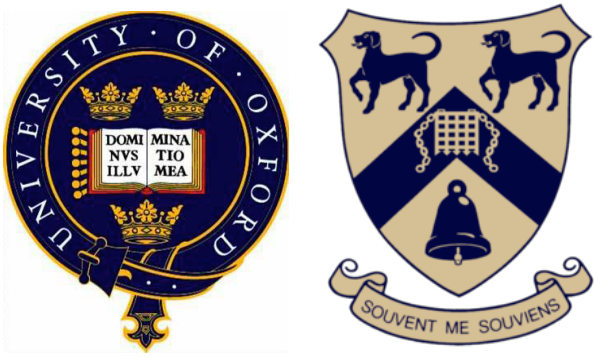
POLYETHYLENEIMINE AS AN ADJUVANT CANDIDATE FOR ENV-BASED HIV-1 IMMUNISATION

A thesis submitted for the degree of Doctor of Philosophy at the University of Oxford,

Hillary Term 2012

Sarah Anna Brinckmann



The Sir William Dunn School of Pathology

and

Lady Margaret Hall

Abstract

Polyethyleneimine as a candidate vaccine adjuvant for Env-based HIV-1 vaccination

Sarah Anna Brinckmann

Sir William Dunn School of Pathology

A thesis submitted for the degree of Doctor of Philosophy at the University of Oxford, Hillary Term 2012.

Almost 30 years after the identification of HIV-1 as the causative agent of AIDS, an effective prophylactic HIV-1 vaccine has yet to be developed. Despite the design of a vast array of therapeutic agents and prevention strategies, which have helped to reduce AIDS mortality and the growth of the pandemic, HIV-1 is still the fourth biggest killer worldwide and an effective vaccine will be needed to halt the pandemic. Over the past three decades, various HIV-1 vaccination strategies have been explored leading to the key understanding that sterile protection against HIV-1 infection will require a combination of cellular and humoral responses, particularly at the mucosal compartment, the common route of infection. Consequently, it has been postulated that this can only be achieved through a potent and effective immunisation strategy involving mucosal immunisation and the application of a heterologous prime-boost regimen. However, the mucosal route of immunisation, in particular for subunit vaccines, has not been well established as a mainstream mode of vaccination in human and results with experimental mucosal immunisation have generally been impeded by a lack of safe and effective mucosal adjuvants. In the work presented herein, I investigated whether the highly cationic polyethyleneimine (PEI), a gold standard DNA delivery agent, has adjuvant activity applied parenterally and mucosally in formulation with glycoprotein antigens, in particular HIV-1 Env gp140, the sole target of neutralising antibodies (nAbs) and therefore the most attractive HIV-1 subunit vaccine candidate. I demonstrated that PEI is a potent inducer of Env-specific Ab responses in a mixed T_h1 and T_h2 context. PEI is particularly potent via the mucosal route and superior to gold standard mucosal adjuvant Cholera Toxin Beta subunit (CTB) as measured by HIV-1 specific IgA responses at the mucosal compartment. PEI showed direct immune stimulatory effects, marked by a significant recruitment of local leukocytes, including inflammatory monocytes and dendritic cells (DCs). Further it associated with antigen to form gp140-PEI particles, which enhanced antigen targeting to, and uptake by, immune cells, in particular monocytes, macrophages, and DCs. The observed adjuvant activity was maintained with the use of various forms of PEI, including linear or branched, and durable or biodegradable, and under a range in molecular weight (MW), suggesting that immune potentiation is a shared attribute of this group of PEI polymers. This is the first demonstration of PEI as a mucosal adjuvant for protein immunisation. These results merit further exploration of PEI in protection models with the implication for clinical trials.

This thesis is dedicated to my parents, Tom and Lilian, and my brother Ruben Tomas.

The real voyage of discovery consists not in seeking out new landscapes, but in having new eyes.
-Marcel Proust, 1871–1922.

Your time is limited, so don't waste it living someone else's life. Don't be trapped by dogma — which is living with the results of other people's thinking. Don't let the noise of others' opinions drown out your own inner voice. And most important, have the courage to follow your heart and intuition. -Steve Jobs, 1955-2005.

It is bad enough that people are dying of AIDS, but no one should die of ignorance.
-Elizabeth Taylor, 1932-2011.

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- **Figure 3.2. Adjuvant activity in a rabbit gp140$_{97CN54}$ immunisation model.**
  Rabbit immunisation was carried out by Dr. Paul Tonks and Dr. Mark Sheppard at Prof. Jonathan Heeney's group.

- **Figure 3.3. Serum gp140$_{97CN54}$-specific IgG responses in a rabbit immunisation model with PEI, FCA/FIA, or Imject®Alum adjuvants.**
  Rabbit immunisation was carried out by Neil Sheppard.

