q β (CpG) versus Q β (o), there are still an overlap in the transcriptional reprogramming triggered by TLR7 and TLR9 engagement (figure III.6).

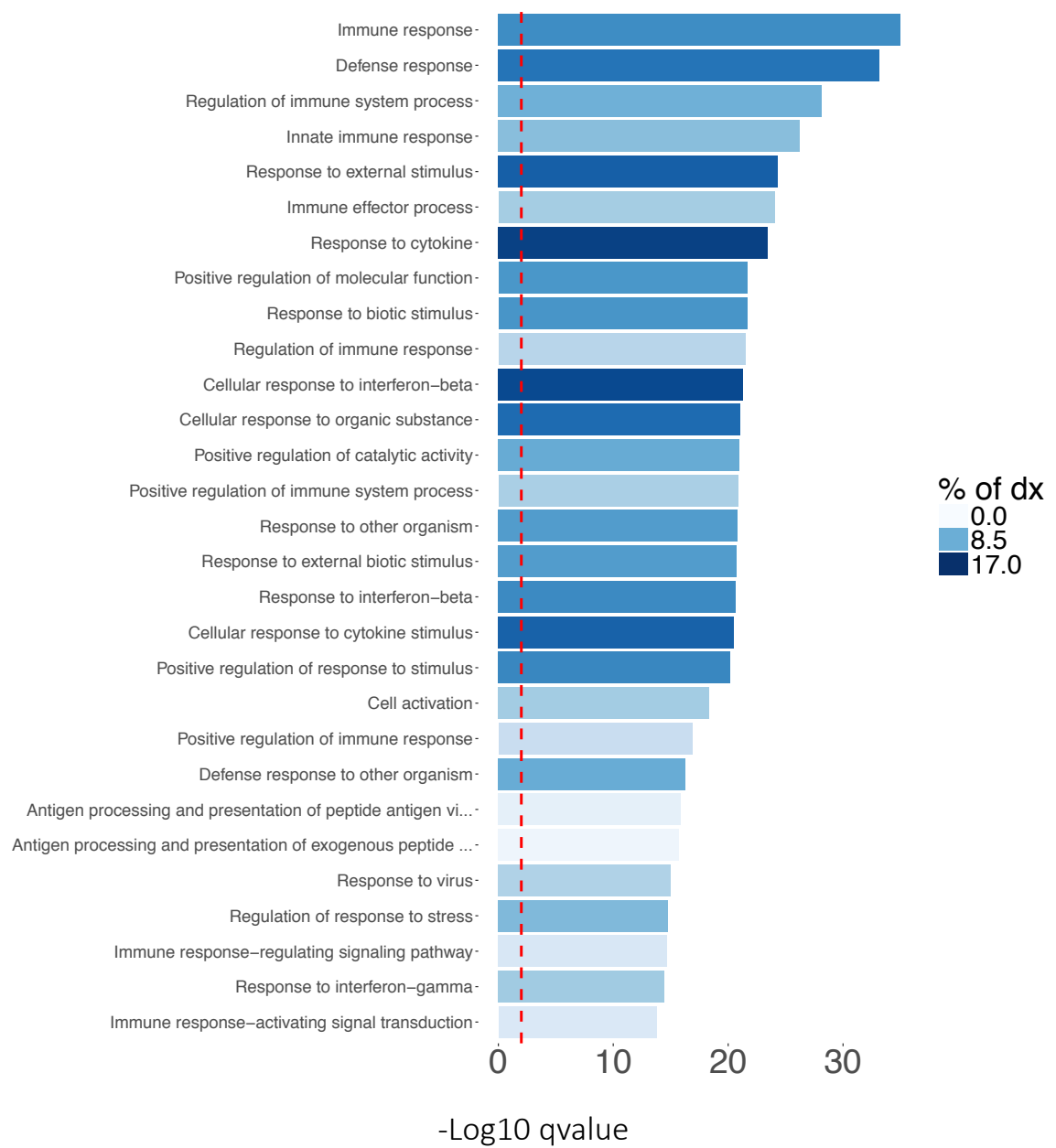
when compared to the control devoid of TLR ligand, Q β (o). All the terms under BP are either related to immune responses or metabolic processes (figure III.7.A and B), confirming the apparent trend observed on analysis of the top 30 genes differently expressed (figure III.4.A and B).

As previously mentioned, upon TLR engagement, cells expressing such receptors undergo a substantial metabolic reprogramming. In accordance to that, when comparing groups that had received either TLR7 or TLR9 agonists (i.e. Q β versus Q β (CpG)), sub-categories related to metabolism were no longer present and all the terms listed are related exclusively to immune responses, including cytokine signalling, migration and responses to stress (figure III.7.C). This was considered an indication that the genes responsible for creating the distinct outcome on the CTL responses to the vaccines were more likely to belong to such category instead of metabolic processes. The two additional GO categories MF and CC can be found in the supplementary materials (figure S4 to S10).

A) Q β vs Q β (o) – 4503 genes



B) Q β (o) vs Q β (CpG) - 3853



C) Q β vs Q β (CpG) – 279 genes

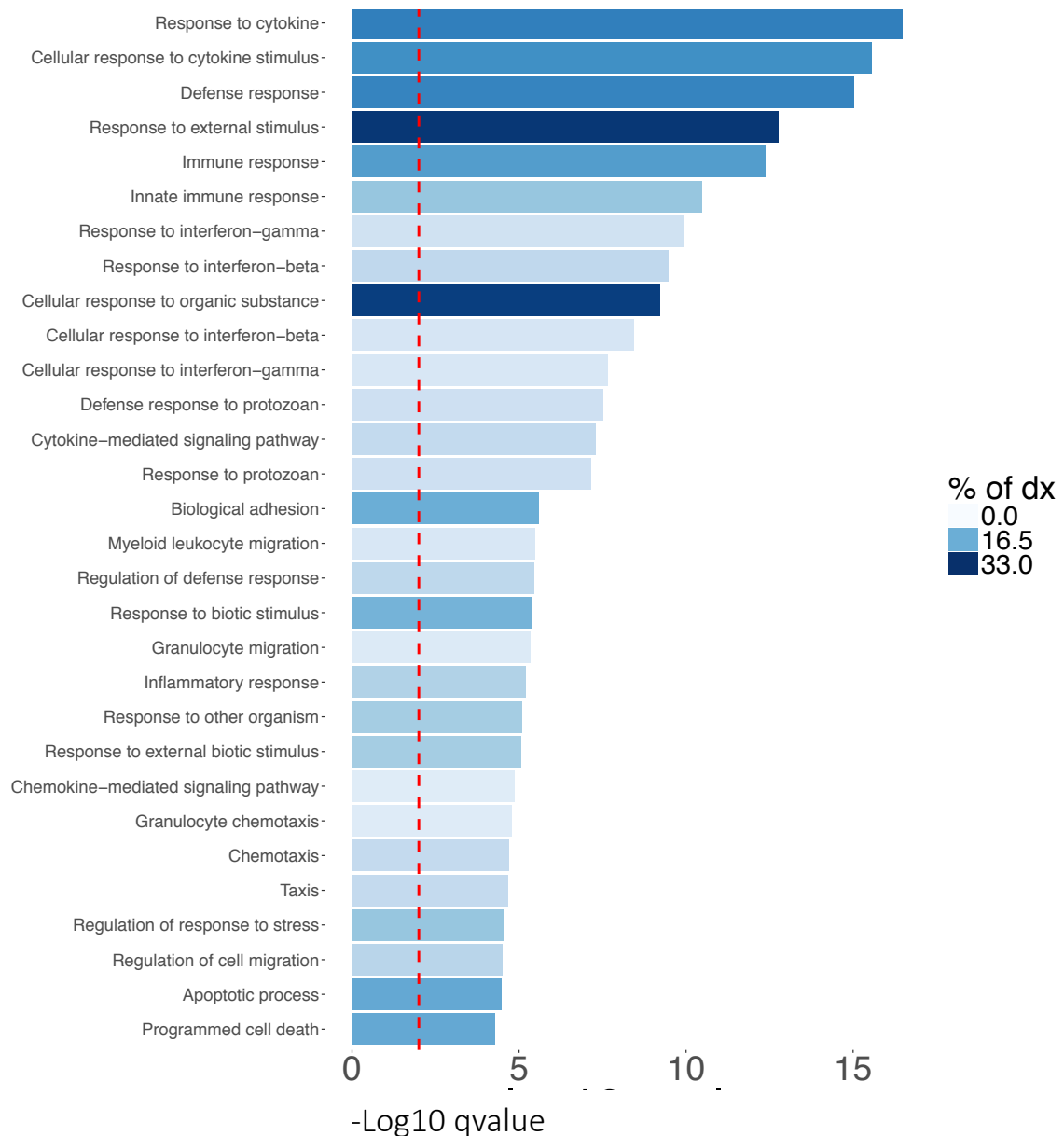


Figure III.7 – Gene Ontology of genes belonging to the sub-ontology “Biological Processes”.

Genes differently expressed between DCs from immunised mice receiving **A)** Q β vs Q β (o) and **B)** Q β (o) vs Q β (CpG), and **C)** Q β vs Q β (CpG). -log 10 of q values. % of DX: percentage of genes differently expressed. Data was analysed using the package DAVID for GO analysis.

Following the initial characterization of the transcriptional regulation, additional data mining methods were employed in order to facilitate the identification of

patterns of gene expression at the system level. To that end, the clusterProfiler tool for statistical analysis of enriched gene sets associated to a given biological function was performed. The clusterProfiler packages relies on the Gene Ontology and the Kyoto Encyclopaedia of Genes and Genomes (KEGG)¹³⁵, a platform that annotates genes and the pathways to which the genes belong to. By combining GO and KEGG pathways, clusterProfiler provides a method to establish themes and biological relevance to the list of genes differently expressed¹³⁶. The analysis had revealed important immune pathways and genes that were differentially regulated across the groups.

To perform the functional enrichment analysis of genes differently expressed, genes with a negative log₂ fold change (FC) value were selected for the down-regulated genes and positive FC for the up-regulated genes. In accordance to the term names shown on the tabular results of the GO analysis (figure III.7), the clusterProfiler had also revealed that Qβ(o) in comparison to the other groups, had pathways down-regulated related either to the immune system or metabolic pathways, such as biosynthesis of amino acids when comparing enriched genes on groups receiving TLR ligands compared to the control (figure S.11).

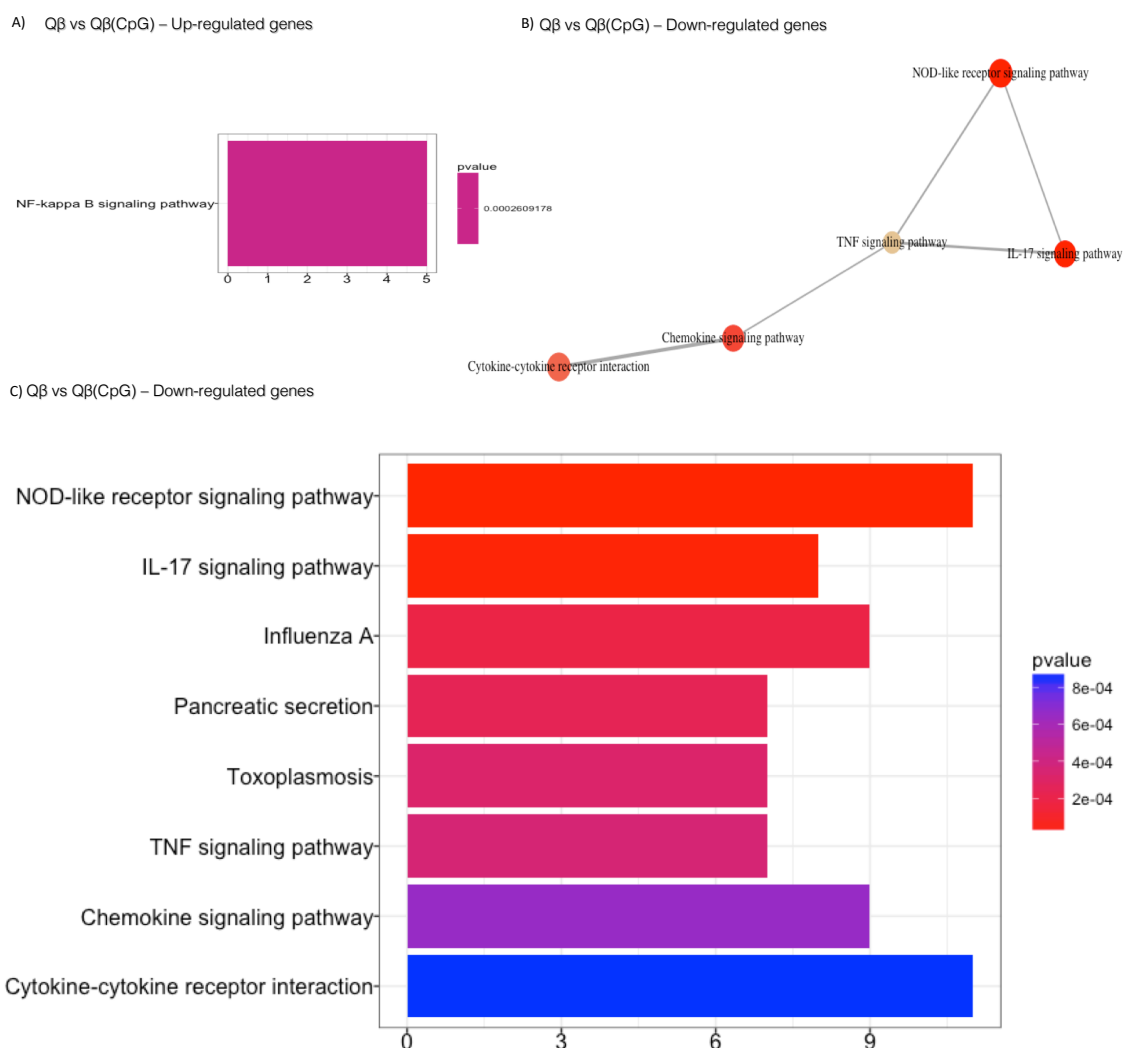


Figure III.8 – Gene set enrichment analysis of differentially regulated genes on Q β vs Q β (CpG)

A) Gene set enrichment analysis of up-regulated genes). **B)** Gene set enrichment analysis of down-regulated genes. Pathways sharing gene sets are connected by a line. **C)** Gene set enrichment analysis of down-regulated genes. Significance displayed as a colour scale as indicated by the p.value. X-axis: number of genes in the dataset per pathway.

The clusterProfiling analysis of Q β (CpG) in comparison to Q β -recipient group (figure III.8) had shown that among the 279 differently expressed genes, relevant immune pathways such as chemokine signalling pathway and cytokine-cytokine receptor interaction were enriched. In addition to those general pathways, genes belonging to the NOD-like receptor pathway such as NLRP12, p38, GBPs, CCL2 and CCL12 were significantly upregulated by the Q β (CpG), other pathways

involving cytokine and chemokine signalling and production were also up-regulated, including TNF- α and IL-17 pathways. Regarding the genes up-regulated on Q β , there was a set of gene belonging to the anti-apoptotic NF κ -B pathway such as Bcl2a1d¹³⁷. We had also performed KEGG module pathways analysis on genes downregulated on the Q β vs Q β (CpG), showing the enrichment of genes belonging to the modules ECS complex, JAK-STAT signalling, and Acylglycerol degradation. The module analysis for up-regulated gene did not return any result. The complete list of differently regulated pathways and the genes contributing to the enrichment are listed below on table III.1.

Table III.1 - KEGG Pathways enriched on genes differently expressed between Q β versus Q β (CpG)

Description	GeneRatio	BgRatio	pvalue	p.adjust	qvalue	Gene Name	Count	FC
NF-kappa B signaling pathway	5/46	104/8263	0.00026092	0.02530903	0.02361993	Relb/Bcl2a1a/Bcl2a1b/Gadd45b/Bcl2a1d	5	Up
NOD-like receptor signaling pathway	11/100	168/8263	5.2401E-06	0.00101658	0.00088806	Gbp2/gbp7/nlrp12/CCL2/CCL2/Gb3/CCL12/Mapk13/Myd88/Oas2/Hsp90ab1	11	Down
IL-17 signaling pathway	8/100	91/8263	1.2895E-05	0.00125084	0.00109271	S100a9/S100a8/CCL2/Lcn2/CCL12/Mmp9/Mapk13/Hsp90ab1	8	Down
Influenza A	9/100	168/8263	0.00018687	0.01144463	0.00999775	CCL2/SOCS3/CCL12/22074/Trv4/Mapk13/Myd88/Furin/Oas2/Hspa8	9	Down
Pancreatic secretion	7/100	103/8263	0.0002361	0.01144463	0.00999775	ctrl/Pnliprp1/Amy2b/Trv4/Cpa2/Bst1/Atp2a2	7	Down
Toxoplasmosis	7/100	108/8263	0.00031631	0.01144463	0.00999775	Igtp/SOCS1/Maok13/CCR5/Irgm1/MyD88/Hspa8	7	Down
TNF signaling pathway	7/100	110/8263	0.00035396	0.01144463	0.00999775	CCL2/ SOCC3/CCL12/Mmp9/Mapk13/Irf1/Irf4	7	Down
Chemokine signaling pathway	9/100	199/8263	0.0006522	0.01807516	0.01579002	CXCR2/CXCR1/CCL2/Pppb/CCR1/CCL12/CCR5/Jak3/Nras	9	Down
Cytokine-cytokine receptor interaction	11/100	296/8263	0.00085723	0.02078771	0.01815964	CXCR2 /CXCR1/CCL2/Pppb/IL-1f9/CCR1/CCL12/CCR5/IL12rb1/Csf3r/16190/IL4ra	11	Down
Prolactin signaling pathway	5/100	72/8263	0.00173004	0.03729202	0.0325774	SOCS3/SOCS1/Mapk13/Irf1/Nras	5	Down

3.4.5 Pro-inflammatory and anti-inflammatory responses are tightly regulated in the early stages of response

DCs undergo a complex maturation process that involves the upregulation of MHC and co-stimulatory molecules. The activation events are accompanied by the secretion of cytokines that will act in an autocrine and paracrine manner impacting not only the DC phenotype, but most of immune cells equipped with the cognate receptors. Considering that most of the pathways differently expressed between Q β and Q β (CpG) were related to immune responses triggered by cytokines the subsequent analysis was focused on assessing cytokine production.

Aiming to have a better understanding of the cytokines being differently regulated in response to immunisation we first analysed the RNA expression level, and we have found marked differences among the groups (figure III.9). Based on the 30 selected cytokines the resulting clustering analysis of the expression level of each cytokine displayed an expression pattern correlated to the type of TLR ligand packaged in the VLP given as a vaccine. The group receiving Q β (CpG) had up-regulated the expression of several pro-inflammatory cytokines such as IFN- γ , IL-2, and the chemokine receptors IL-1R and IL-12R, whilst down-regulating IL-4, a cytokine known to promote Th2 polarization. The group receiving Q β clustered closer to Q β (o) instead of Q β (CpG). Differences were especially seen for the cytokines IL-10, CCL2, and IL-1 to name a few.

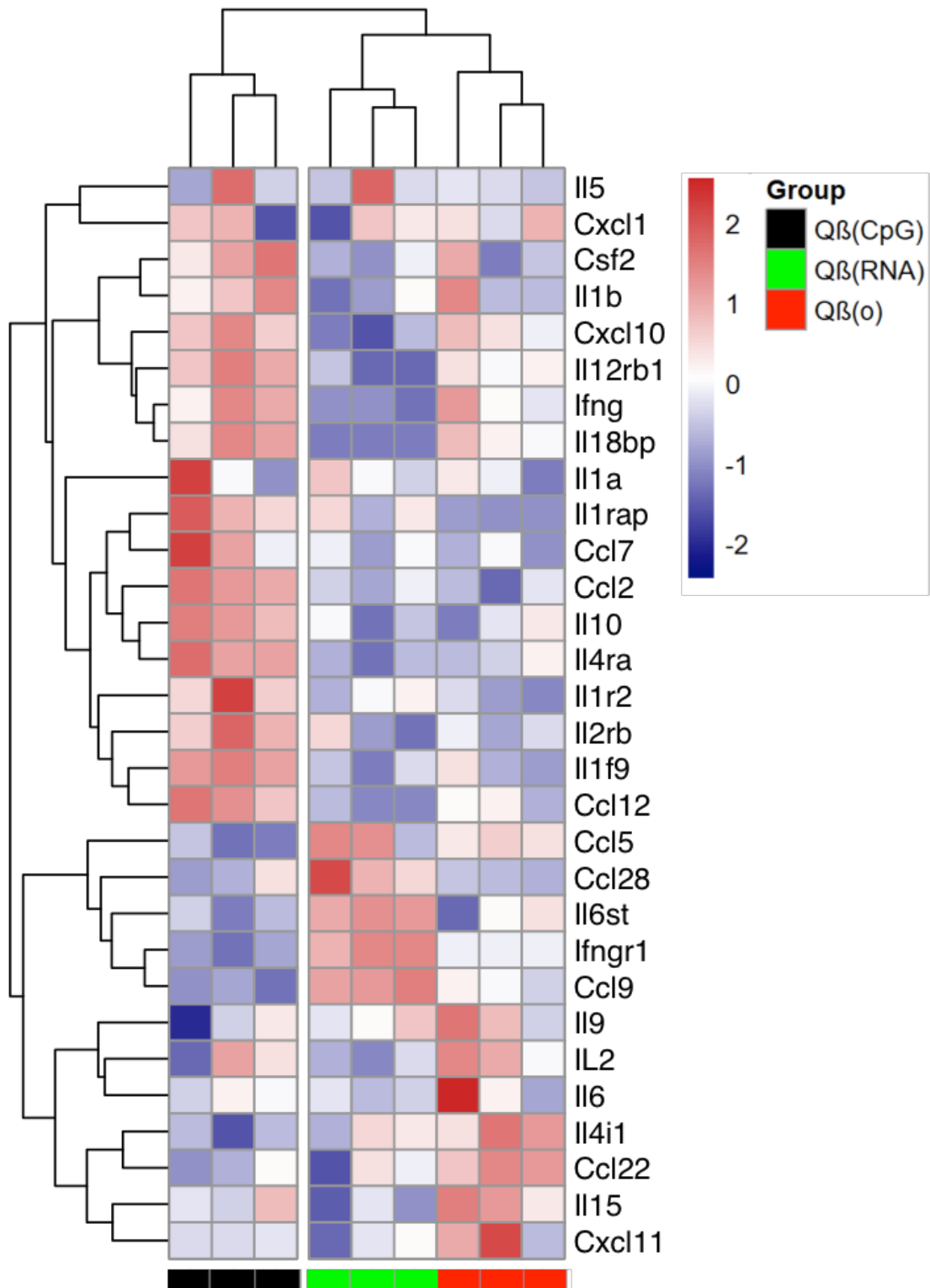


Figure III.9: Immunisation with the different VLPs leads to distinct Cytokine production

Expression data (multi mapped counts per million) of selected Cytokines 24h post immunisation in splenic DCs. Hierarchical clustering of top 30 differently expressed cytokines. Clustering calculated based on the Euclidean distances using complete linkage clustering.

In order to validate the mRNA expression of cytokines, key cytokines secreted by splenocytes 24h following *in vivo* immunisation were then measured by Milliplex (figure III.10). It was observed that overall, Q β (CpG) induces higher levels of secretion of several inflammatory and some anti-inflammatory cytokines. Most of the cytokines followed the same trend seen in the RNAseq data, others such as IFN- γ had shown an opposite pattern among the groups. Likewise the RNA expression data, pro-inflammatory and Th1 chemokines such as CCL2, IL1- α IL1-b, IL-6 were secreted in higher levels by Q β (CpG) as well as anti-inflammatory cytokines such as IL-10. The Th9 promoting cytokine IL-9 on the other hand, was produced in higher levels by the Q β and Q β (o) groups, confirming the RNA expression data and showing the dominating Th1-biased response induced by CpG. As previously mentioned, contrary to the RNA expression data, IFN- γ was mostly produced in response to Q β containing RNA, which could be an indication that there are other cells than DCs producing IFN- γ in response to the RNA compared to CpG. However, the mRNA expression had not been independently confirmed by other method.