- **Figure 5.3. Peritoneal leukocyte recruitment 4 or 24 h after i.p. PEI injection.**
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- **Appendix 5.I. Cytokine induction in the peritoneum after i.p. administration.**
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Abbreviations

• 2-ME	2-mercaptoethanol
• Ab	Antibody
• $Al(OH)_3$	Aluminium hydroxide
• Approx.	Approximately
• 3-D	Three-dimensional
• Ad(x)	Adenovirus serotype (x)
• ADCC	Antibody Dependent Cell-mediated Cytotoxicity
• ADCMI	Antibody Dependent Complement-Mediated Inactivation
• AIDS	Acquired Immunodeficiency Syndrome
• APOBEC	apoliprotein B editing complex
• APC	Antigen Presenting Cell
• ART	Antiretroviral Therapy
• AS	Adjuvant Systems
• ASC	Apoptosis-associated speck-like protein containing a carboxy-terminal CARD
• ATP	Adenosine Triphosphate
• AZT	Zidovudine
• B12	IgG1B12
• BMDC	Bone Marrow Derived DC
• bNAb	broadly neutralising antibody
• bnmAb	broadly neutralising monoclonal antibody
• bPEI	Branched PEI
• BSA	Bovine Serum Albumin
• C region	conserved region
• C-terminus	Carboxy-Terminus
• CAPRISA	Centre for AIDS Programme of Research in South Africa
• CCL	CC Chemokine Ligand
• CCR	CC Chemokine Receptor
• CD markers	Cluster of Differentiation marker
• CD4bs	CD4 binding site
• CD4i	CD4-induced
• CHO	Chinese Hamster Ovary
• CLN	Cervical Lymph Node
• CLRs	C-type Lectin Receptors
• CpG (ODN)	CpG Oligodeoxynucleotides
• Cpm	Counts Per Minute
• CRbs	Coreceptor binding site
• CRF	Circulating Recombinant Form
• CTB	Cholera Toxin Beta Subunit
• CTL	Cytotoxic T cell
• CXCL	Chemokine (C-X-C motif) Ligand
• CXCR	Chemokine (CXC) Receptor

- DAMP — Damage-Associated Molecular Pattern
- DAPI — 4',6-diamidino-2-phenylindole
- DC — Dendritic Cell
- DC-SIGN — Dendritic Cell-Specific Intracellular adhesion molecule 3 Grabbing Non- integrin
- DMSO — Dimethyl Sulphoxide
- DNA — Deoxyribonucleic acid
- DNases — Deoxyribonuclease
- (D)PBS — (Dulbecco's) Phosphate Buffered Saline
- DOL — Degree Of Labelling
- EDC — 1-Ethyl-3-(3-Dimethylaminopropyl)Carbodiimide hydrochloride
- EDTA — EthylenedDamineTetraacetic Acid
- ELISA — Enzyme-Linked ImmunoSorbent Assays
- Em. — Emission (maximum)
- Env — Envelope
- ER — endoplasmatic reticulum
- EU/mL — Endotoxin Units/mL
- ESN — Exposed SeroNegative
- Ext. coefficient — Extinction coefficient
- Exc. — Excitation (maximum)
- Fab — Antigen Binding Fragment
- FACS — Fluorescence-Activated Cell Sorting
- FCA — Freund's Complete Adjuvant
- FCS — Fetal Calf Serum
- FcR — Fc Receptor
- FIA — Freund's Incomplete Adjuvant
- FITC — Fluorescein Isothiocyanate
- FSC — Forward Scatter
- h — hour(s)
- G-CSF — Granulocyte Colony-Stimulating Factor
- g/mg/µg — gram/milligram/microgram
- G2-HD-OEI — Biodegradable form of PEI
- GALT — Gut-Associated Lymphoid Tissue
- GCN4 — General Control Nondepressible
- GM-CSF — Granulocyte-Macrophage Colony-Stimulating Factor
- GMP — Good Manufacturing Practice
- GRID — Gay-Related Immuno Deficiency
- GSK — GlaxoSmithKline
- HAART — Highly Active AntiRetroviral Therapy
- HBG — HEPES Buffered Glucose
- HBS — HEPES Buffered Saline
- HBSS — Hanks Balanced Salt Solution
- HBV — Hepatitis B Virus
- HEL — Hen Egg Lysozyme
- HIV — Human Immunodeficiency Virus