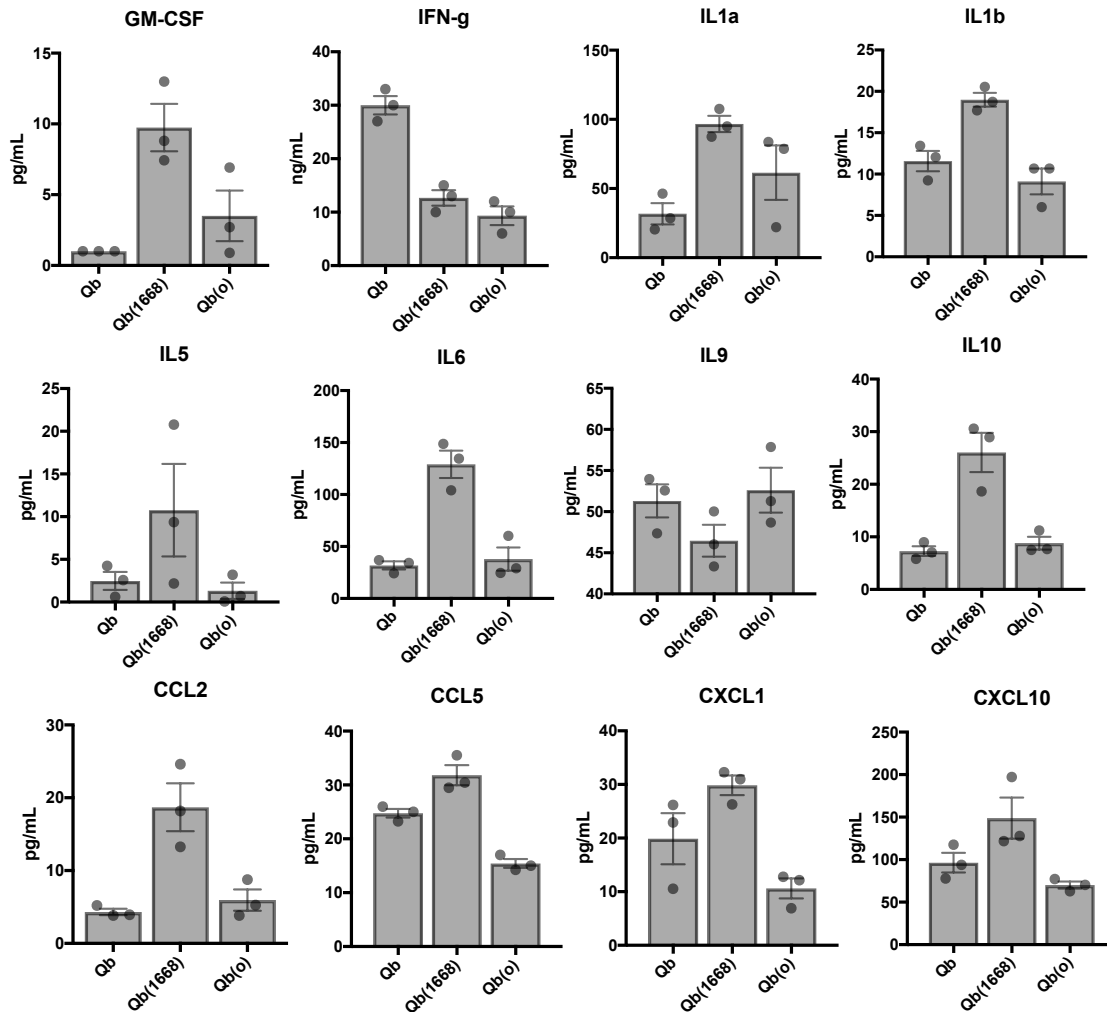


Figure III.10: Immunisation with the different VLPs leads to distinct Cytokines production

Secreted cytokines by splenocytes 24h post immunisation. Values represented as mean + SEM. N=3 mice per group.

Aiming to identify cytokines produced 24h post immunisation that could be correlated to T cell responses, a pairwise correlation analysis of the average cytokine production and outcome of T cell responses as measured by tetramer staining was performed. The correlation matrix (figure III.11) showed that the tested variable of tetramer staining was positively correlated to IL-6, IL-10, IFN- γ , CXCL10, CCL2, IL-1a, IL-1b, CXCL1, and TNF- α . The cytokines IL-9 was negatively correlated to tetramer staining while the cytokines CXCL2, CCL3,

CCL4, G-CSF did not reach statistical significance ($p < 0.01$). Cytokines IL-2, IL-4, IL-7, IL-12p40, and IL-12p70, IL-15 had also been measured but were below the detection limit of the assay.

Interestingly, B type CpG had been consistently described as an inducer of IL-12 secretion¹³⁸, namely when testing in an *in vitro* setting. In our live experimental setting however, we were unable to detect IL-12 at this time point. It was also observed higher production levels of GM-CSF in the Q β (CpG) receiving group, a cytokine that is critical for DCs activation and is typically produced by naïve T cells and NK cells¹³⁹. This data shows the complexity of the orchestration of T cell responses in response to vaccines and the complex cross-modulation of innate and adaptive immune system.

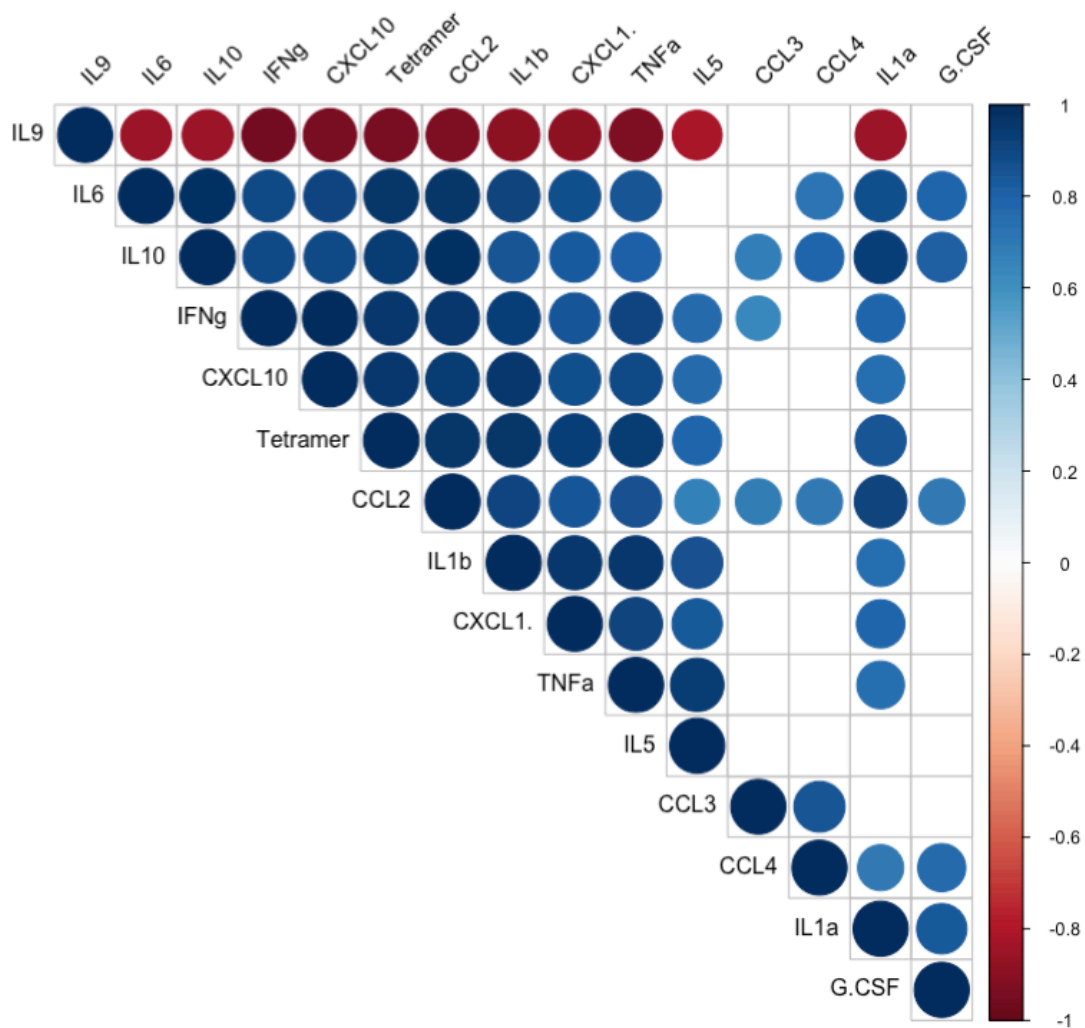


Figure III.11 – Correlation matrix of cytokine production and CTL responses

Correlation matrix of cytokines secreted by splenocytes 24h post immunisation. Positive correlations are displayed in blue and negative correlations in red colour. Colour intensity and the size of the circle are proportional to the correlation coefficients. Legend colours shows the correlation coefficients and the corresponding colours. Correlation calculated using Parsons coefficient. Correlations with significance $p > 0.01$ are depicted in white.

3.4.6 CCL2 modulates CD8⁺ T cell responses induced by Q β (CpG)

Considering the evident up-regulation of CCL2 mRNA on DCs, the higher levels of CCL2 in the supernatant of splenocytes from mice immunised with Q β (CpG) (figure III.8 A and B, respectively) and the positive correlation of CCL2 production and T cells responses (figure III.11) we sought to investigate the role of CCL2 in CTL responses.

First we had investigated activation makers in BMDCs from CCL2^{-/-} mice in response to VLPs. CD86 upregulation was reduced in response to both Q β and Q β (CpG). To evaluate the impact of CCL2 in CTL responses, sex and age matched CCL2^{-/-} mice and WT counterparts were immunised with Q β -p33 or Q β (CpG)-p33. T cell responses were subsequently assessed 7 days later. CCL2^{-/-} mice had a marginally reduced CTL response with a reduction in the clonal expansion of p33 specific T cells (figure III.12.D). In addition to that, the capacity of those cells to produce IFN- γ was reduced compared to the WT (figure III.12.E). The reduced response was interpreted as an evidence of a modulatory effect of CCL2 on the T cell response.

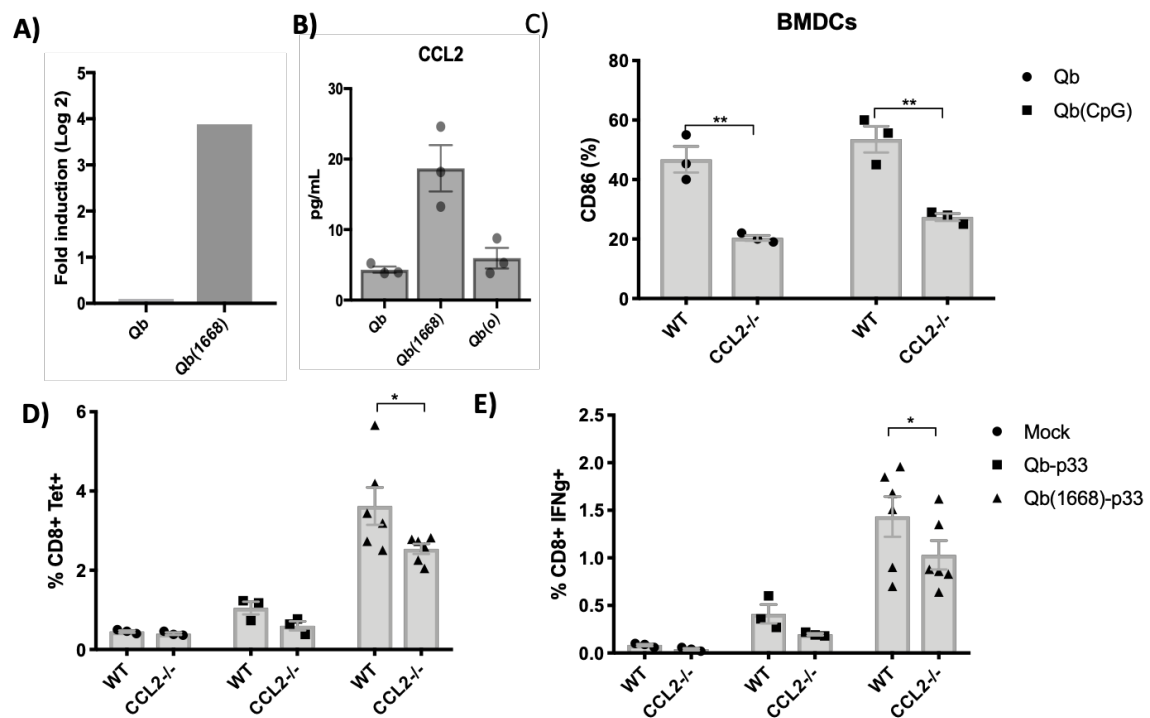


Figure III.12: CCL2 is up-regulated in DCs immunised with Q β (CpG) that modulates CTL responses

A) Fold induction mRNA expression of CCL2 in comparison to Q β (o). Data is represented as mean fold change. N=3 mice per group. **B)** CCL2 secreted by splenocytes 32h post immunisation. N=3 mice per group. **C)** CD86 expression on BMDCs pulsed with VLPs for 3h p33-specific tetramer staining of CD8+ T cells. BMDCs were generated from pooled bone marrow from 2 mice. 3 individual replicates per condition. **D)** Tetramer staining of CD8+ T cells 7 days post immunisation. **E)** ICS staining of CD3⁺CD8⁺ T cells 7 days post vaccination. 6h stimulation with p33 peptide. Data represented as Mean + SEM of 2 combined experiments. Statistical analysis was performed by calculating the Z score of the combined p-values of the two independent experiments. *p<0.04.

CCL2 acts as a pro-inflammatory chemokine regulating the trafficking and homeostasis of immune cells such as DCs and T cells²⁷. Firstly, the trafficking of immune cells to the spleen following immunisation was investigated. Spleens were collected on day 1 in order to assess the percentage of activated DCs, and T cells counts were measured on day 7 by flow cytometry. There were no observable differences in the percentage of DCs on day 1 and T cells on day 7 of spleens from

CCL2 KO and WT. Therefore, ruling out inefficient trafficking of DCs and T cells as the cause for the differences observed in the T cell responses of CCL2 KO and WT (not shown).

3.4.7 Q β and Q β (CpG) induce low-levels of IFN- α *in vivo*

The essential role of the type I IFN in the orchestration of VLP-induced CTL responses has been previously demonstrated by Keller et *al.* In the referred paper, CTL responses induced by VLPs containing either RNA or CpG in mice lacking the IFN- α and β receptor (IFN $\alpha\beta$ R) were completely abolished⁹, indicating a role for type I IFN responses. With that in mind, we wanted to investigate the role of type I IFN in our system hoping to understand how immune responses triggered by B-type CpG were dependent of IFN $\alpha\beta$ R, considering that B-type CpGs have been consistently shown to be poor inducers of IFN- α . Our initial assumption was that differential endosomal trafficking of the free B type CpG in comparison to the packaged version would result in IFN- α production, as previously discussed in section 3.3.1. However, we found no IFN- α production induced by Q β (CpG) in comparison to the free form of the CpG *in vitro* (not shown). In the pertinent literature, IFN- α production in response to CpGs are often measured using *in vitro* techniques, which may not represent the complexity of the *in vivo* responses that govern vaccination outcome. With those limitation in mind, we set ourselves to attempt measuring type I IFN responses *in vivo*.

Analysis of the transcriptional reprogramming of DCs has demonstrated a characteristic type-I IFN signature induced by both VLPs containing TLR ligands (figure III.14). Both Q β and Q β (CpG) induced upregulation of several interferon stimulated genes (ISG) such as Ifi44, Ifit1, Ifit2, ISG15, GBP family, Oas11, and

Oasl2 in comparison to Qβ(o). To assess IFN-α production *in vivo*, we have immunised mice and collected serum in the early time points of the response (6h, 12h, 24h), however, circulating levels of IFN-α in the serum of immunised mice were below the detection limit of commercially available ELISA Kits of 31.5 pg/mL (not shown).

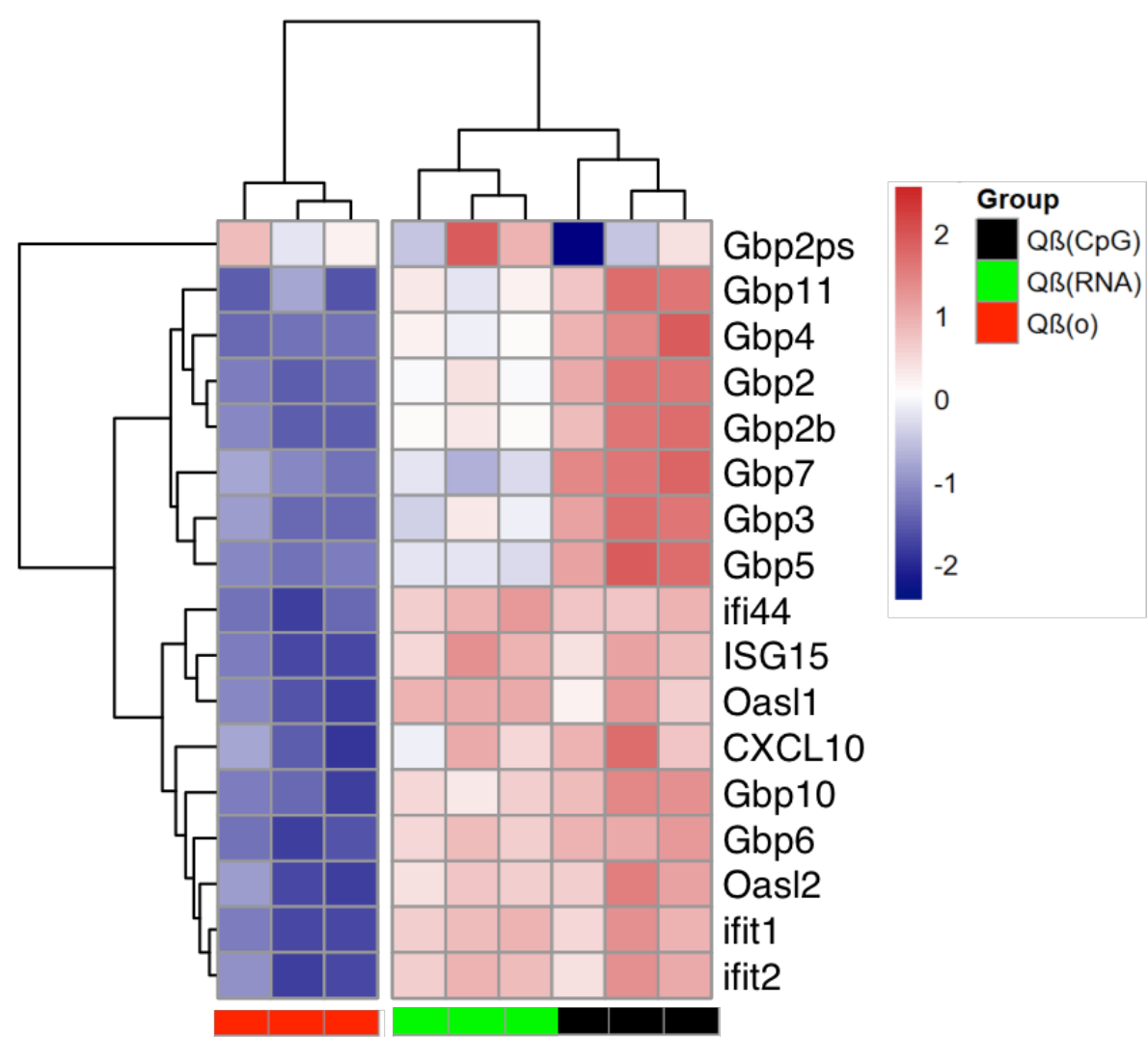


Figure III.14: Type I IFN response induced in DCs by Qβ

A) Expression data (multi mapped counts per million) of ISG genes post immunisation in splenic DCs with Qβ, Qβ(CpG) and Qβ(o). Clustering calculated based on the Euclidean distances using complete linkage clustering.

Considering the evident up-regulation of ISGs, it was then speculated that IFN- α could either be produced and detected only at the local level, such as in lymphoid organs including LNs and spleen, or that the circulating levels were simply too low to be measured, which does not necessarily rule out a role for IFN in VLP-induced responses. To test this hypothesis, the ability of serum from immunised mice to induce ISG up-regulation in other cell types was measured. 3T3 cells treated with serum were then tested for Ifti1 induction, showing that both VLPs are indeed able to induce the IFN (figure III.15.A), supporting the assumption of low levels of systemic type I responses. Moreover, by generating BMDCs and treating such cells with VLP we had further confirmed that ability of VLPs to trigger IFN- α production, with Q β containing RNA inducing higher levels of IFN-alpha in the time-points of 24h and 32h (figure III.15.B). Of note, it is important to state that *in vitro* IFN- α production by BMDCs in response to Q β (CpG) were consistently close to the detection limit of the kit in previous experiments following consistent techniques, with the exception of this experiment where values were slightly above of the detection limit (31.5pg/mL). Nonetheless, the expected trend of Q β inducing higher levels of IFN- α was observed.

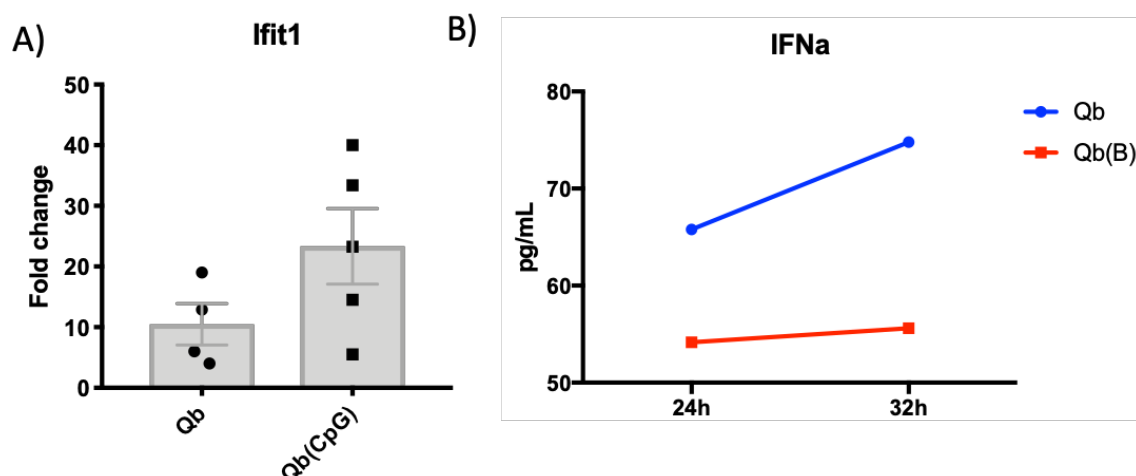


Figure III.15: *In vivo* type I IFN response induced Q β and Q β (CpG) and *in vitro* IFN- α production by DCs.

Type I IFN response in WT mice as measured by the induction of the ISG Ifit1 in 3T3 cells post exposure to serum of immunised mice. N= 5 mice per group. Serum was collected 24h following immunisation. Fold change of gene expression in comparison to group receiving Q β (o). **B)** IFN- α production by BMDCs in response to Q β and Q β (CpG) measured by ELISA. BMDCs derived from three mice. N=2 replicates per condition. IFIT1 induction and IFN α production experiments were performed twice.

3.4.8 Type I IFN induced by Q β but not Q β (CpG) is modulated by the adaptor molecule STING

Q β and Q β (CpG)-induced responses are strictly TLR dependent and are completely abolished in a MyD88 KO as previously demonstrated by former member of the group³⁹. However, recent literature had demonstrated a role for other nucleic acid sensors in the modulation of the response triggered by TLRs and such receptors are increasingly being targeted for the development of adjuvants for vaccines^{141–144}. To evaluate the overall immune response and possible impact of other PRRs in the immune response raised by VLPs, type I IFN and T cell responses

were evaluated in mice lacking either the downstream adaptor of cytoplasmic sensors of nucleic acid (i) Stimulator of IFN genes (STING) or the (ii) Mitochondrial antiviral-signalling protein (MAVS). The molecule STING is an adaptor molecule of cyclic guanosine monophosphate (GMP)–adenosine monophosphate (AMP) synthase (cGAS), a cytoplasmic DNA sensor that upon activation leads to type I IFN production (STING is also known as Tmem373 and Myps)¹²⁵. MAVS, is the adaptor of the receptor Retinoic-acid inducible gene (RIG-I), a cytoplasmic RNA-helicase involved in the recognition of intermediary species of RNA replication, such as dsRNA and the 5'phosphates of the RNA strand⁶³. RIG-I activation also leads to type I IFN production⁶³.

Mice lacking either STING or MAVS molecules were immunised with Q β -p33 or Q β (CpG)-p33 following the same immunisation protocols used throughout this study. Serum was collected after 24h following immunisation and CTL responses assessed on day 7. Serum from immunised WT and KO mice was tested regarding the ability to induce up-regulation of ISG genes. We found that mice lacking STING had a great reduction of type I IFN responses induced by Q β , while groups receiving Q β (CpG) had similar responses to the WT (figure III.16.A). On the other hand, no difference was found in type I IFN responses triggered in the MAVS KO (figure III.16.B).

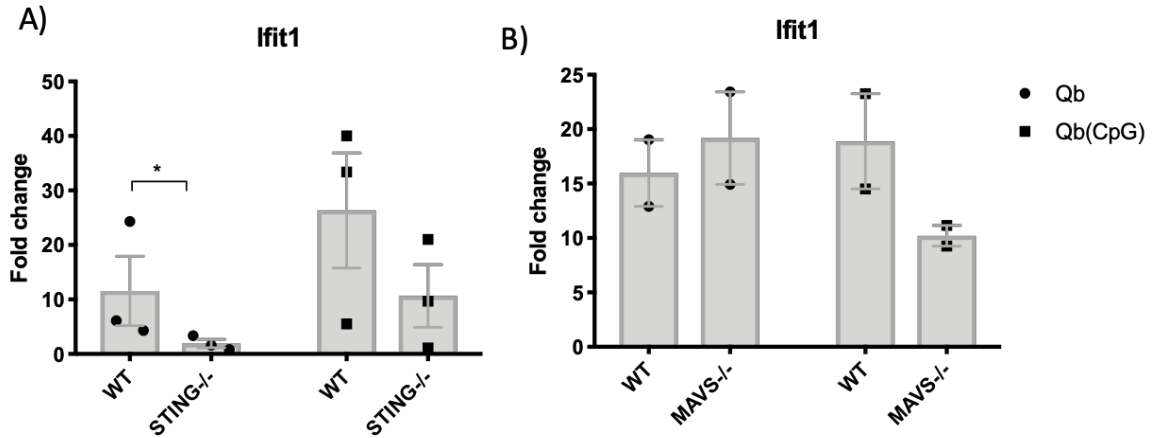


Figure III.16: Type I IFN response induced in DCs by Q β containing prokaryotic RNA is modulated by STING.

A) Type I IFN response on the STING KO and B) Type I IFN response on the MAVS KO 24h following immunisation, as measured by the induction of the ISG Ifit1 in 3T3 cells. Data is represented as Log2 scale of fold change and statistical differences analysed by t test. ** p>0.02. Data is representative of two independent experiments.

p33-specific CTL responses were measured by tetramer staining and cytokine production 7 days after immunisation. Unexpectedly, CTL responses in the knockouts were found to be marginally increased (figure III.17). Both MAVS and STING KO had a consistent but not significant tendency of producing higher levels of IFN- γ (figure III.17.B) and TNF- α (figure III.17.C) in accordance to an increase on the p33-specific CD8⁺ T cells (figure III.17.A).

Hoping to investigate the seemingly increased CTL responses in the KOs, we wanted to address the interplay between TLR-induced responses and STING. Pathogen-derived RNA has been shown to activate type I IFN pathway in a STING-dependent manner in a *Staph. aureus* infection model, and a pro-inflammatory program via TLR, which was found to result in IL-1 β secretion. In this model, STING-mediated IFN responses were found to reduce IL-1 β secretion

and protection was improved in the absence of STING¹⁴¹. Considering that Q β (CpG) induces higher levels of IL-1 α and IL-1 β (figure III.10) and lack of STING only influenced type I IFN production in response to Q β but not Q β (CpG), we wanted to investigate whether STING was influencing IFN- α , IL-1 β production, and CTL responses in a similar fashion. Taking into account the low systemic levels of cytokines in response to VLPs, BMDCs from WT and STING KO mice were generated. BMDCs were then pulsed with VLPs and IL-1 β and IFN- α production were measured 24h later. We found no differences in IL-1 β production between KO and WT (figure III.17.D), IFN- α levels were lower in the BMDCs derived from the STING KO; however this was not statistically significant. (figure III.17.E).

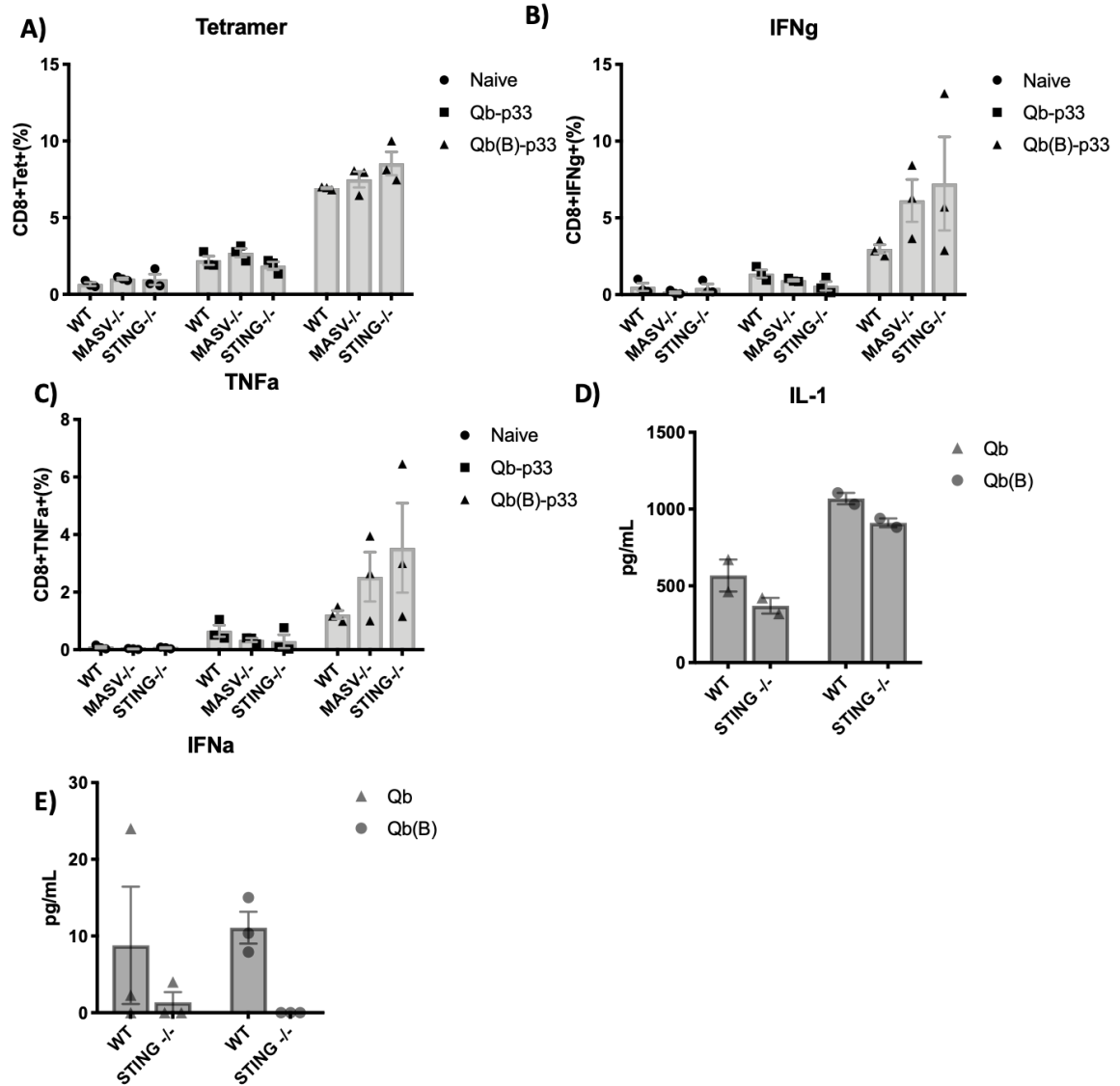


Figure III.17 - CTL responses induced by VLPs is augmented in the absence of STING and MAVS

A) p33-specific tetramer staining of CD8⁺ T cells and **B)** IFN- γ and **C)** TNF- α as measured by ICS staining of re-stimulated CD3⁺CD8⁺ T cells 7 days post vaccination. Data represented as Mean + SEM. Data is representative of 3 independent experiments. Depicted experiment had 3 mice per group. **D)** IL-1 β production and **E)** IFN- α production by WT and STING BMDCs pulsed with 100 μ g/mL of Q β or Q β (CpG) for 24h. Data represented as mean + SEM. BMDCs derived from 3 mice. N=2 replicates per condition. Statistical significance was measured by two tailed *t*-tests followed by Bonferroni-Dunn's correction for multiple testing. **p*<0.01.

3.4.9 Immunisation with Q β (CpG) reduces the expression of inhibitory molecule on tumour infiltrating lymphocytes

As previously discussed, in order to induce appropriate T cell responses it is necessary to provide concomitantly to activation signals, co-stimulation and pro-inflammatory cytokines. In addition to that, regulatory network of T cells also counts with inhibitory molecules that can hamper CTL responses even in the presence of stimuli. To date, several T cell inhibitory molecules have been identified and such molecules are often targeted for pharmacological interventions. An example of that is the superior T cell responses induced by immunotherapy administered in combination to checkpoint inhibitors such as the monoclonal antibodies developed against the programmed death receptor 1 (PD-1) and the cytotoxic lymphocyte associated protein 4 (CTLA-4)³³. Considering the great therapeutic value and the importance of inhibitory molecules in the overall CTL responses we wanted to evaluate the expression of such molecules that might inhibit T cells in our model.

To that end, based on the current literature, the expression of the known T cell inhibitory molecules CTLA4, BTLA, PDCD-1, Lag3, CD274, and CD101 that are expressed on DCs were analysed in response to the three vaccines Q β , Q β (CpG) and Q β (o) (figure III.18).

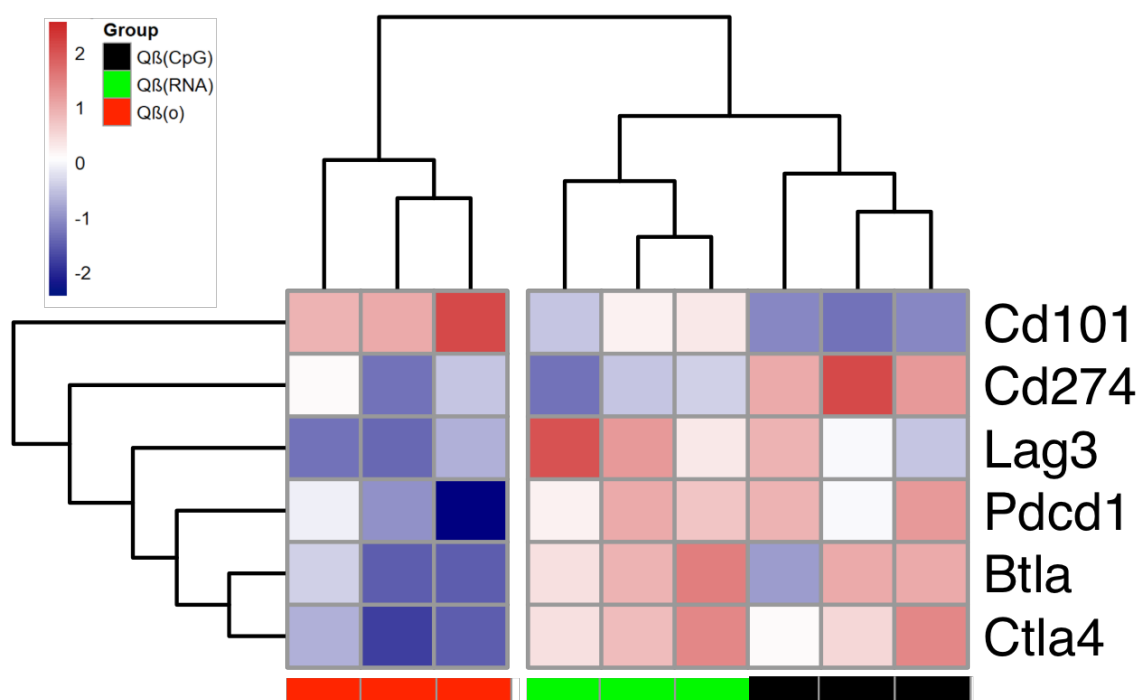


Figure III.18 – Expression of inhibitory molecules of T cell activity on DCs is altered in response to immunisation

Expression data (multi mapped counts per million) of genes coding for inhibitory molecules post immunisation in splenic DCs with Qβ, Qβ(CpG) and Qβ(o). Clustering calculated based on the Euclidean distances using complete linkage clustering.

From the inhibitory molecules analysed, CD101 (also known as V7) was markedly down-regulated on DCs immunised with Qβ(CpG) (figure III.18. and III.19.A). Next, it was sought to validate the expression of CD101 *in vivo*. To that end, WT mice were immunised with the three formulations of VLPs and the expression of CD101 24h after immunisation was assessed. CD101 expression was tested on splenic and lymph node (LN) resident DCs and no differences on the expression of this molecule could be observed (figure III.19.B).

Considering that CD101 inhibits T cell responses by two distinct mechanism – *i.e.* either through direct CD101 activation on DCs, which results in IL-10 production

or yet, through direct activation on T cells, by blocking Ca^{2+} influx in response to TCR/CD3 engagement¹⁴⁵ –the expression of this molecule on CD8⁺ T cells was also investigated. Moreover, considering the previously discussed therapeutic value of blocking inhibitory molecules in immunotherapy, the expression of CD101 on tumour infiltrating lymphocytes (TIL) and tumour resident DCs was assessed.

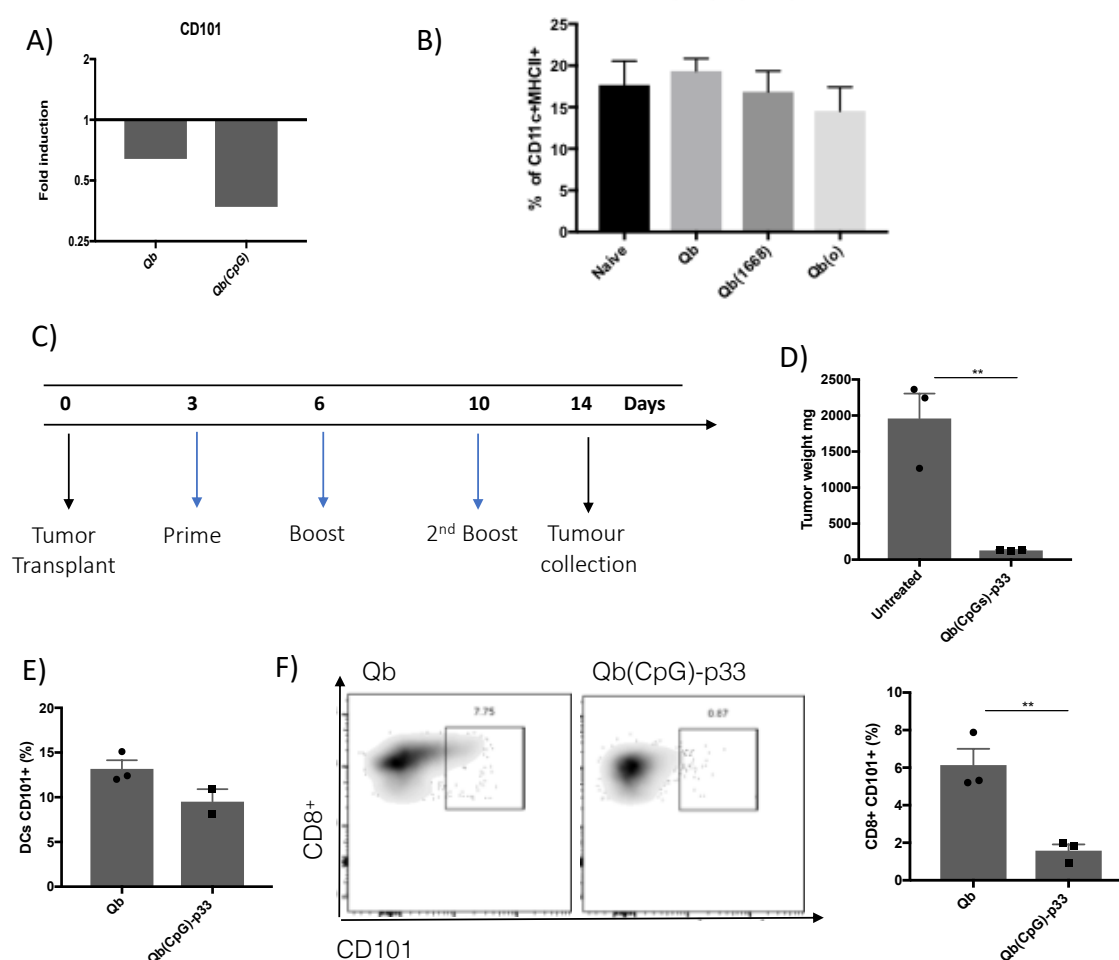


Figure III.19 – CD101 expression and T cell responses

A) CD101 mRNA expression on DCs 24h post-immunisation. Data expressed as the mean fold change in relation to the control (Qβ(o)). n=3. Experiment was performed only once. **B)** CD101 expression on splenic DCs 24h AND 32h post-immunisation. Data is representative of 3 independent experiments. **C)** Adapted tumour transplantation and immunisation schedule. 3 days following tumour transplantation, mice were immunised with 120μg of Qβ(CpG)-p33 or Qβ as control. **D)** Tumour growth expressed in weight (mg). Data

represented as mean + SEM. N=3. **E)** Percentage of CD101+ DCs in the tumour. **F)** Plot of CD101 expression on tumour-infiltrating CD8+ T cells of immunised mice. Data represented as mean + SEM. N=3. **p<0.03. Data on tumour is representative of 2 independent experiments.

To that end, Q β (CpG)-p33 was employed as a therapeutic vaccine in a murine B16F10p33 melanoma tumour model. The experimental schedule is outlined in figure III.19.C.. 3 days following implantation of tumours (tumours previously grown on RAG-/- mice – see figure S.10), mice were primed with either Q β (CpG)-p33 or control VLP. A homologous boost was given on day 6 and 10. On day 14, control groups reached severity limited, determining the end point of the experiment, tumours were then collected for further analysis. Mice receiving Q β (CpG)-p33 controlled tumour growth and were fully protected (figure III.19.D). Freshly isolated tumour infiltrating lymphocytes and DCs were stained for CD101 expression and analysed cytometry. Roughly 10% of tumour resident DCs were CD101 positive, the same frequency found on spleen-derived DCs from healthy mice (figure III.19). About 8% of CD8+ TILs from control mice were expressing CD101 while mice receiving the therapeutic vaccine had a marked reduction on CD101 expression (figure III.19.F). This indicates that TILs recovered from tumours controlled by immunotherapy may be more active to TILs from tumours that grew unrestrained.

3.5 Discussion

Here, the modulation of the innate immune response mediated by VLPs packing TLR ligands was investigated and characterised in the context of vaccinations. The

outcome of immunisations employing PRR ligands was evaluated and correlated to the early events promoted by the activation of DCs that are translated in the orchestration of the subsequent adaptive responses mediated by T cells.

We have shown that packing CpGs into VLP alters the endosomal trafficking of CpG. Packed CpG is retained for longer periods of time in endosomal compartments before reaching lysosomes. This “re-routing” may mean that CpG gets to interact with TLR9 for longer in the endosomes before reaching lysosomes, which is shown to recruit different types of IRFs compared to set of IRFs associated with lysosomes⁷⁹. Ultimately, the altered endosomal sorting may impact the immune response as seen by differential IL-12 production by DCs and total splenocytes in response to packed CpG. In addition to that, packing CpG guarantees the delivery of the molecule to the relevant cells, namely APCs, reducing side-effects and reducing the dose of CpG used in the formulations, as previously discussed (section 1.6.1) and demonstrated by others⁷⁸.

The initial innate immune response mediated by DCs influences subsequent adaptive immune responses. Here, the transcriptional reprogramming that DCs undergo in response to immunisation was characterised and we attempted to correlate such findings to T cell responses. Functional analysis of the genes expressed in response to the different formulations of VLP indicated important immune pathways being differently regulated 24h post immunisation. This analysis had provided a plethora of data that will likely support further work beyond this thesis. Early cytokine production was correlated to T cell responses and from the most relevant cytokines, knockout models were selected in order to further evaluate those findings in a vaccination context.

From those pathways, CCL2 was selected due to its marked distinct expression in response to CpG and its relevance to the overall immune response. CCL2 chemokine produced by DCs 24h after immunisation modulates the quality of the cytokines produced by T cells in a later stage of the response. We were however, unable to establish whether the effect was a result of CCL2 signalling on DCs or a direct CCL2 signalling on T cells. CCL2 has been shown to support DCs maturation¹⁴⁶ and the recruitment and modulation of IFN- γ production by T cells¹⁴⁷. We reasoned that the small but significant reduction of cytokine production by T cells may be a result of the redundancy of the chemokine system. CCL2 signals through CCR2, a receptor shared with several other chemokines. The lack of CCL2 may have been compensated by re-modelling of the milieu of cytokines that supports T cell responses. In addition to CCL2, other cytokines were positively correlated to CTL responses in our model, including pro-inflammatory cytokines and IL-10, a cytokine known for its anti-inflammatory activity on T cells. Although, IL-10 has inhibitory activity of the immune response, our data supports recent findings demonstrating a more complex behaviour of IL-10¹⁴⁸. IL-10 analysed in the complex the milieu of cytokine induced *in vivo* seems to support CTL responses, by either modulating to response to avoid excessive immune activation or yet, by controlling suppressive subsets of CD4⁺ T cells, as demonstrated by others¹⁴⁹.

Both Q β and Q β (CpG) induce a robust type I IFN signature 24h post immunisation, however BMDCs pulsed with Q β produced higher levels of IFN- α in comparison to Q β (CpG). We reasoned that type I IFN responses are important for the immune response raised by Q β and Q β (CpG). However, considering the similar pattern of ISG expressed by DCs, type I IFN is likely to be a common

regulator of the immune responses raised by both VLPs and therefore not responsible for the major differences in the T cell responses raised by each vaccine. This assumption is based on the previously published findings that T cell responses against VLPs containing RNA or CpG are completely abolished on a IFN α R KO⁷⁸, which indicates the essential role of type I IFN for T cell responses induced by both RNA and CpG.

Cytoplasmic nucleic acid receptors are often associated with recognition of intermediates of pathogen replication such as dsRNA and DNA in the cytoplasm, which are often not present during immunisations containing VLPs, as VLPs are replication incompetent. In addition to that, VLPs mostly deliver their nucleic acid content to the endosomal compartment instead of the cytoplasm, which can shield the recognition of the nucleic acids by cytosolic sensors. Our data however, revealed a role for STING in the orchestration of type I IFN responses elicited by Q β containing RNA. STING being the downstream molecule of cGAS, a DNA sensor, has been shown to not influence the production of IFN in response to RNA *in vitro*¹⁵⁰. Interestingly however, mice lacking cGAS were more susceptible to a lethal challenge by the RNA virus West Nile, showing that cGAS-STING may play an indirect role in the *in vivo* immune response elicited by RNA¹⁵¹. Furthermore, different reports had demonstrated a role for cGAS and RIG-I in response to non-replicative pathogens, including VLPs^{68,152}. The suggested mechanism for RIG-I and cGAS engagement even in the absence of their ligands is through the recognition of cellular and mitochondrial stress⁹³. We reasoned that the reduced type I IFN responses in the STING KO in response to Q β is unlikely to be a result of direct activation of the RNA packed into the VLP, but instead a secondary response triggered by the TLR. Several reports have shown the interplay and cross-modulation between TLRs and cytosolic sensors in other models.

The previously described T cell inhibitory molecules CD101 was shown to be downregulated at the RNA level on DCs upon immunisation with Q β (CpG). However, we have found no differences of the expression of the molecule on the surface of DCs post immunisation. Interestingly however, we have shown that the expression of this molecule is reduced in TIL from tumours that are controlled by therapeutic immunisation. CD101 expression on DCs leads to IL-10 production, an anti-inflammatory cytokine. On T cells on the other hand, CD101 engagement reduces the strength of TCR/CD3 signalling¹⁴⁵. By demonstrating that this inhibitory molecule is differently expressed in tumours that are controlled by therapeutic immunisation compared to untreated tumours, we may have found one of the mechanisms by which Q β (CpG) controls tumour growth, however this should be assessed in future work. This observation may contribute for future work on characterising the expression in tumours and the use as a therapeutic target to boost T cells fitness and responses in immunotherapy against tumours.

In summary, this work contributes to the understanding of DC-mediated immune responses and further establishes the potential of VLPs to modulate the response and adequate it to the different types of offenses that the immune is subjected to. Finally, we have contributed to the notion that immune responses are orchestrated by a complex milieu of cytokines and immune cells and that there are several processes happening in parallel that interact and shape the overall response. Understanding such process is a challenge but rewarding endeavour as many therapeutic targets and basic knowledge can be derived from research of this kind. Moreover, VLPs and CpG are currently being employed for clinical use, especially

in vaccine formulation. We hope to contribute to the understanding of the immune activity of both compounds and facilitate their use as vaccines.

Chapter IV: Innate Pattern Recognition Receptors and IgG class Switching – Implications for vaccine design

4.1 Introduction

Influenza is a worldwide concern for public health and, in spite of widespread immunisation programs in place, annual epidemics and occasional pandemics still occur. Around 3 to 5 million cases of the severe form of the disease are registered every year with estimated mortality rates between 6 to 20%¹. Out of the four types of seasonal influenza only type A and B are of public health concern according to the WHO Influenza surveillance program. Type A is the most common circulating strain and is further classified according to the envelope proteins of the virus; neuraminidase (NA) and hemagglutinin (HA). Currently, there are two caveats regarding the vaccines developed against type A Influenza. Firstly, all the vaccines approved are based on inactivated, split or live-attenuated viruses. The live attenuated vaccine may carry the particular problem that some of the risk groups for influenza virus infection are also groups with heightened risks of side effects upon immunisation with live vaccines, such as immunocompromised subjects. Secondly, the circulating strains used for vaccine formulation are often unable to protect against emerging strains of the virus. Indeed, the deadly Influenza pandemics thus far resulted from emerging viruses that underwent antigenic shift usually by exchanging HA with other circulating strains².

Antigenic targets for Influenza vaccine development

In addition to HA, the influenza virus envelope contains two other major proteins, NA, and the M2 ion channel. HA is the immunodominant protein of the viral envelope and in natural Influenza infections, the response is dominated by anti-HA antibodies compared to the other proteins. Nonetheless, M2 has been shown in preclinical models to be similarly protective as HA-based vaccines³. Considering the high mutation rate of HA, it would be beneficial to generate vaccines able to induce a protective response against more conserved structures such as the fusion loop of HA and the extracellular domain of M2, that unlike the globular domain of HA are shown to promote protection against multiple strains and across different clades. Moreover, by developing recombinant vaccines instead of inactivated or attenuated ones, the associated risk of tolerability issues is likely to be reduced.

M2 is a homo-tetramer integral membrane protein and its extracellular domain (M2e) is highly conserved across most of the Influenza A strains. Moreover, M2e has remained relatively unchanged throughout the major influenza pandemics, which renders M2 an antigenic alternative for a vaccine with broader spectrum of protection against a broad range of Influenza strains. Importantly, M2 is poorly immunogenic during natural infection and antibodies raised by whole virus vaccines are mostly dominated by anti-HA antibodies and anti-M2 antibodies remain largely absent. Hence, there is basically no pressure on M2e to generate antibody escape mutants, partially explaining why the extracellular domain of M2 is so well conserved. In contrast to conventional vaccines and natural infection, active immunisation employing M2 as antigen has been shown to confer protection when administered in combination with adjuvants in murine, ferrets, and macaques models^{3,4}. The main argument in favour of developing M2-based

vaccines does not lie in replacing the annual vaccination programs against influenza epidemics with the whole virus vaccines. Rather, developing M2-based vaccines could stand as the best alternative to control newly emerging strains in particular for pandemic threats that may not be controlled by HA-based vaccines as they usually are only released for use in humans after the peak of the pandemic. Thus, M2e-based vaccines may be a valuable and perhaps pivotal addition to conventional flu immunisation programs.

It has been previously demonstrated by different groups that heterologous Virus-like particle (VLP) displaying M2e confers protection in an mouse adapted influenza challenge and promotes cross-protection to virtually all the circulating Influenza strains^{155,157,158}. Therefore, with the aim of producing an universal vaccine against pandemic Influenza, we wanted to further investigate optimal strategies to increase the immunogenicity and induction of protective IgG subclasses of M2-based vaccines by using VLPs.

For M2-based vaccines, it has been demonstrated that protection is mediated not through direct viral neutralisation, but rather through Fc- γ receptor interaction of M2e-specific antibodies with innate immune cells. Therefore, the quality of the humoral responses (*e.g.* dominant IgG subclasses) and not only the magnitude of the response induced by M2-vaccines may be crucial to provide protection⁴. The importance of IgG subclasses has been demonstrated using M2e specific monoclonal antibodies with identical variable regions but fused to murine IgG1 or IgG2a. The IgG2a monoclonal antibodies were much more potent at protecting against mortality and morbidity in an influenza virus challenge, owing to the distinct effector interaction capacities of the Fc- γ portions of the IgG subclasses. In

addition, it was demonstrated that isotype switching to the protective form IgG2 is strictly TLR7 dependent, and TLR dependent isotype switching to IgG2 was crucial for protection and reduction in morbidity in influenza vaccines. The same findings regarding the importance of TLR stimulation by influenza vaccine has been shown with other vaccine formulations employing HA as an antigenic target⁷, further establishing that TLR stimulation is important for the control of the disease and not a requirement restricted to VLP-based vaccines⁷.

VLP based vaccines against Influenza

As extensively discussed throughout this thesis, VLPs are an attractive vaccine platform due to their excellent safety profile associated with high immunogenicity and relatively low production costs under GMP standards⁹. Their high immunogenicity is due to the virus-like surface structure and, in some cases, the presence of nucleic acids derived from the host cell expression system. RNA is often co-packaged into VLPs derived from RNA phages as the poly-anionic molecules are involved in the assembly and stability of the particle¹⁰. During process development, the choice of expression system usually revolves around yield, cost and sometimes the convenience of familiarity with the system and techniques in place. This strategy, however, usually fails to evaluate the impact of the production process on the immunogenicity of the particle. For instance, the most common platforms for protein production are *E. coli* or eukaryotic systems such as insect cells, yeast or mammalian cells. However, the secondary modifications in the nucleic acid of each of the expression system vary greatly, thus possibly resulting in different immune responses due to differential TLR7 and

RIG-I/MVAS agonistic capacity, thus impacting the subsequent efficacy of the vaccine¹¹.

The ability to recognise pathogens as foreign and potentially dangerous is an important feature of the immune system and it is promoted by several pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and cytoplasmic nucleic acid receptors, such as the retinoic acid inducible gene I (RIG-I) and cGAS. These sensors orchestrate the immune response by triggering transcriptomic programs that result in cytokine production, cell recruitment and maturation, and ultimately promotes inflammation or tolerance. VLP-induced isotype switching from IgM to IgG2b/c is promoted by dual engagement of BCR and TLR in B cells following uptake of VLPs containing RNA^{12,13}. There is evidences that prokaryotic and eukaryotic RNAs of different classes (*e.g.* rRNA, mRNA, and tRNA) despite all being TLR7 agonists, have distinct capacities to activate and induce cytokine production in DCs *in vitro*^{11,14}. However very little evidence for the same has been gathered *in vivo* and even less is known whether these observations would impact the protective immune response induced by vaccines. Considering the pivotal role of TLR7 signalling in B cells for efficacy of vaccines against influenza we wanted to evaluate the impact of different classes of RNA in the formulation of VLP based vaccines.

In the present study we demonstrate that not only the presence of the TLR7 ligand is important to promote isotype switching to IgG2 but also the level of secondary modifications of the TLR ligand employed. We observed that RNA secondary modifications affected B cell responses in a strictly TLR7-dependent fashion, while cytoplasmic receptors were not involved in the process. The results presented here

support M2-based vaccine as a highly efficacious influenza vaccine. Moreover, we further elucidate the versatile nature of VLPs as a vaccine platform able to rationally modulate and skew the humoral immune response.

4.2 Experimental approach and Research

4.2.1 Experimental approach

A panel of VLPs containing RNAs with different levels of secondary modifications was developed. Mice were immunised with Q β VLP containing different types of RNA and an universal influenza epitope. Immune response and vaccine efficacy were assessed.

4.2.2 Research questions

1. What is the impact of the source of RNA in the quality and magnitude of the immune response elicited by VLPs?
2. Do different types of RNA impact markers of activation of B cells and DCs?
3. Do the differences observed in the immune response impact the efficacy of the vaccine in an Influenza challenge model?

4.3 Material & Methods

A complete list of materials and reagents, suppliers and catalogue codes utilised on this thesis can be found on the supplementary materials (Table S.3 to S.7).

4.3.1 Q β preparation

VLP derived from the coat protein of the bacteriophage Q β were recombinantly expressed in *E. coli* and purified as described on section 1.3.1.

Disassembly of Q β VLPs - Q β VLP was disassembled by reducing disulphide bonds with 10 mM DTT and RNA was precipitated with 0.7M MgCl₂. The solution was centrifuged 10 min at 14000xg at 4 °C (Eppendorf 5810 R, in fixed angle rotor A-4-62) to remove the precipitated RNA from the solution. The supernatant, containing the released, dimeric Q β coat protein, was used for the chromatographic purification steps.

Purification of Q β coat protein - The supernatant of the disassembly reaction was loaded onto a cation exchange SP-Sepharose FF column (GE Healthcare) and eluted in ionic gradient. In a second step, the isolated Q β coat protein was loaded onto a size exclusion Sephacryl S-100 HR (GE Healthcare) column equilibrated with 20 mM sodium phosphate / 250 mM sodium chloride, pH 7.2.

RNA extraction – Total eukaryotic RNA was extracted from confluent HEK 293 cells, total prokaryotic RNA from *E. coli* and tRNA purchased from yeast (Sigma). Extraction was carried out using the phenol-chlorophorm method. Quality of RNA was assessed by 1% agarose gel and quantified by Qubit according to manufacturer's instructions.

Re-assembling Q β VLPs - Purified coat protein in reducing conditions was mixed with 100 nmol of RNA in 50% DMSO. The solution was then dialysed at room temperature against 500 ml of PBS, pH 7.2, using a 3.5 kDa cut off dialysis membrane. H₂O₂ was added to 7 mM final concentration and the solution incubated for 1h to induce the formation of disulphide bonds. After 1h, VLPs were

dialysed against 500mL of PBS at RT. Re-assembling was assessed by agarose gel, DLS and EM.

Vaccine preparation and peptide sequence- M2 peptide SLLTEVETPIRNEWGC-azide were coupled to Q β VLPs through the copper-free click chemistry crosslinker DBCO following instructions of the product (Sigma).

4.3.2 Electron microscopy and Dynamic light scattering

Suspensions of VLPs were adsorbed on carbon-formvar coated copper grids, 400 mesh (Agar Scientific) and stained with 1% uranyl acetate (pH 6.8). The grids were examined with a Tecnai T12 microscope. VLP samples were diluted to 0.3 mg/mL with in 20mM HEPES buffer and analysed at room temperature with a Malvern Zetasizer Nano ZS (Malvern) equipped with a He-Ne laser (633 nm). Data analysis was with DTS software (version 4.2), using a non-negatively constrained least squares (NNLS) fitting algorithm. Dispersant refractive index and viscosity of the dispersant were assumed to be 1.33 and 1.02 cP, respectively.

4.3.3 LPS and RNA content of preparations.

Final preparations of the vaccine were tested for endotoxin content using the Pierce LAL chromogenic LPS test (Thermo Scientific) following the manufacturer's instruction.

RNA was measured by densitometry on a 1% agarose gel stained with SYBR safe and confirmed by Quant-It RNA HS kit and Qubit fluorimeter (Thermo Scientific) according to manufacturer's instructions.

4.3.4 Animals and immunisations

All mice used in this study were bred in specific pathogen-free (SPF) conditions. This study was carried out in accordance with the recommendations of the Animals (Scientific Procedures) Act 1986 (ASPA) and European Directive 2010/63/EU on the protection of animals used for scientific purposes and in accordance with the Swiss Animals Act (455.109.1) (September 2008, 5th, University of Bern). Wild-type C57BL/6J mice were purchased from Envigo.

The C57BL/6 MAVS^{-/-} (also known as *Cardif*^{-/-}) mice were provided by Jan Rehwinkel and it have been described elsewhere¹⁶³.

For antibody responses, mice were immunised s.c. with 50µg of Qβ(pRNA)-M2e, Qβ(eRNA)-M2e, or Qβ(tRNA)-M2e and serum collected on day 10 from the lateral tail vein.

4.3.5 ELISA

M2e-specific antibodies were measured by coating plates with 5µg/mL of the M2e peptide crosslinked to the carrier protein RNase. Peptide and carrier protein were crosslinked using SMPH and following the manufacturer's instructions. VLP-specific antibodies were measured by coating Immunoplates (NUNC) with Qβ VLPs at 2.5µg/mL, diluted in carbonate-bicarbonate coating buffer overnight and blocked with 2% BSA in PBS-T. Immune serum was serially diluted 12x (2-fold dilution) starting at 1:50 for IgG1 and 1:100 for IgG, IgG2b and IgG2c. Subtypes were detected with HRP-conjugated goat anti-mouse IgG1, IgG2b and IgG2c antibodies (all from Thermo Fisher), diluted to 1:3.000. TMB solution was added and reaction halted with 0.1M H₂SO₄. Absorbance was immediately read at a wavelength of 450 nm.

4.3.6 Type I IFN response measurement

ELISA – Serum from immunised mice was tested following manufacturer's instruction. The ELISA Kit was purchased from Thermo Fisher with the sensitivity range of 31-2,000 pg/mL.

IFIT induction on 3T3 cells - Mouse type I IFN levels in the serum was measured by incubating 3T3 cells seeded with 25µl of serum from immunised mice. Cells were incubated for 3h with serum. Following incubation period, cells were immediately prepared for RNA extraction.

4.3.7 qPCRs

Total RNA was isolated using the RNAeasy Mini Kit (Qiagen) with in column DNA digestion. cDNA and non-RT controls were synthesised using the iScript cDNA Synthesis kit (BioRad). Quantitative RT-qPCR was performed in duplicate using SYBR Green Master Mix (BioRad) on an CFX connect machine (BioRad). Results were analysed with a CFX Maestro Software (BioRad) employing the comparative Ct methods GAPDH as the endogenous normalisation control.

Table IV.1 – Primer sequence

Gene	Forward	Reverse	Supplier
IFIT1	5'AGAACAGCTACCACCTTTAC	5'TTCTTGATGTCAAGGAACTG	Sigma
GAPDH	qMmuCEP0039581 (Assay ID)	sequence not provided by the supplier	BioRad

4.3.8 Cytometry

VLP uptake - Q β VLPs were labelled with AlexaFluor 647 or PE containing the NHS reactive group as instructed by the manufacturer (all from Thermo Fischer). C57BL/6 mice (9–12 weeks old; Envigo) were injected s.c. with 30 μ g of fluorescent VLP and peptide into the hind leg.

Popliteal and inguinal LNs were isolated and a single-cell suspension was prepared by incubating LN with 1 mg/mL collagenase D (Roche) and 0.04 mg/mL DNase I (Boehringer) in 5% FSC containing DMEM, for 30 min at 37°C. Organ pieces were passed through a 70- μ m cell strainer and stained for cell-specific markers. The following fluorochrome-labelled antibodies were used: anti-CD11c, anti-CD317, anti-CD8a (all from eBioscience), anti-F4/80, Live/Dead Aqua cell stain (all from Life Technologies, Thermo Fisher Scientific).

4.3.9 Influenza challenge

C57BL/6 mice were immunized s.c. on days 0 and 14 with 50 μ g with either Q β (pRNA)-M2e or Q β (eRNA)-M2e or as a control with 50 μ g of Q β . On day 21, mice were bled in order to determine anti-M2 IgG or subclass antibody titres by ELISA. Mice were then challenged at the indicated time point with a lethal dose (2 $\times$ LD50) of mouse-adapted influenza A virus PR8 (H1N1 strain was kindly provided by Dr. Manfred Kopf, ETH Zurich). Following the challenging, body weight and temperature of was monitored daily at the same time of the day. Experimental end-point was determined based on body temperature reaching 30°C or loss of 30% of their initial body weight, when mice were euthanized.

Aiming to reduce the number of animals used in this study and the potential suffering imposed to the animals, we made the decision not to include VLPs containing tRNA in the challenge, as the overall IgG titres were lower and specially owing to the similar IgG2/IgG1 ratio between VLP containing eukaryotic RNA (eRNA). Considering that our primary null hypothesis was that the ratio of IgG2/IgG1 does not affects the protection conferred by the vaccine, it was reasoned that Q β (eRNA)-M2e versus Q β (pRNA)-M2e would provide enough evidence to assess the validity of the hypothesis.

Influenza challenge models were performed in collaboration with Elisa Roesti (University of Bern).

4.3.10 Statistical analysis

Antibody titres were represented as OD50 (dilutions that reached half the maximal OD observed for the assay). Data was examined by ANOVA (whenever ANOVA F statistics were significant, $p < 0.05$) followed by multiple T test corrected by Bonferroni correction. Differences were considered statistically significant at $P < 0.03$.

Survival rate was analysed by Log-rank (Mantel-Cox) test.

4.4 Results

4.4.1 VLPs packaging eukaryotic mRNA fail to promote isotype switching at the same levels as prokaryotic mRNA

VLPs and other classes of nanoparticles induce a humoral response dominated by IgG1 in the absence of packed RNA in mice^{112,158}. Whilst in the presence of ssRNA derived from *E. coli* the humoral immune responses are dominated by the IgG2 subclass. To confirm the capacity of other classes of RNA to promote IgG2-skewed responses, we have generated VLPs derived from the bacteriophage Q β packing prokaryotic RNA from *E. coli*, eukaryotic RNA from HEK 293T cell line, and yeast derived tRNA by *in vitro* re-assembly of the VLPs. The integrity of VLPs was assessed by electron microscopy, dynamic light scattering (DLS) and native agarose gels demonstrating that Q β re-assembled with the three classes of RNA. Each having similar icosahedral shape, sizes of approximately 30 nm, as measured by EM (figure IV.2.A) and DLS, and similar negative net charge as measured by ζ potential. Moreover, similar amounts of RNA were packaged regardless of the class of VLP (figure IV.2.B) as assessed by the charge-dependent migration pattern in a native agarose gel. After assessing the quality of VLPs, the three formulations were coupled to the M2e peptide derived from the extracellular domain of the conserved M2 protein from Influenza A strain¹⁵⁵. The three VLPs are henceforth represented as Q β (pRNA) for prokaryotic RNA, Q β (eRNA) for eukaryotic messenger RNA (mRNA) and Q β (tRNA) for eukaryotic transfer RNA (tRNA).

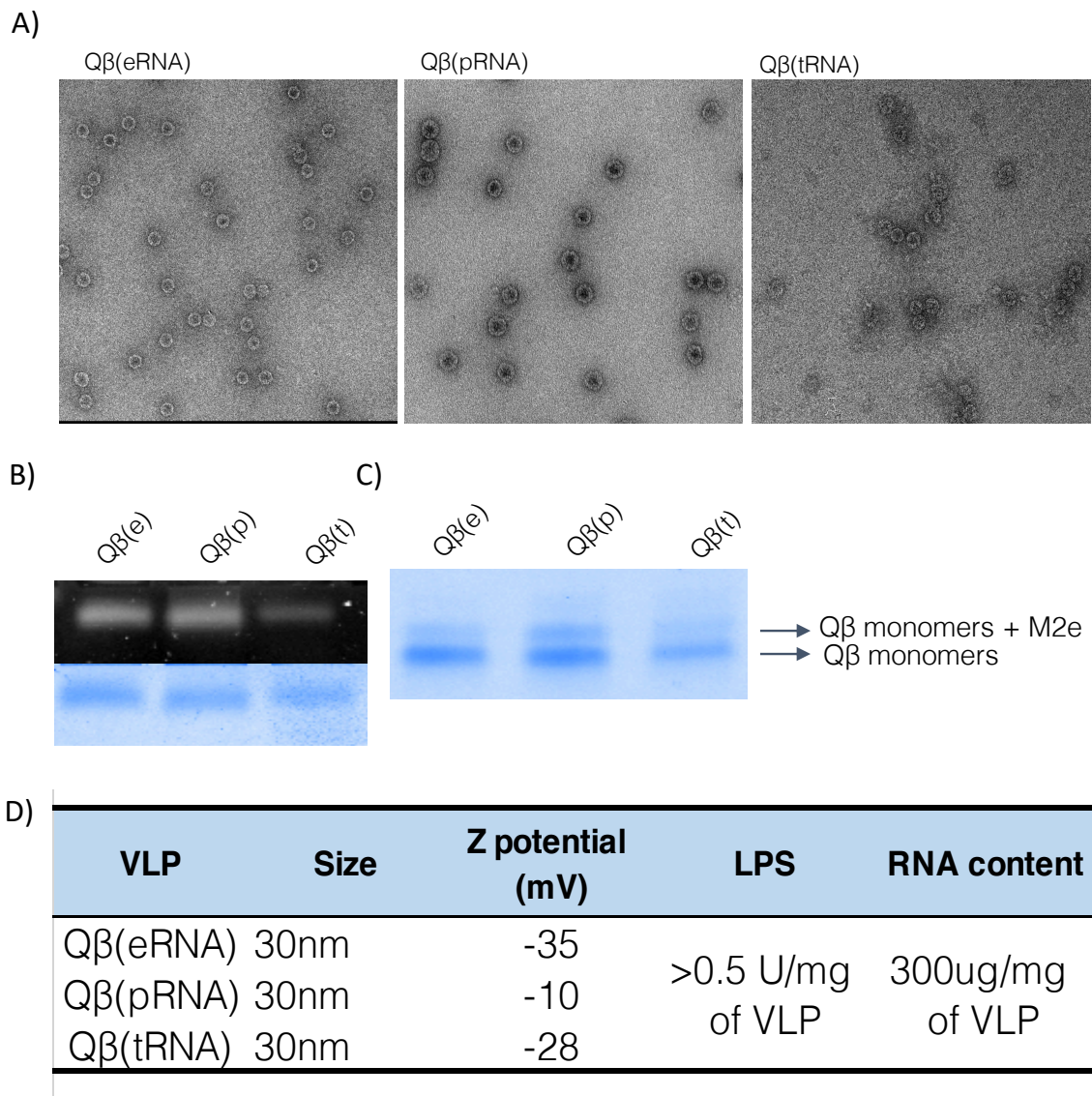


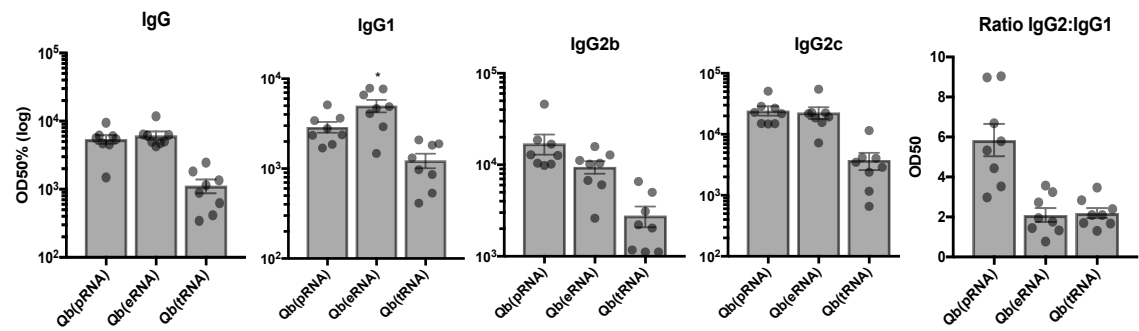
Figure IV.2: Vaccine preparation and characterisation

A) Electron micrograph of re-assembled VLPs. Q β VLP containing eukaryotic RNA (eRNA), prokaryotic RNA (pRNA) and tRNA (tRNA) have similar structure and size. **B)** Native analysis of RNA and protein content of re-assembled VLPs. Upper panel showing SYBR staining and bottom panel showing protein staining of same agarose gel. **C)** Similar coupling efficiency of different VLPs and M2 peptides on a reduced SDS-PAGE gel electrophoresis. **D)** Characterisation of VLPs. Size was taken as an average of values obtained in the EM images and by DLS. Z potential was measured by DLS and is expressed as mV. LPS content expressed on endotoxin units (EU) per milligram of VLP. RNA content per mg of VLP.

Vaccines had also been characterised in terms of the LPS content to rule out additional TLR stimulation. All the formulations had similar LPS concentration lower than <0.5 EU dose of vaccine, excluding LPS as variable affecting the relevant immune responses.

To evaluate the impact of the packaged RNA on the IgG2/IgG1 ratio after immunisation, C57Bl/6 female mice were immunised s.c. with 50ug of the respective Q β VLP and the total IgG and indicator subclasses against VLP and M2 peptide were assessed 10 day later by ELISA (figure IV.3). VLPs containing different classes of RNA had induced distinct humoral responses with different ratios of isotypes. Likewise, the type of response against the peptides coupled to the VLPs followed the same pattern. Q β (eRNA)-M2e and Q β (pRNA)-M2e immunised mice had similar circulating levels of total IgG, while Q β (tRNA)-M2e had approximately a 10-fold reduction in the circulating IgG titres. To further characterise the humoral responses, IgG1 and the two isotypic forms of IgG2 in the C57BL/6 mice IgG2b and IgG2c were measured. In accordance with the significantly lower total IgG levels, Q β (tRNA)-M2e had lower levels in comparison to the other groups, while Q β (eRNA)-M2e and Q β (pRNA)-M2e induced similar levels of IgG2b/c but significantly different levels of IgG1 ($p < 0.03$). Moreover, the ratio IgG2/IgG1 was 3 times higher in the Q β (pRNA)-M2e group compared to both Q β (eRNA)-M2e and Q β (tRNA)-M2e demonstrating the superior capacity of prokaryotic RNA at promoting the isotype switching to IgG2 over IgG1.

A) Q β -specific Antibodies



B) M2-specific Antibodies

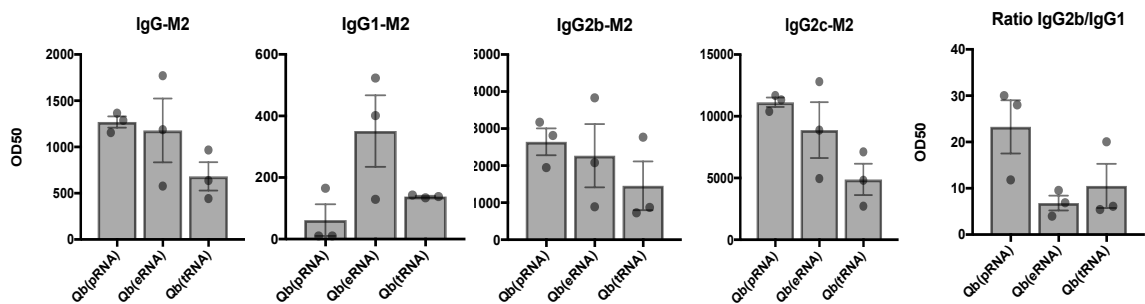


Figure IV.3 - Total IgG and subtypes and IgG2/IgG1 ratio are influenced by the type of RNA packaged by VLPs.

IgG, IgG1, IgG2b and IgG2c were measured by ELISA 10 days after s.c. immunisation with 50ug of VLP. Q β (eRNA)-M2e induces significantly higher levels of antigen-specific IgG1 compared to the two other vaccines ($p > 0.03$) against VLP and M2. Ratios of IgG2b:IgG1 are represented as isotype geometric mean titres. **A)** VLP-specific antibodies. Mean + SEM $n = 8$ mice per group. **B)** M2-specific antibodies. Mean + SEM $n = 3$ mice per group. OD50 with titres defined as those dilutions that reached half the maximal OD observed for the assay. The results are representative of 2 independent experiments.

4.4.2 Isotype switching promoted by VLPs is independent of MAVS

The isotype switching promoted by RNA recognition is attributed to direct TLR7 engagement in B cells. Recent literature however, suggests a role for cytosolic RNA sensors in the modulation of the humoral response¹⁶⁴. Secondary modifications in the RNA is a prominent feature distinguishing prokaryotic RNA from eukaryotic RNA recognized by cytoplasmic RNA sensor RIG-I. To investigate a possible role

for RIG-I in driving IgG2 responses, mice lacking the RIG-I downstream adaptor molecule MAVS were immunised following the same regimen and the IgG subtypes were evaluated 10 days after immunisations (figure IV.4). For all the parameters evaluated, MAVS knockout had similar levels of total IgG, IgG1 and IgG2b/c, ruling out a role for RIG-I in the isotype switching mediated by prokaryotic RNA in VLPs.

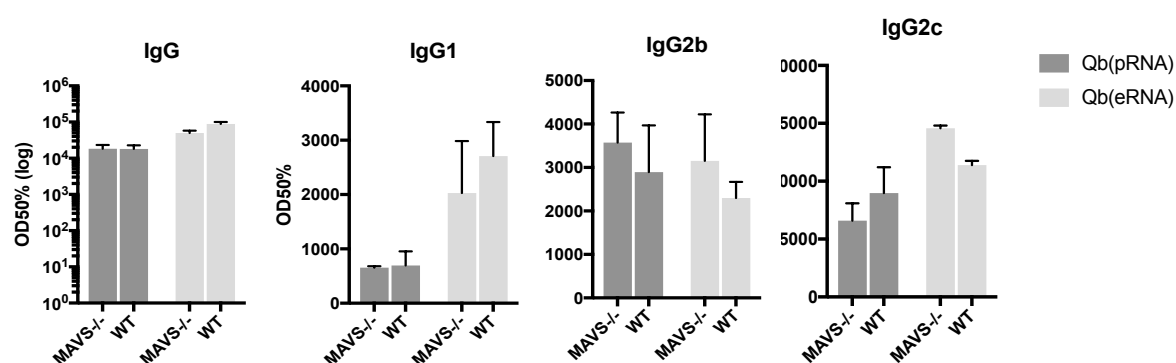


Figure IV.4 – Isotype switching promoted by VLPs is independent of MAVS.

A) IgG, IgG1, IgG2b and IgG2c were measured by ELISA 10 days after s.c immunisation with 50ug of VLP in age and sex-matched MAVS knock-out and WT counterparts. VLP-specific antibodies. Mean + SEM n=5. OD50 with titres defined as those dilutions that reached half the maximal OD observed for the assay.

4.4.3 Activation of DCs and B cells

Although isotype switching is dependent of direct TLR7 engagement in B cells, TLR7 and MyD88 expression on naive B cells has been shown to be modulated by IFN- α produced by pDCs¹⁶⁵. Considering that DCs also respond differently according to the class of RNA, we wanted to assess whether DC responses would be influencing B cells and isotype switching in our model.

First, to confirm the concomitant uptake of Q β VLP by different subsets of DCs and B cells, mice were injected with VLPs coupled to a fluorescent label. 24h later, draining lymph nodes were processed and immune cells analysed for VLP uptake (figure IV.5). 4% of total macrophages (CD11c+F4/80+) were positive for the VLP. Similar percentage of pDCs (CD11c+CD317+) and cDCs (CD11c+F4/80- CD317-) had taken up the VLP 20h post immunisation. Up to 1% of B cells were positive for the VLP confirming the capacity of the VLP to interact with the different subsets of DCs and B cells.

To test the early immune response mediated by DCs and B cells, mice were immunised with 50 μ g of Q β (pRNA), Q β (eRNA) and Q β (tRNA) s.c. and 20h later serum was collected to evaluate circulating levels of IFN- α . Serum levels of IFN- α were first assessed by commercially available ELISA kits; however IFN- α levels were below the detection limit of the assay (31 pg/mL). Considering that nucleic acid containing VLPs are shown to induce IFN- α production *in vitro*, a more sensitive test to determine type I IFN responses was employed. The 3T3 murine cell line was incubated with serum from immunised mice and the IFN-dependent induction of the ISG IFIT1 gene was measured by qPCR. Serum of immunised mice induced different levels of IFIT1 gene depending on the type of vaccine received (figure IV.4.B), with Q β VLP containing prokaryotic RNA inducing comparatively higher levels of the expression of the IFIT1 gene on 3T3 cells.

Next, we wanted to evaluate whether the type I IFN response was regulating key markers of the immune response. TLR7 and CD69, two receptors that are up-regulated in response to IFN- α were measured on splenic B cells 20h following immunisation in order to analyse the activation state of B cell *in vivo*. We found

that TLR7 was up-regulated on B cells from immunised mice (figure IV.4.C) when compared to the naïve, but there were virtually no differences in the expression of TLR7 between Q β (pRNA) compared to Q β (eRNA) (figure IV.4.D) despite strikingly different levels of IFIT1 induction.

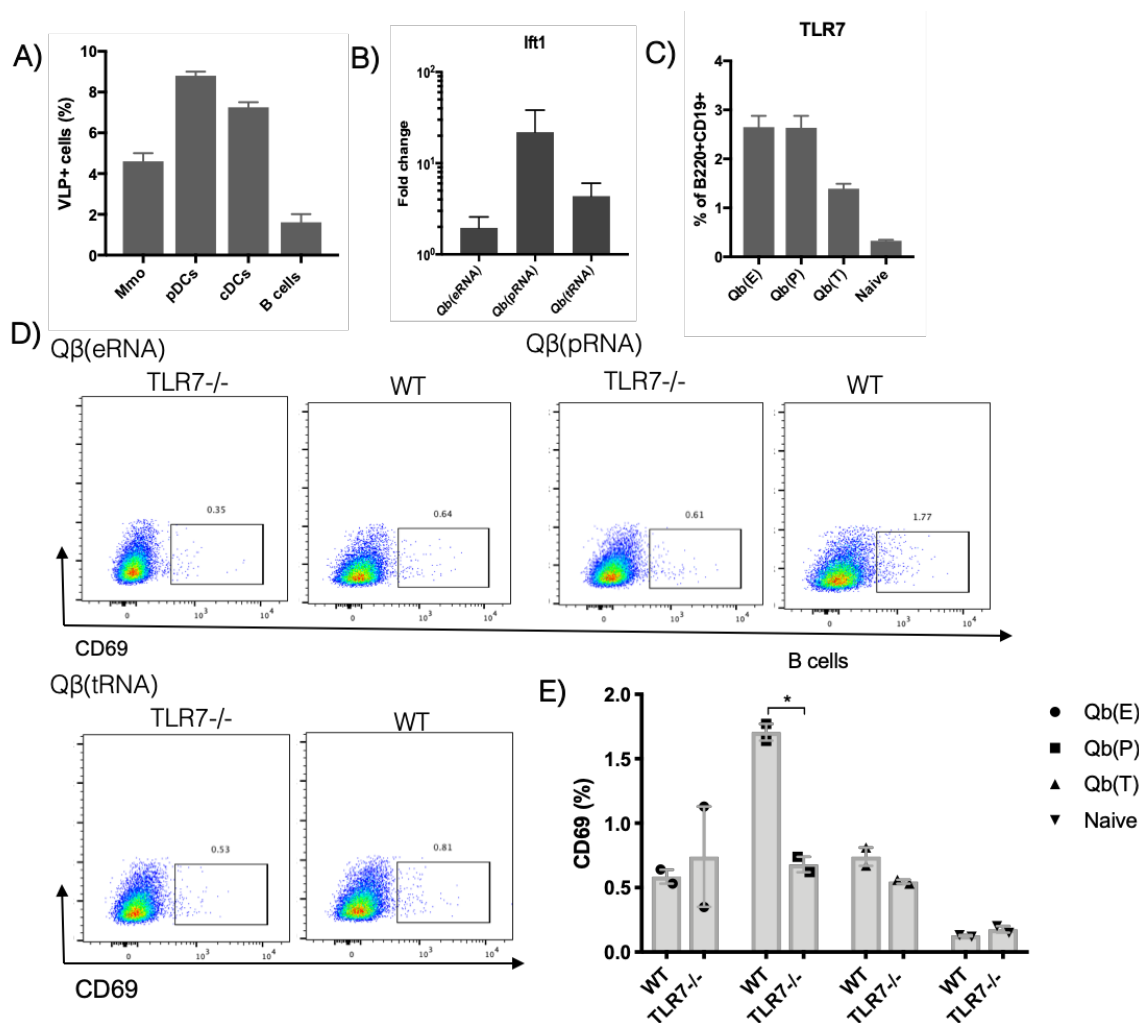


Figure IV.5 – TLR7 dependent immune response of B cells and DCs.

A) Interaction of VLPs with immune cells in vivo. Axis indicates the percentage of macrophages (Mmo), pDCs, cDCs and B cells positive for *E. coli*-expressed Q β 20h post injection. Experiment is representative of 3 independent experiments. **B)** Induction of the *ifit1* gene on 3T3 cells treated with serum of mice immunised with VLPs loaded with different RNAs 20h post immunisation, measured by RT-qPCR. Data are presented as fold changes compared to the average cells treated with sera of naïve mice. Experiment was performed only once. **C)** TLR7 expression in B cells (B220⁺CD19⁺) from immunised mice 24h post immunisation. Experiment was performed only once. **D)** CD69 expression on splenic

B cells 24h post immunisation of WT and TLR7 knock out mice. Gated on B cells (B220⁺CD19⁺). Experiment was performed only once. **E)** Barplot of CD69 expression on B cells and splenic monocytes 24h post immunisation. Experiment was performed only once. Data represented as mean + SEM n=3 mice per group. * P < 0.04.

In accordance with the literature, CD69 expression was dependent on TLR7 and up-regulation was completely abolished on the TLR7 knockout in response to VLP. In addition to that, CD69 was expressed in higher levels in B cells and monocytes from mice immunised with Q β (pRNA). This may be correlated to the higher levels of I IFN responses induced by Q β (pRNA) (figure IV.4 C-G).

4.4.4 Flu challenge

Following the assessment of the humoral immune responses, we wanted to investigate whether the ratio of isotype switched antibodies would influence the protection conferred by the Q β -M2e universal influenza vaccine. To that end, mice immunised in a prime-boost regimen with Q β VLPs coupled to M2e packaging either prokaryotic RNA or eukaryotic RNA were then challenged on day 28 (figure IV.6.A) with the previously determined lethal dose (2 $\times$ LD50) of the mouse-adapted influenza A virus PR8 (H1N1 strain). Temperature and weight were monitored daily until control group reached the severity limit of 30% weight loss or temperatures lower than 32°C, when mice were euthanised. All the procedures conformed to local ethical frameworks in place aiming for the reduction of suffering and numbers of animals used. Mice immunised with both formulations were protected against the lethal dose of influenza but there were some differences in morbidity. Mice immunised with Q β (pRNA)-M2e had marginally reduced weight loss during the challenge and better temperature control when compared to Q β (eRNA)-M2e, although there were no differences in overall mortality as all

immunised mice survived.

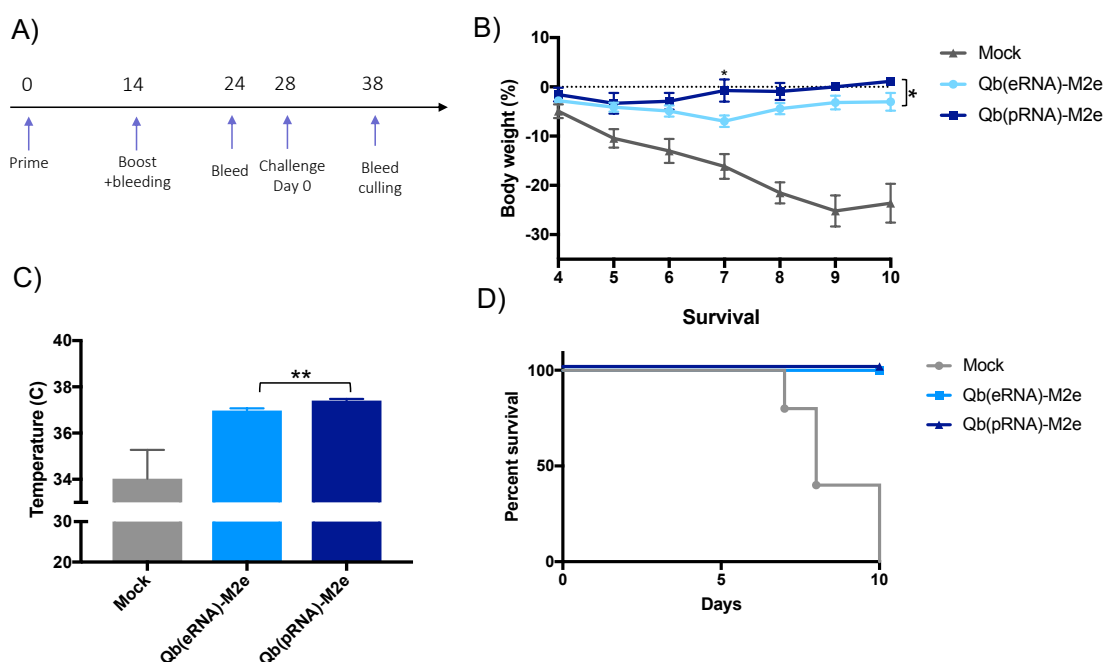


Figure IV.6 – Reduced morbidity symptoms of mice immunised with formulations containing prokaryotic RNA in an Influenza challenge.

A) Immunisation and challenge schedule. C57Bl/6 mice were immunized s.c. with 50 µg of Qβ(pRNA)-M2e (prokaryotic RNA) or Qβ(eRNA)-M2e (eukaryotic RNA), or PBS as a control on day 0 and 14. Mice were then challenged with a lethal dose of the mouse-adapted influenza A/PR/8/34 virus (2×LD₅₀) on day 28. **B)** Body weight (%) represented as the mean +/- SEM percentage of weight loss (n=5). Statistical significance measured by Welch's T test (p>0.007). **C)** Average temperature from day 4 to 11 post challenge. **D)** Percentage of survival of immunised and control groups following challenge. Experiment was performed only once.

*One mouse from the Qβ(pRNA) group was prematurely culled sick due to unrelated sickness. Mice that reached the limit severity score were euthanised and the last observed parameter was used for the following days and average calculations (Last observation carried forward, LOCF).

Humoral responses in challenged mice were assessed following immunisation and challenge (figure IV.5). Antibody titres were greatly boosted by a second dose of the vaccines and the ratio of IgG2/IgG1 was the same as in mice immunised in following a one-dose regimen (figure IV.3). Interestingly, M2-specific humoral responses were not detected in the mock group even post challenge, confirming that influenza virus infection does not normally trigger an anti-M2 response.

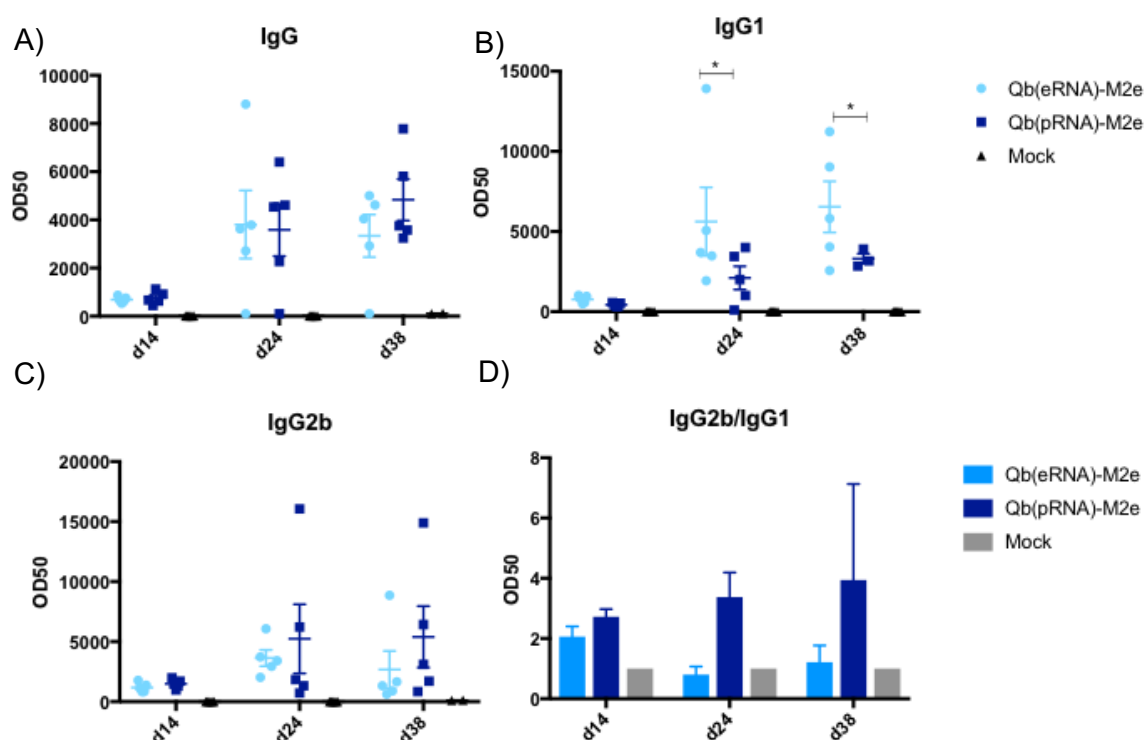


Figure IV.7 – Humoral responses against M2e.

Circulating levels of IgG, IgG1, IgG2b were measured by ELISA on day 14, day 24 (10 days after boost) and day 38 (10 days after challenge). Vaccine dose was 50ug of VLP, injected s.c.. Qβ(eRNA)-M2e induces significantly higher levels of antigen-specific IgG1 compared to the other vaccines (* p<0.04) against VLP and M2. Ratios of IgG2b:IgG1 are represented as isotype geometric mean titres. Data represented as mean + SEM, n=5 mice per group. Experiment was performed only once.

4.5 Discussion

In the present study, we provide evidence for an important role of the type of RNA packed into VLPs in driving the quantity and quality of immune responses induced by vaccination. Our results also contribute to the understanding of PRR stimulation as mediators of adjuvant activity in vaccines. The ability to differentiate between self and foreign nucleic acids by TLRs is attributed to 1) endosomal localisation of the receptors that guarantees spatial segregation of receptor and its host-derived ligands in the cytosol and 2) distinct agonistic capacities of different classes of RNA. Indeed, the immunogenicity of VLPs is in part dependent on the origin of the nucleic acid packaged and packing prokaryotic RNA leads to better protection against disease induced by against influenza virus infection.

With respect to regulation of isotype switching, it is known that dual signalling provided by BCR engagement and TLR signalling is required. VLPs containing nucleic acids are able to provide both signals, contributing to the quality of the humoral responses without the requirement of additional adjuvants. The presented evidence that the type of RNA influences the protective capacity of M2e based vaccines may therefore contribute to the rational design of vaccines in the future.

IFN- α is known to play a role in modulating TLR7 and MyD88 expression in B cells *in vitro*. In the present study, however, we were unable to replicate such finding *in vivo*. Even though Q β (pRNA) was superior at inducing type I IFN responses *in vivo*, these differences did not translate to differences in TLR7

expression in B cells. Recent literature also suggests a role for type III IFN produced by NK cells in modulating TLR7 sensitivity and expression by B cells²⁰; however, this was not addressed directly in this study. Furthermore, we found no role for MAVS in the antibody response elicited by VLPs in disagreement to the findings described by Zeng *et al*; however this paper has recently been retracted. Although we do not rule out a role for such receptors in isotype switching, our data argue that in the context of VLP-mediated TLR stimulation, receptors such as RIG-I may not be essential to contribute to isotype switching or protection.

An example of how the findings of this study may be translated into clinical solutions can be illustrated by the VLP-based HPV vaccines. All three currently available prophylactic HPV vaccines do not contain packaged nucleic acid in their final formulation, but rather benefit from the inclusion of adjuvants including some engaging TLR4²¹. This supports the role of TLR in driving immunogenicity and efficacy of vaccines. Another supporting example of the major role of TLR ligands in the immunogenicity of vaccines is exemplified by a VLP-based vaccine against the influenza strain H5N1. The VLP was expressed in A549 cells and failed to protect against H5N1 challenge. The immunogenicity of the vaccine was enhanced by the addition of an adjuvant targeting the RIG-I receptor, which resulted in protection. Taking our findings into consideration, it is not unreasonable to speculate that use of VLPs packaging RNA and expressed in prokaryotic systems would be a more efficient and effective platform, eliminating the need to include additional adjuvants that may result in increased costs and unforeseen side-effects for the vaccine. Collectively, our data support the growing trend of employing ligands for PRRs as adjuvants and provides further evidence to optimise the design of adjuvants of this kind. We demonstrated that even

though both vaccines (i.e. containing pRNA or eRNA) perform similarly in terms of protection against lethal infection, there were significant differences in the relative morbidity experienced during the acute phase and recovery from the disease.

In conclusion, our results demonstrate *in vivo* that manufacturing differences resulting in distinct types of packaged RNA in VLPs, has an impact on the quality of the vaccine. We propose that during the development phase of VLPs, the type and quality of RNA packed should be assessed and thoroughly considered while evaluating which VLP should be employed as vaccine platform. Furthermore, when expressing VLPs in either prokaryotic or eukaryotic cells, RNA may be used as means of modulating the immune response to achieve the desired outcome. As in certain situations antibodies with increased or reduced effector capacity may be desired, depending on the disease and target structure, choosing the right expression system and RNA quality may be an important point of consideration.

Chapter V - Conclusion

1. Summary of conclusions

Chapter I – Literature review

Based on the literature review, it became evident the success of immunisation as an intervention to prevent (prophylactic vaccines) and also to treat (therapeutic vaccines) a variety of diseases. However, in order to develop superior subunit vaccines, it seems necessary to have a better understanding of the immune system. Thereby providing a rational framework for improved vaccine platforms that are able to modulate the response adequately to the disease target, especially when aiming to develop T cell vaccines.

Chapter II

- It was demonstrated that employing VLPs as an adjuvant for subunit vaccines can be beneficial even in the absence of conjugation of VLP and antigen. Moreover, we have demonstrated that the rules for improving T and B cell responses in a VLP-based vaccine are different depending of the manner as by the antigen is presented.
- We have provided pre-clinical data supporting the development of a therapeutic vaccine against HPV, employing VLPs packing CpG and presenting the heterologous antigen E7. The vaccine relies on T cell responses for protection and has the advantage of not requiring coupling of VLP and antigen, which renders the production process cheaper and faster.

- The adjuvant mechanisms of VLPs in the induction of humoral and CTL responses are distinct, and have differential requirements based on the level of organisation of the antigen as well as the manner of uptake into the relevant immune cells.

Chapter III

- The activation reprogramming of DCs that influence CD8⁺ T cell responses was characterised and thoroughly investigated. The data presented provided insights that may support further investigation on the topic of induction and establishment of T cell responses.
- We have shown a modulatory effect of CCL2 in the immune responses generated by T cells. However, we were unable to elucidate the precise mechanism of action.
- We also observed that the inhibitory molecule CD101 is down-regulated in tumours controlled by therapeutic vaccination. Thus, we propose CD101 as a possible target to help improve immunotherapy against tumours.

Chapter IV

- The presence of nucleic acid in VLPs is essential for optimal immune responses. Moreover, the source and secondary characteristics of the encapsulated nucleic acid greatly influences the quality of the response. These findings would suggest that greater attention to detail regarding the nature of the nucleic acid should be considered during the design phase of vaccines. The implication being selecting the optimal host system for expression.

- We have developed and further refined an influenza vaccine. Demonstrating how basic immunology research can also assist the development of superior vaccines, and guide scientists into making decisions based on evidence and not solely on convenience.

2. Limitations

In chapter III we have sequenced total DCs and not only DCs that had taken up the VLP. This may have either diluted the effects or it may have added the confounding factor of genes that were differently regulated in response to direct uptake of the VLP and genes that had its expression modified due to paracrine signalling of cytokines produced by the activated VLPs. However, considering that the aim of this thesis was to find general immune correlates of the innate immune response to the later adaptive response, we believe that this did not negatively impact our conclusions. However, this limitation should be considered when analysing the response at the cell population level.

Several murine immune cells express TLR9 and respond to CpG. We focus on DCs due to their role in presenting antigens and shaping the response of T cells. However, we recognise that confounding factors may have interfered in the outcomes observed as a result of other cells responding to CpG such as B cells.

We observed significant differences in the immune response depending on the cell type used or the experimental setting (primary cDC and pDCs versus BMDCs or *in vitro* versus *in vivo*). Our main priority while designing experiments was to guarantee consistency of conditions across the tests and whenever possible, we

aimed to run in *in vivo* test, however different approaches had to be used in order to answer our questions.

In chapter III, given the large number of relevant genes differently expressed and pathways enriched between Q β and Q β (CpG), it would be nearly impossible due to time and resources constraints to test the relevance or impact of all differently expressed genes. However, the data set and results generated in this thesis will be made publicly available and will support future projects. In addition to that, certain relevant targets were proven very challenging to test due to lack of reagents or mouse models.

Cytokines and targets chosen were biased towards analytes that could be measured using commercially available kits.

3. Final considerations

In this thesis I have presented data investigating the immune response elicited by subunit vaccines employing VLPs. Our focus was to find targets and immune correlates of vaccine efficacy that could be translated into therapies or pharmacological targets. Considering the scope, some topics were not thoroughly investigated, but we expect that our findings will support further studies on mechanisms and more detailed studies on DC, B, and T cell biology.

An important consideration is that, literature has consistently shown that several activation molecules of DCs and T cells, that had been shown crucial for the induction of cellular responses against modular antigens seem to have a redundant role in the immune response triggered by whole viruses or bacteria^{14,167}. This seem

also to apply to VLPs containing TLR ligands. Due to the several PAMPs and DAMPs that VLPs possess, and considering the data shown on this thesis, it is reasonable to assume that VLPs associated with TLR ligands activate a myriad of pathways and immune mediators that together, coordinate the immune responses having sometimes redundant roles. This explains the difficulty that we have found in isolating genes that would completely abrogate the immune response. This conclusion also corroborates the use of VLPs as an universal vaccine platform as for by relying on several mechanisms to induce and establish an immune response, the vaccine is more likely to be efficient across different populations and individuals with diverse immune response.

Supplementary materials

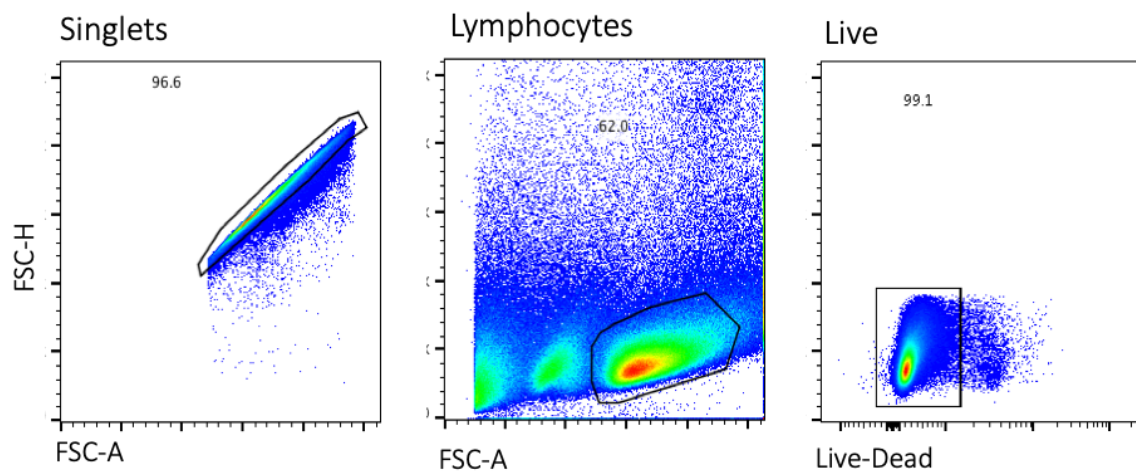
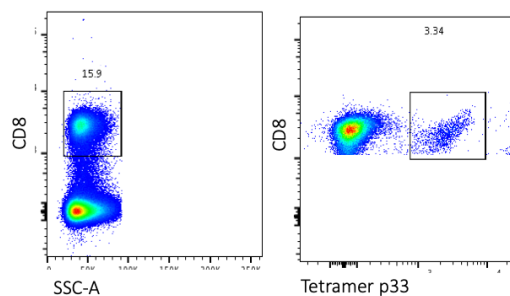


Figure S1 – General gating strategy

General initial gating of every analysis. Singlets by analysing the forward scattering by height (FSC-H) and area (FSC-A), followed selection of live cells.

A) Tetramer positive CD8+ T cells



B) CD3+CD8+ lymphocytes and IFN γ production

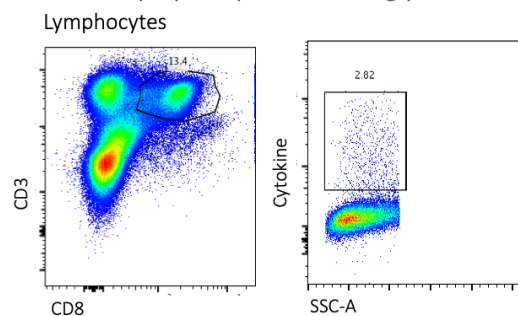


Figure S2 – Gating strategy for lymphocytes.

A) Tetramer staining, selection of CD8+ followed by Tetramer positive. C) ICS gating strategy. Starting in a similar way as described in A and then as outlines in C, with CD3+CD8+ double positives selected and then the cytokine of interest.

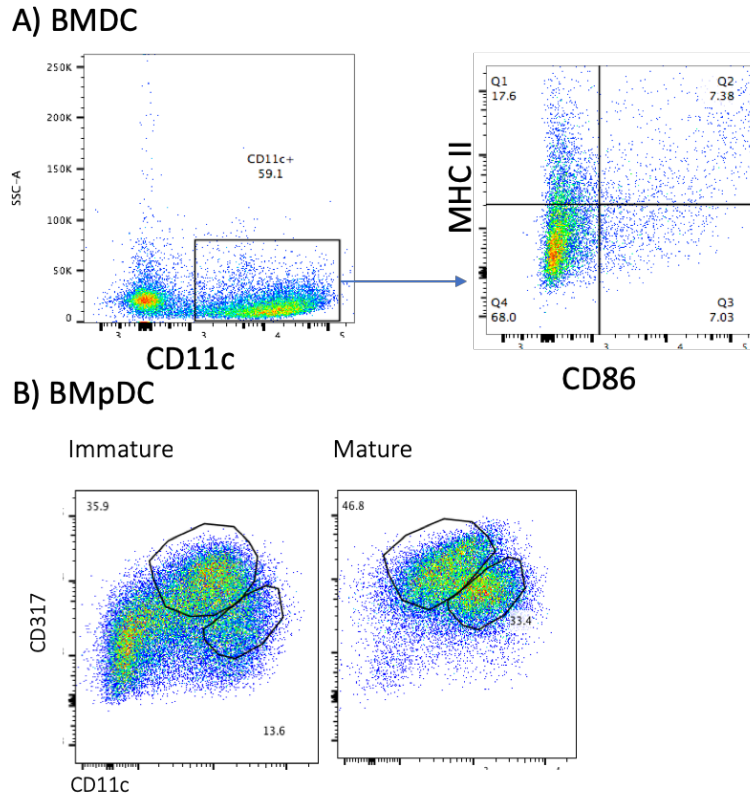
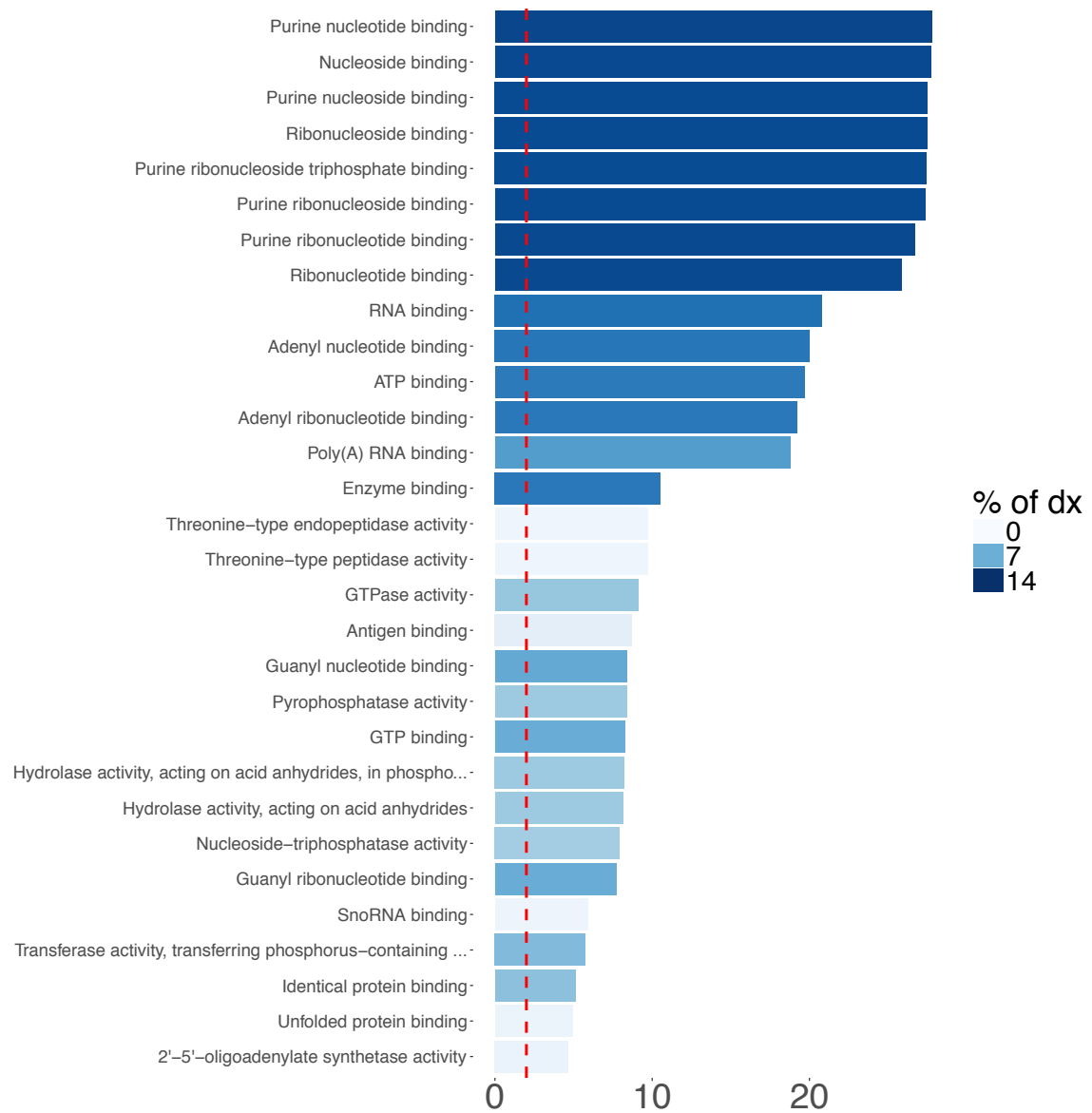


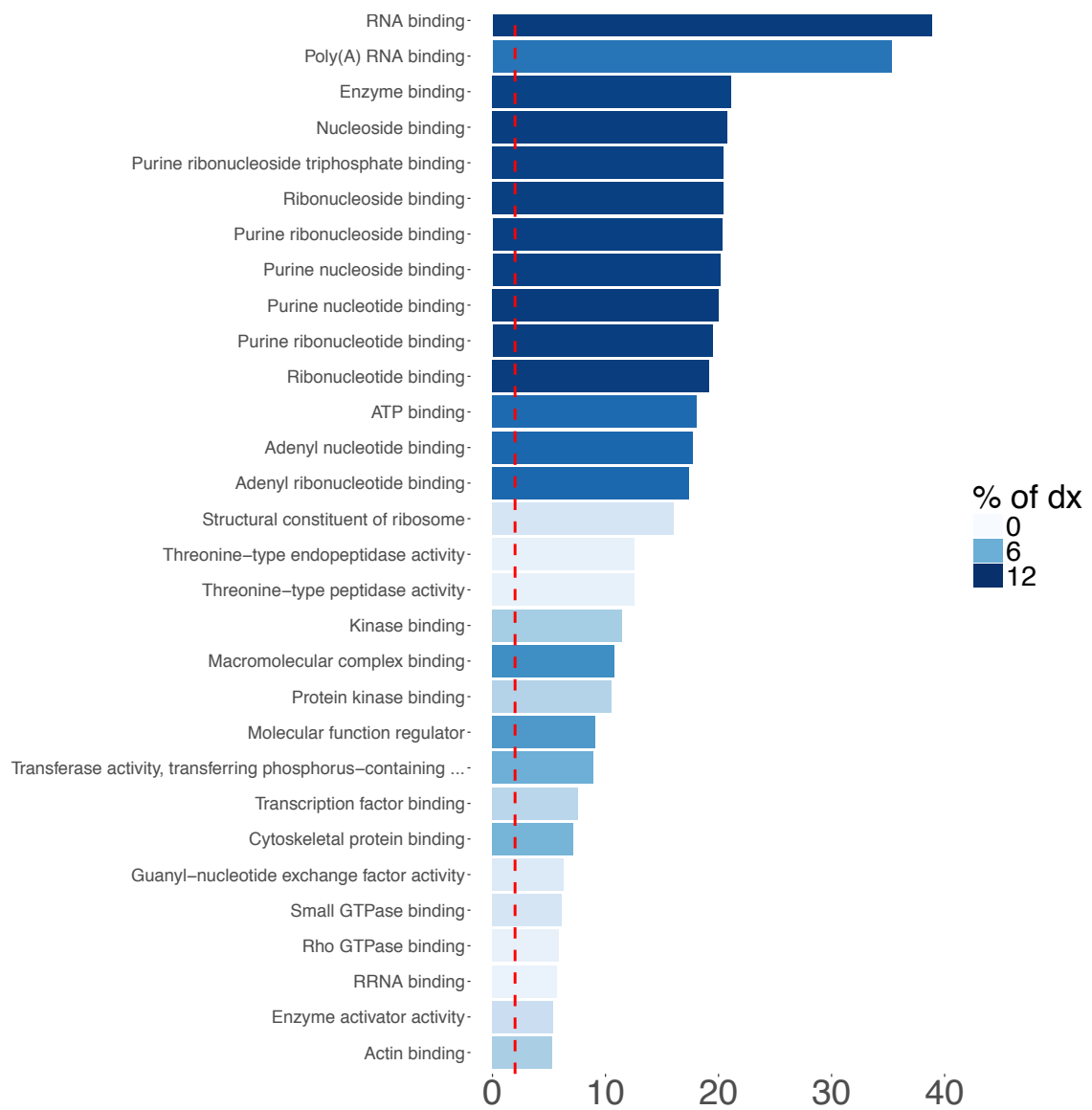
Figure S3 – Gating strategy for BMDCs.

A) Differentiation of BMDCs. Expression of CD11c and maturation markers MHCII and CD86. **B)** Differentiation of BMpDC. CD317 and CD11c expression pre and post maturation with CpG.

A) Q β (o) vs Q β (CpG) – 4503 genes



B) Q β vs Q β (o)



B) Q β vs Q β (CpG)

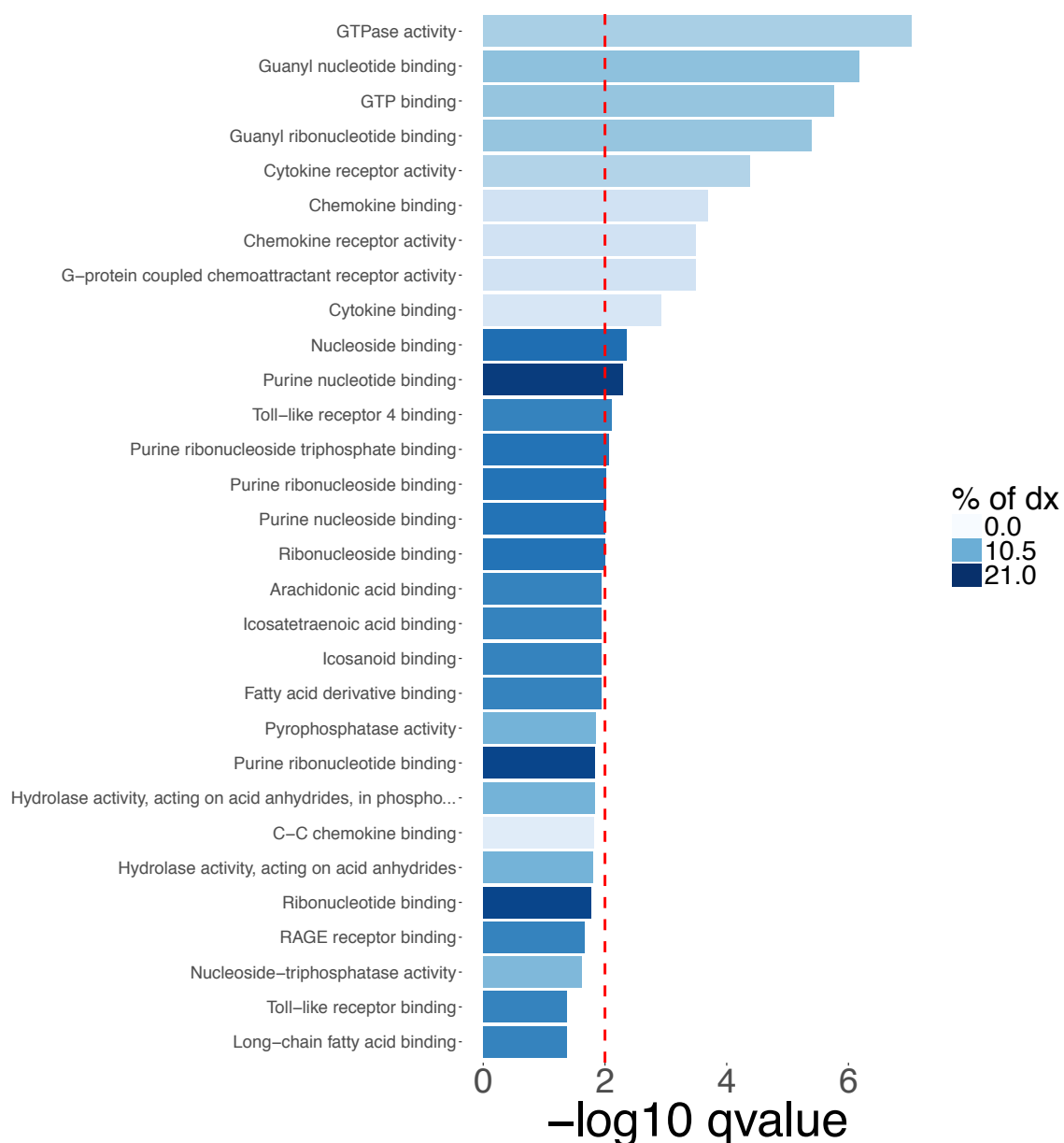
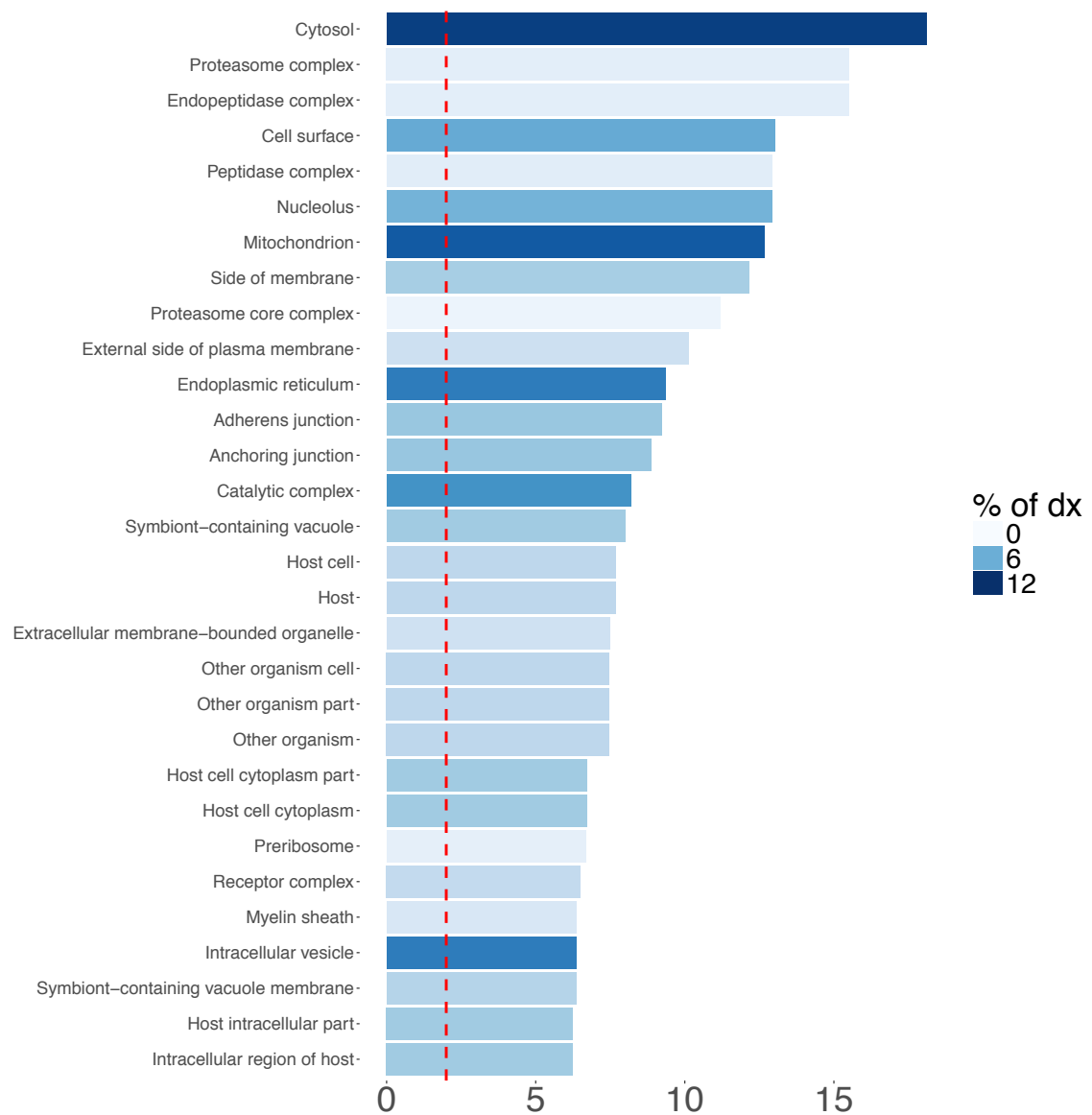


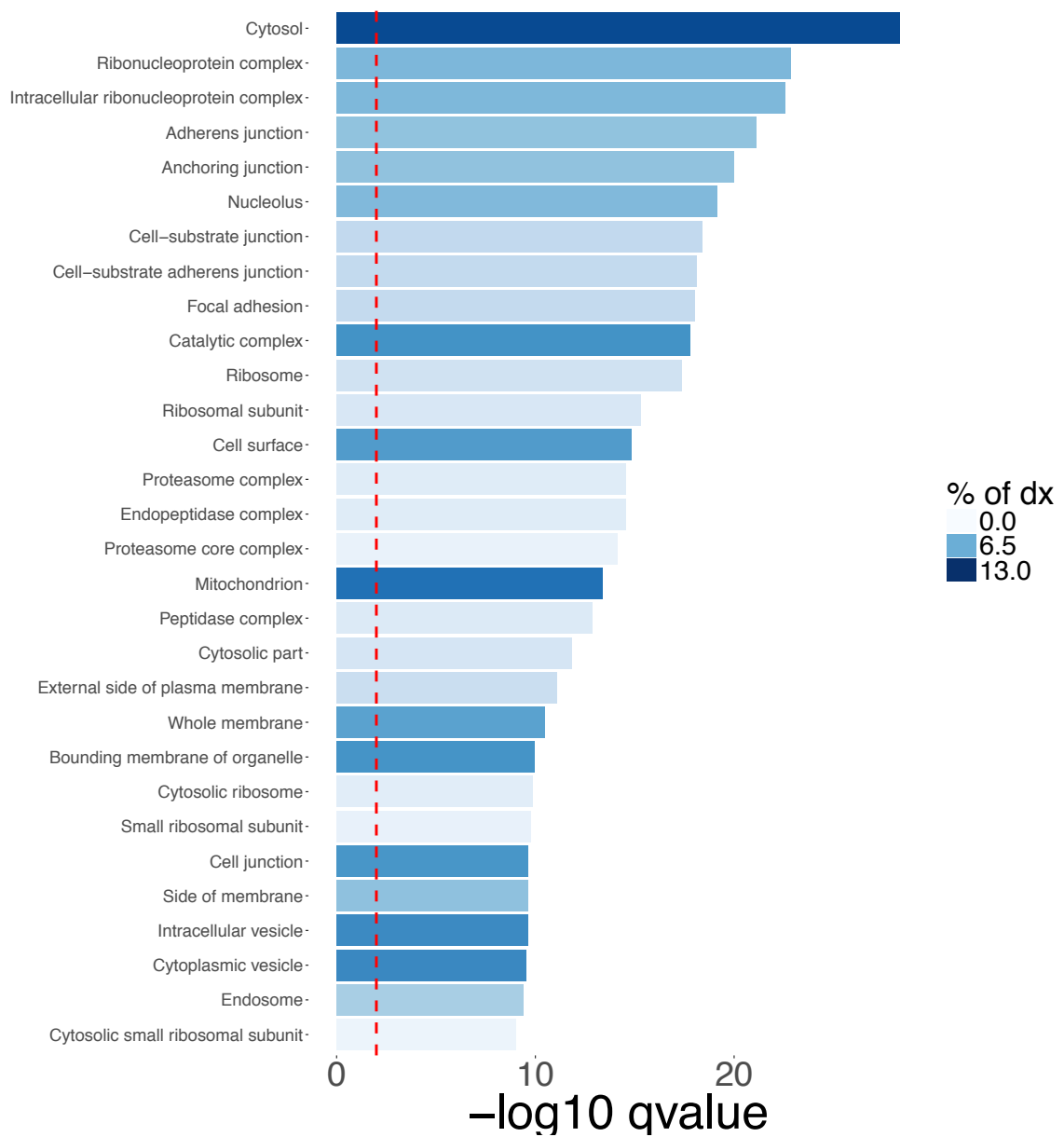
Figure S4 – Gene Ontology analysis of molecular function

A) Q β (o) vs Q β (CpG), B) Q β vs Q β (o) , C) Q β vs Q β (CpG).

A) Q β (o) vs Q β (CpG) – 4503 genes



B) $Q\beta$ vs $Q\beta(o)$



C) Q β vs Q β (CpG)

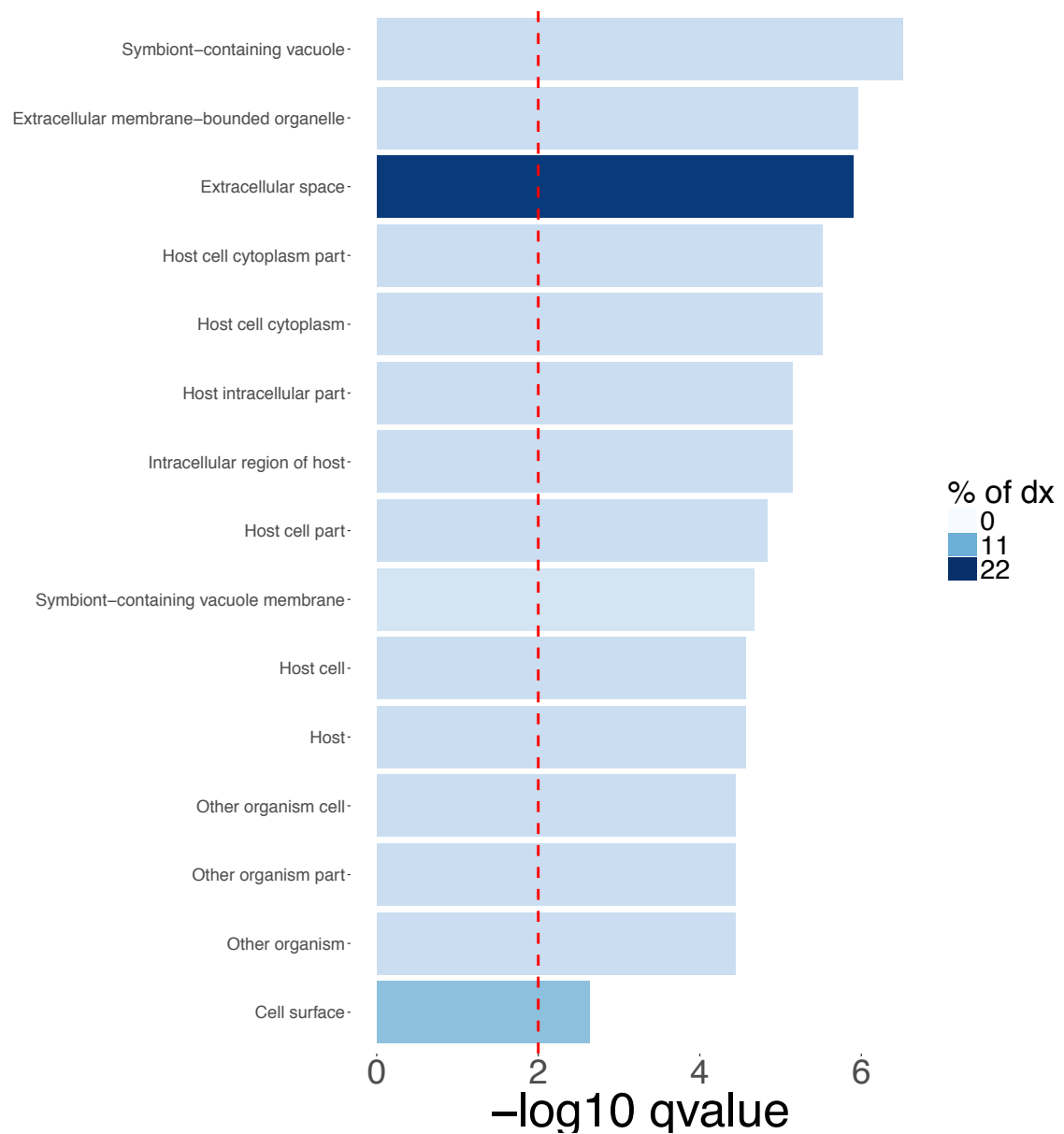
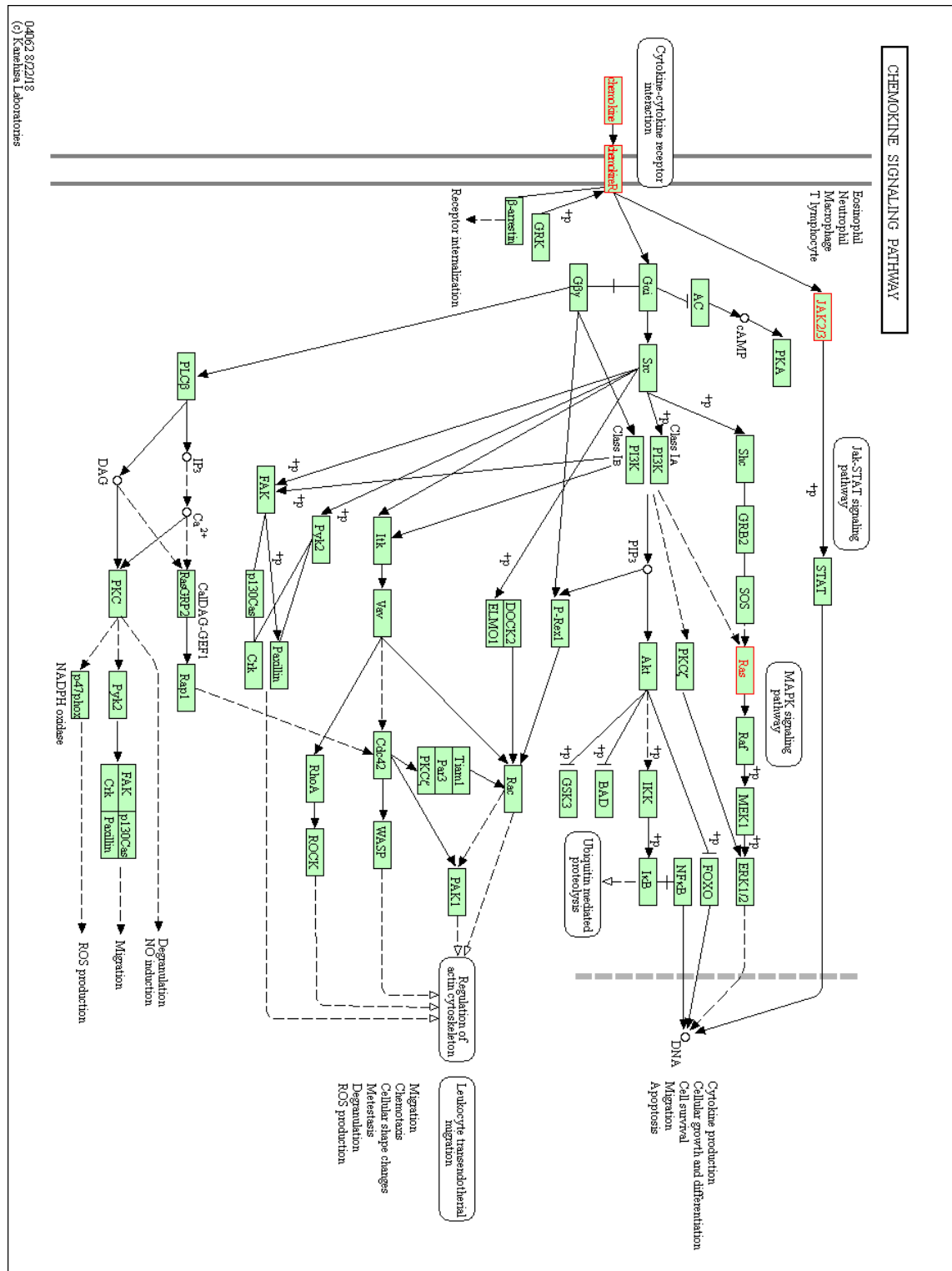
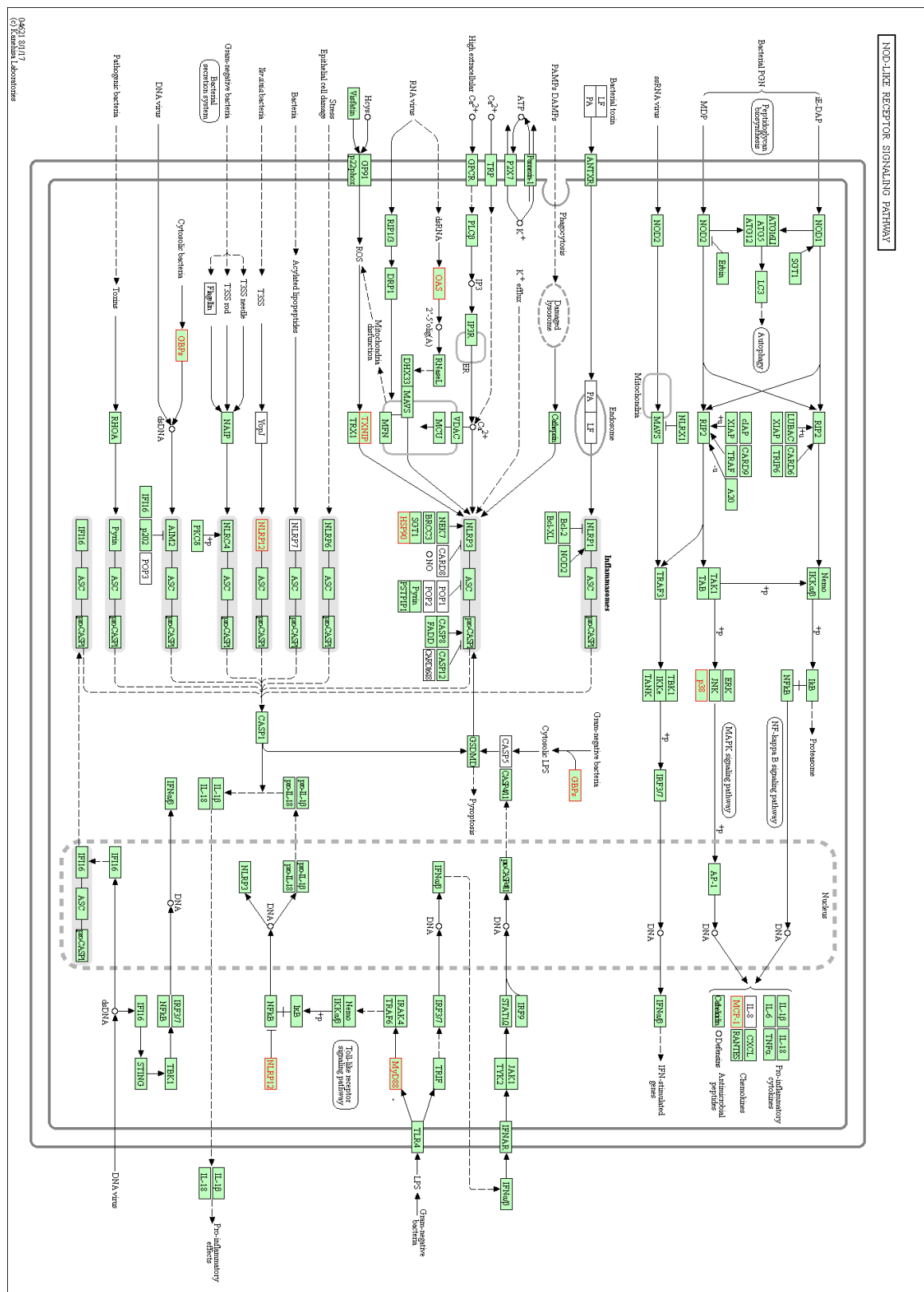
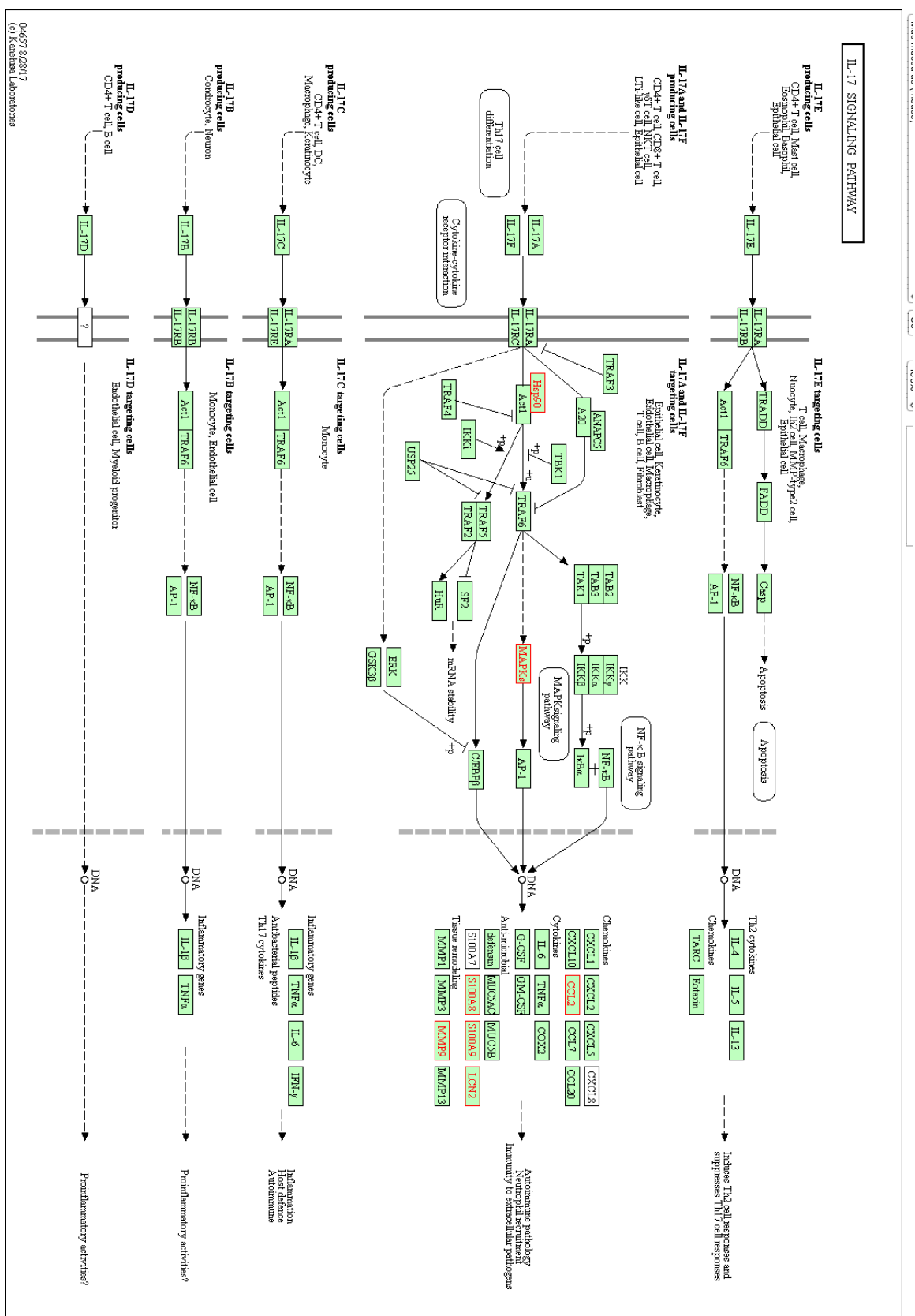


Figure S5 – Gene Ontology analysis of cellular component

A) Q β (o) vs Q β (CpG), B) Q β vs Q β (o) , C) Q β vs Q β (CpG).







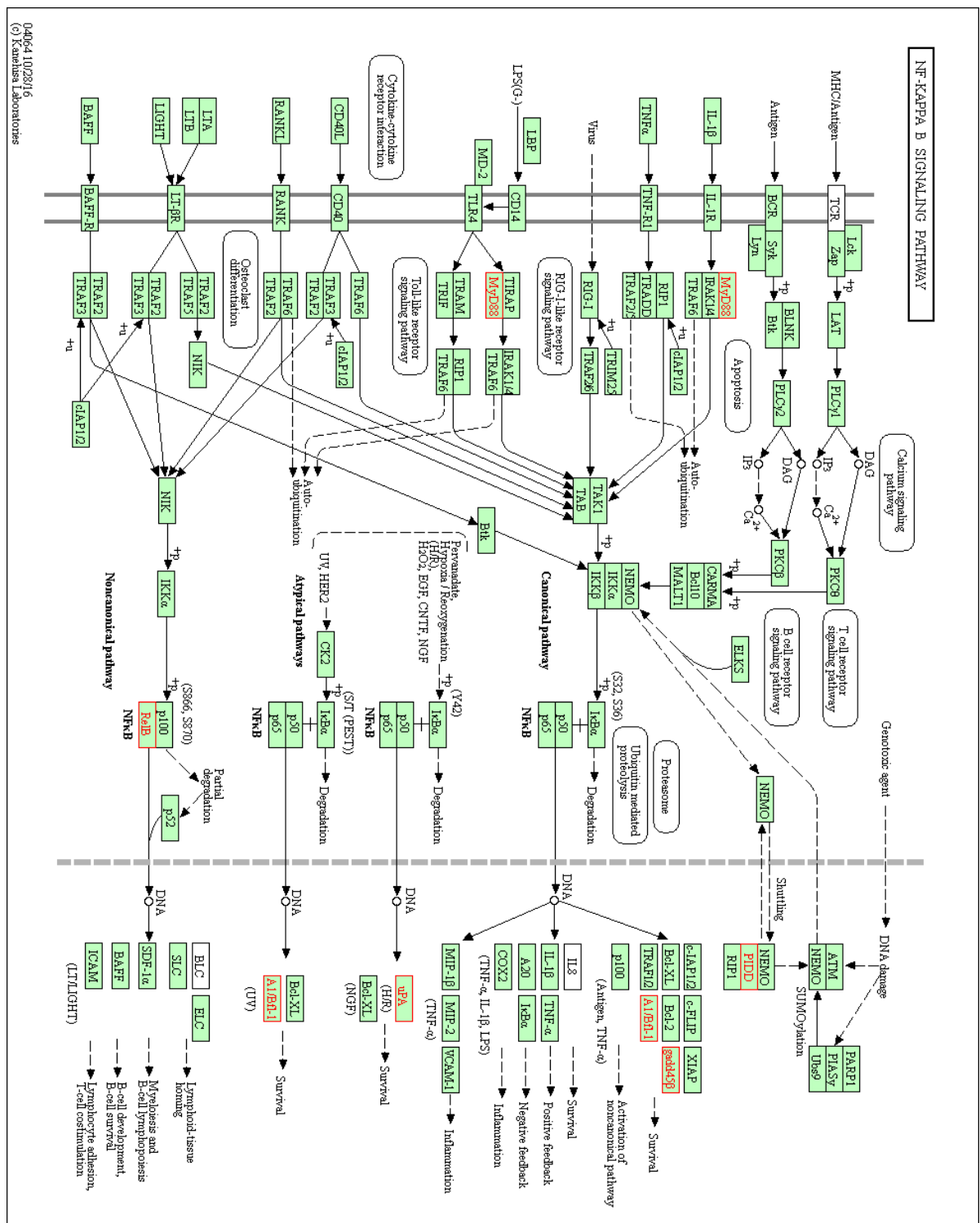


Figure S10 - KEGG pathway enrichment Qβ versus Qβ(CpG): NF-κB signalling pathway

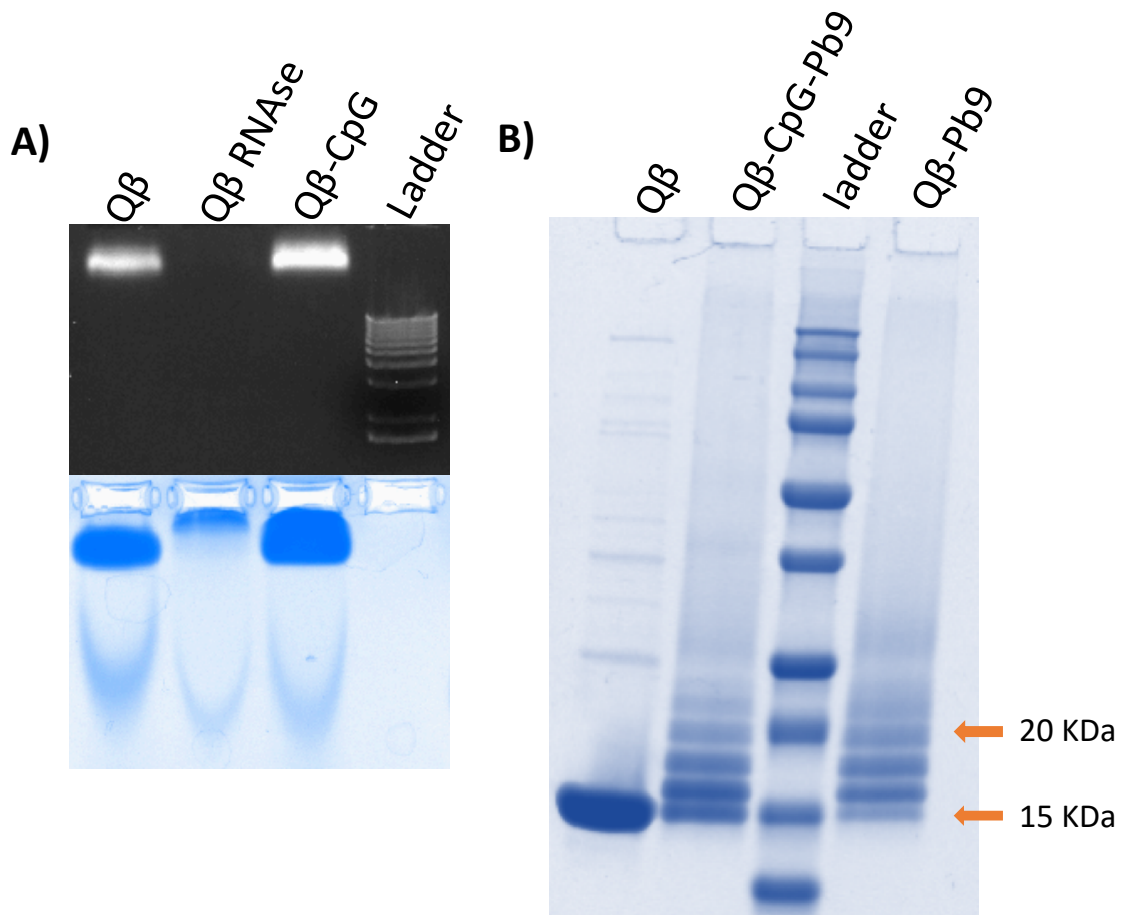


Figure S11 – Re-packing of VLPs and coupling efficiency analysis

A) Repacking of VLP with CpG was accessed by native agarose gel analysis followed by double staining with SYBR safe to detect nucleic acid and Coomassie stain to detect protein. The co-localisation of nucleic acid and protein, in addition to other techniques, indicates packing of nucleic acid and stability of the particle. **B)** Coupling efficiency analysis of VLPs and peptide performed on a reduced SDS-PAGE gel. The 14kDa monomer when coupled with the copies of a peptide of approximately 1.2kDa migrates less on the gel creating this “ladder” effect, which indicates covalent coupling of antigen and monomer of the VLP.

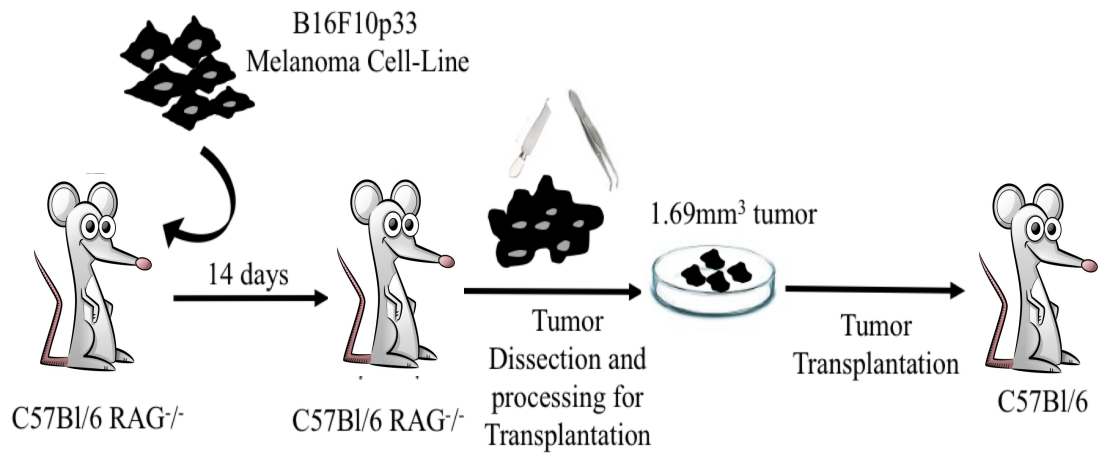


Figure S12 – Re-packing of VLPs and coupling efficiency analysis

Schematic representation of the adapted tumour experimental method. 1×10^6 B16F10p33 melanoma cell line were injected into one flank of RAG^{-/-} mice (6-8 weeks old; Harlan). Fourteen days later the growing tumours were collected and dissected into $\sim 2\text{mm}^3$ size and transplanted into the flank of C57BL/6 mice (6-8 weeks old; Harlan).

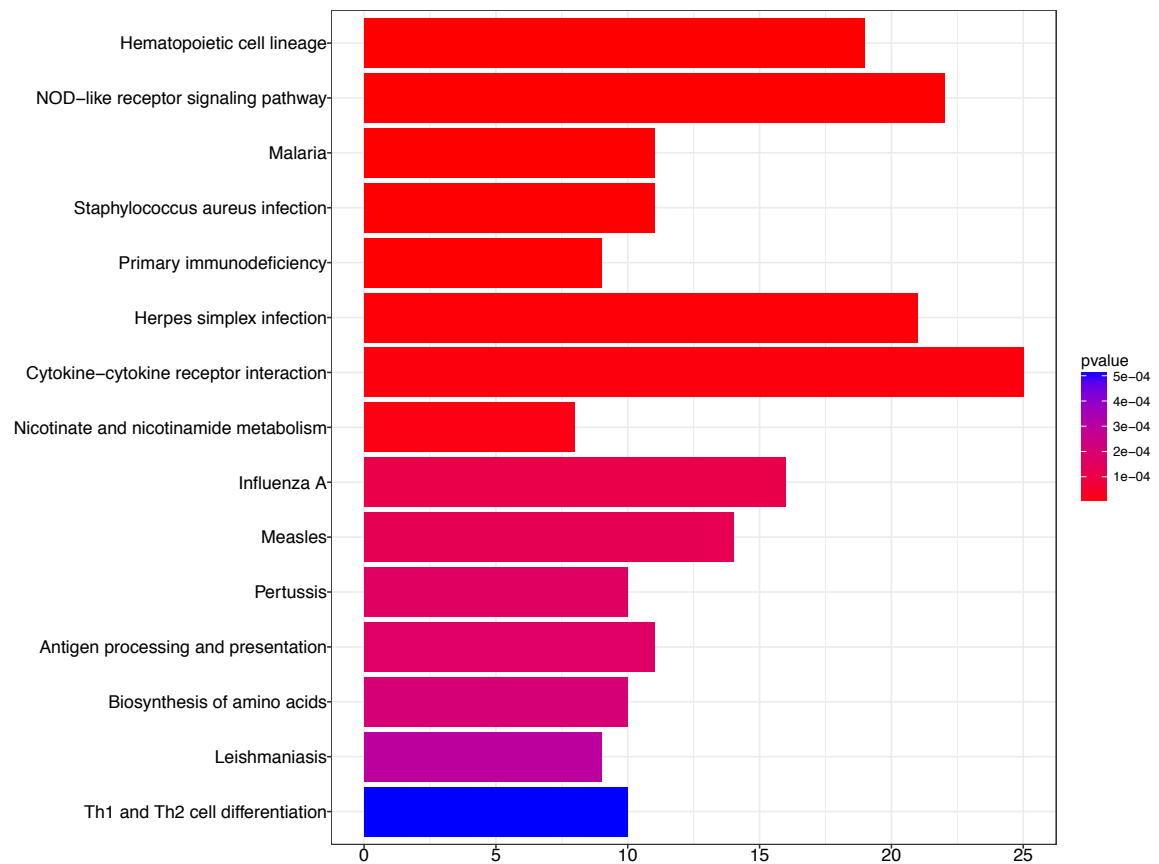


Figure S13 – ClusterProfiler analysis of genes downregulated on Q β (CpG) compared to Q β (o).

Gene set enrichment analysis of down-regulated genes between Q β (CpG) versus Q β (o). Significance displayed as a colour scale as indicated by the p.value. X-axis: number of genes in the dataset per pathway.

Table S.1 - Vaccines developed

Vaccine	Disease target	TLR ligand	Antigen	Intervention type
Q β (eRNA)-M2e	Influenza A	eukaryotic RNA from HEK cells	M2e:	Prophylactic
Q β (pRNA)-M2e	Influenza A	Prokaryotic RNA from E. coli	SLLTEVETPIRNE	
Q β (tRNA)-M2e	Influenza A	tRNA from yeast	WGC	
Q β (G10)-p33	LCMV related tumours	G10 A type CpG	p33: KAVYNFATC	Therapeutic
Q β (pRNA)-p33	LCMV related tumours	Prokaryotic RNA from E. coli		
Q β (CpG)-p33	LCMV related tumours	1668 B type CpG		
Q β -E7	HPV related tumours	Prokaryotic RNA from E. coli	E7: RAHYNIVTF	Therapeutic
Q β (CpG)-E7	HPV related tumours	1668 B type CpG		
Q β (o)-E7	HPV related tumours	no TLR ligand		

Table S.2 – Mouse strains

Mouse strain	Source
C57BL/6 WT	Envigo
Cardif-/-	Jan Rehwinkel (University of Oxford)
Myps-/-	John Cambier (University of Texas)
RAG-/-	Harlan
TLR7-/-	Manfred Kompf (ETH Zurich)
CCL2-/-	The Jackson Laboratory

Table S.3 – List of Reagents

Reagent	Supplier	Catalogue Number
SMPH (succinimidyl-6-(X-maleimidopropionamido) hexanoate NHS ester)	Thermo Fisher SCIENTIFIC	22363
DMSO	Sigma-Aldrich	M81802
DMEM Medium	Sigma-Aldrich	D6046
Poly-L-Lysine	Sigma-Aldrich	P4707
IL-4	Peprtech	214-14
GMCSF	Sigma	PMC2014
LysoTracker	Life Technologies	L-7526
EDTA	Sigma-Aldrich	E7889-100ml
Foetal Calf Serum (FCS)	Sigma-Aldrich	F7524
L-Glutamine	Sigma-Aldrich	G-7513
Monensin	BD Bioscience	554724
BrefeldinA	BD Bioscience	555029
IC Fixation Buffer	Fisher-Scientific	00-8222-49
Permeabilization Buffer 10x	Fisher-Scientific	00-8333-56
Collagenase D	Roche	11 088 858 001
DNase I	Sigma-Aldrich	4716728001
SYBR Safe DNA gel stain	Thermo Fisher Scientific	S33102
Agarose	Sigma-Aldrich	A9539
LB Media	Fisher-Scientific	1282-1670
M9 Media	Sigma-Aldrich	M6030
Casaminoacids	Fisher-Scientific	1274-7109
Vitamine B (Thiamine Hydrochloride)	Sigma-Aldrich	T4625
Ampicilin	Sigma-Aldrich	10835242001
Lysonaze	Merck Novagen	2701776

Table S.4 – List of Antibodies

Target (mouse)	Code	Conjugate	Brand
IgG	A3682	HRP	Thermo
IgG1	4 6120	HRP	Thermo
IgG2a	M32207	HRP	Thermo
IgG2b	M32407	HRP	Thermo
IgG2c	PA1-299288	HRP	Thermo
IgG3	M32607	HRP	Thermo
CD3	45-0037-41	PercP Cy5.5	eBioscience
CD4	56-0042-82	AF700	eBioscience
CD8	56-0081-82	AF700	eBioscience
CD11c	A18639	APC-Cy7	Life
CD11b	25-0112-81	PE-Cy7	Affymetrix
CD69	MA1-207	PE	eBioscience
CD86	17-0862-81	APC	eBioscience
F4/80	MF48028	Pacific blue	Life
IFNg	11-7311-82	APC	Life
MHC-II	12-5321-81	PE	eBioscience
TNFa	12-7321-82	PE	Affymetrix
Fc Block antibody	553142	NA	eBioscience
Tetramers H2-Db p33	Custom made	APC	NIH tetramer facility

Table S.5 – List of Materials

Materials	Supplier	Catalogue Number
Amicon centrifuge tubes 100 kDa MWCU	Thermo Fischer	UFC501008
Optically clear PCR seal	4titude	
Any kD mini-protein TGX precast protein gel	Bio-RAD	4569033
Glass bottom plates	MatTek	
PCR plates		
ELISA plates Nunc-Immuno (96 well)	Sigma	M9410-1CS
70 μ M cell strainer	BD Bioscience	352350
Fractogel EMD TMAE	Merck	116881
HiPrep 16/60 Sephacril S500-HR	Sigma	GE28-9356-06

Table S.6 – List of Reagents used for vaccine preparation

Reagents for vaccine preparation		
Item	Code	Brand
Yest derived tRNA	15401011	Thermo
CpG 1668	tlrl-1668	Invivogen
CpG G10	custom made	Eurogentec
CpG 1668 AF-567	custom made	Eurogentec
CpG G10 Af-567	custom made	Eurogentec
RNAse A	70856	Merck
SMPH	22363	Thermo
DTT	10197777001	Sigma
p33 H2-Db peptide (33-41)	custom made	Eurogentec
E7 H2-Db peptide (49-57)	custom made	Eurogentec
M2 H2-Db peptide	custom made	Eurogentec
Hydrogen Peroxide	106097	Sigma

Table S.7 – List of commercial Kits for cytokine measurement

Kit	Supplier	Catalogue Number
IFN alpha mouse ELISA	Thermo	BMS6027
Mouse IL-1b/IL1-F2 DuoSet ELISA	R&D Systems	DY401-05
Mouse IL-12 p70 Quantikine ELISA	R&D Systems	M1270
Luminex Cytokine 20-Plex Mouse P	Thermo	MCYTOMAG- 70K

Table S8 - Correlation score of cytokines produced 24h and p33 Tetramer positive T cells.

Tetramer	IFNg	TNFa	CCL2	IL1a	IL1b	CXCL10	IL6	IL9	IL10	CXCL1.	G-CSF	CCL3	CCL4	IL5
1	0.95693146	0.94502932	0.96702101	0.85700008	0.96325947	0.95594918	0.96731815	-0.942181	0.94567639	0.93140025	0.64099104	0.55637382	0.59880742	0.78846308
IFNg	0.95693146	1	0.90765739	0.95044856	0.77168571	0.93032343	0.8924536	-0.954462	0.88605922	0.84981487	0.46188124	0.64477598	0.55225993	0.76865175
TNFa	0.94502932	0.90765739	1	0.86024249	0.7456818	0.9572805	0.85094948	-0.9246303	0.80303413	0.91969443	0.43200761	0.36922349	0.34944394	0.94350096
CCL2	0.96702101	0.95044856	0.86024249	1	0.91003651	0.90204517	0.94828073	-0.9259777	0.98124136	0.84908273	0.69695893	0.68649042	0.69780541	0.66110073
IL1a	0.85700008	0.77168571	0.7456818	0.91003651	1	0.74140059	0.87963459	-0.8503521	0.92560129	0.772341	0.83147372	0.63259031	0.69902328	0.54538509
IL1b	0.96325947	0.93032343	0.9572805	0.90204517	0.74140059	1	0.95279505	-0.8981628	0.85835736	0.95746584	0.51780588	0.35810658	0.39426291	0.86309618
CXCL10	0.95594918	0.99183672	0.89907535	0.94828073	0.75738829	0.95279505	1	-0.93547	0.88751016	0.87769535	0.48313426	0.58101734	0.51794832	0.76237191
IL6	0.96731815	0.8924536	0.85094948	0.9659967	0.87963459	0.90205656	1	-0.8506292	0.98488248	0.87796235	0.78099682	0.58025512	0.72017586	0.63913768
IL9	-0.942181	-0.954462	-0.9246303	-0.9259777	-0.8503521	-0.93547	-0.8506292	1	-0.8588771	-0.8819676	-0.4959098	-0.5943193	-0.484187	-0.8160066
IL10	0.94567639	0.88605922	0.80303413	0.98124136	0.92560129	0.85835736	0.88751016	-0.8588771	1	0.82624934	0.81038072	0.67988307	0.78203195	0.56791253
CXCL1.	0.93140025	0.84981487	0.91969443	0.84908273	0.772341	0.95746584	0.87769535	-0.8819676	0.82624934	1	0.60284743	0.26200802	0.35525574	0.83348868
G-CSF	0.64099104	0.46188124	0.43200761	0.69695893	0.83147372	0.51780588	0.48313426	-0.4959098	0.81038072	0.60284743	1	0.44028327	0.76166332	0.16930797
CCL3	0.55637382	0.64477598	0.36922349	0.68649042	0.63259031	0.35810658	0.58101734	-0.5943193	0.67988307	0.26200802	0.44028327	1	0.85715742	0.11381272
CCL4	0.59880742	0.55225993	0.34944394	0.69780541	0.69902328	0.39426291	0.51794832	-0.484187	0.78203195	0.35525574	0.76166332	0.85715742	1	0.02781425
IL5	0.78846308	0.76865175	0.94350096	0.66110073	0.54538509	0.86309618	0.76237191	-0.8160066	0.56791253	0.83348868	0.16930797	0.11381272	0.02781425	1

Table S9 - P values of correlation score of cytokines produced 24h and p33 Tetramer positive T cells.

	Tetramer	IFN γ	TNF α	CCL2	IL1 α	IL1 β	CXCL10	IL6	IL9	IL10	CXCL11	G-CSF	CCL3	CCL4	IL5
Tetramer	#N/A	2.3464E-08	1.1134E-07	4.2413E-07	4.2413E-09	4.4734E-05	8.4812E-09	2.7103E-08	4.0019E-09	1.5354E-07	1.0325E-07	4.5439E-07	0.01002362	0.03124309	0.01834097
IFNγ	2.3464E-08	#N/A	2.9584E-06	5.7463E-08	0.00075186	5.0147E-07	5.1514E-13	7.6724E-06	3.3511E-08	1.0991E-05	6.0413E-05	0.08305009	0.00945483	0.0327846	0.00081274
TNFα	1.1134E-07	2.9584E-06	#N/A	3.8855E-05	0.00141546	2.2274E-08	5.1605E-06	5.7673E-05	8.2394E-07	0.00031234	1.2294E-06	0.10781553	0.17561453	0.20171108	1.3257E-07
CCL2	4.2413E-09	5.7463E-08	3.8855E-05	#N/A	2.5115E-06	4.2813E-06	7.5504E-08	5.1614E-09	7.3521E-07	1.1211E-10	6.2238E-05	0.00388244	0.00470607	0.00382122	0.00728419
IL1α	4.4734E-05	0.00075186	0.00141546	2.5115E-06	#N/A	0.0015599	0.00107472	1.545E-05	5.9103E-05	7.5915E-07	0.00073921	0.00012194	0.01138141	0.00373449	0.03548725
IL1β	8.4812E-09	5.0147E-07	2.2274E-08	4.2813E-06	0.0015599	#N/A	4.2161E-08	2.4733E-06	5.459E-06	4.2189E-05	2.1663E-08	0.04803006	0.18999347	0.14589446	3.4224E-05
CXCL10	2.7103E-08	5.1514E-13	5.1605E-06	7.5504E-08	0.00107472	4.2161E-08	#N/A	4.2782E-06	3.084E-07	1.015E-05	1.7058E-05	0.06809884	0.02312162	0.04795789	0.00095148
IL6	4.0019E-09	7.6724E-06	5.7673E-05	5.1614E-09	1.545E-05	2.4733E-06	4.2782E-06	#N/A	5.8436E-05	2.7813E-11	1.6829E-05	0.00058772	0.02334574	0.0024616	0.01031169
IL9	1.5354E-07	3.3511E-08	8.2394E-07	7.3521E-07	5.9103E-05	5.459E-06	3.084E-07	5.8436E-05	#N/A	4.1247E-05	1.3684E-05	0.06010679	0.01946803	0.06741279	0.0002074
IL10	1.0325E-07	1.0991E-05	0.00031234	1.1211E-10	7.5915E-07	4.2189E-05	1.015E-05	2.7813E-11	4.1247E-05	#N/A	0.00014673	0.00024863	0.00529336	0.00057143	0.02720999
CXCL11	4.5439E-07	6.0413E-05	1.2294E-06	6.2238E-05	0.00073921	2.1663E-08	1.7058E-05	1.6829E-05	1.3684E-05	0.00014673	#N/A	0.0173699	0.34550542	0.19379986	0.00011335
G-CSF	0.01002362	0.08305009	0.10781553	0.00388244	0.00012194	0.04803006	0.06809884	0.00058772	0.06010679	0.00024863	0.0173699	#N/A	0.1004974	0.00096827	0.54636161
CCL3	0.03124309	0.00945483	0.17561453	0.00470607	0.01138141	0.18999347	0.02312162	0.02334574	0.01946803	0.00529336	0.34550542	0.1004974	#N/A	4.4432E-05	0.68630784
CCL4	0.01834097	0.0327846	0.20171108	0.00382122	0.00373449	0.14589446	0.04795789	0.0024616	0.06741279	0.00057143	0.19379986	0.00096827	4.4432E-05	#N/A	0.92161739
IL5	0.00047829	0.00081274	1.3257E-07	0.00728419	0.03548725	3.4224E-05	0.00095148	0.01031169	0.0002074	0.02720999	0.00011335	0.54636161	0.68630784	0.92161739	#N/A

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