- HLA Human Leukocyte Antigen
- HPV Human Papilloma Virus
- HRP HorseRadish Peroxidase
- HSA Human Serum Albumin
- HSPGs Heparin Sulphate Glycoproteins
- HTLV Human T-cell Leukaemia Virus
- i.n. intranasal
- i.p. intraperitoneal
- i.v. intravenous
- IC_{50} Half maximal Inhibitory Concentration
- IDU's Intravenous Drug Users
- IFN Interferon
- IL Interleukin
- ISCOM Immune Stimulating Complex
- Jet-PEI lPEI 22kDa form
- Jet-PEI SS disulfide-linked biodegradable form of Jet-PEI
- kDa/Da kilodalton/dalton
- LAMP-1 Lysosomal
- LAV Lymphoadenopathy-associated virus
- LC Langerhans cells
- LN Lymph Node
- lPEI Linear PEI
- LPS Lipopolysaccharide
- LSCM Laser Scanning Confocal Microscopy
- LTNPs Long-Term Nonprogressors
- LTR Long Terminal Repeat
- M cell Membranous cell (part of MALT)
- mAb monoclonal antibody
- MCP Monocyte Chemoattractant Protein
- MFI Mean Fluorescence Intensity
- MHC I/II Major Histocompatibility Complex class I/II
- MIP-1 Macrophage Inflammatory Protein-1
- mL milliliter
- m million
- mM millimolar
- M Molar
- MPER Mebrane-Proximal External Region
- MPL-A Monophosphoryl Lipid A
- MR Molar Ratio
- MSU Monosodium Yrate crystals
- MTCT Mother-To-Child Transmission
- MVA Modified Vaccinia Ankara
- MW Molecular Weight
- MYD88 Myeloid differentiation primary response gene (88)
- N/P ratio Ratio of nitrogen groups (PEI) to phosphate groups (DNA)

- nAb neutralising antibody
- NALT Nasal-Associated-Lymphoid-Tissue
- NHP (model) Non-Human Primate (model)
- NHS N-hydroxysulfosuccinimide
- NK cells Natural Killer cells
- NKT cells Natural Killer T cells
- NKp44 L NKp44 Ligand
- NLR (NOD)-Like Receptor
- NLRC4 NLR family CARD domain-containing protein 4
- NLRP3 NLR family, pyrin domain containing 3
- NMR Nuclear Magnetic Resonance
- NNRTI Non-Nucleoside Reverse Transcriptase Inhibitor
- NOD Nucleotide-Oligomerisation Domain
- NRTI Nucleoside Reverse Transcriptase Inhibitor
- NZW New Zealand White
- OEI oligoethyleneimine
- OEI-BAA-OEI actetal-linked biodegradable form of PEI
- OVA Ovalbumin
- p38 MAP p38 Mitogen-Activated Protein Kinase
- PAMP Pathogen-Associated-Molecular-Patterns
- PBMCs Peripheral Blood Mononuclear Cell
- PEI Polyethyleneimine
- PEIm Linear Mannobiosylated PEI
- PeP Post-Exposure Prophylaxis
- PGE2 Prostaglandin E2
- pI isoelectric point
- PI Propidium Iodide
- PPI PolyProlyleneImine
- PR Protease
- PRR Pattern Recognition Receptor
- P/S Penicillin/Streptomycin
- RIG-I Retinoic Acid-Inducible Gene I
- RLH (RIG-I)-like RNA Helicase
- RNA Ribonucleic Acid
- RT Reverse Transcriptase
- RT Room Temperature (in M&M Section)
- RU Response Units (pg/mm^2)
- s.c. subcutaneous
- sCD4 soluble CD4
- scSIV single-cycle SIV
- SDF-1 Stromal Cell-Derived Factor-1
- SDS Sodium dodecyl sulphate
- SEC Size Exclusion Chromatography
- SHIV Simian-Human Immunodeficiency Virus
- siRNA Small Interfering RNA

- SIV Simian Immunodeficiency Virus
- SLPI Secretory Leukocyte Protease Inhibitor
- Span 85 sorbitan triolate
- SPR Surface Plasmon Resonance
- STD Sexually Transmitted Disease
- TCR T Cell Receptor
- TEM Transmission Electron Microscopy
- TFP tetrafluorophenyl
- TGF Transforming Growth Factor
- T_h cell T helper cell
- TIP47 Tail Interacting Protein of 47 kDa
- TLR Toll-Like Receptor
- TMB 3,3',5,5',-tetramethylbenzidine
- TNF-**α** Tumour Necrosis Factor- **α**
- tPA tissue plasminogen activator
- TRIF TIR-domain-containing adapter-inducing interferon-**β**
- TRIM-5**α** TRIpartite Motif-5 **α**
- tRNA transfer RNA
- UA Uric Acid
- URF Unique Recombinant Form
- UV Ultraviolet
- V loop Variable loop
- VLPs Virus-Like particles

CHAPTER 1

Introduction

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1.1. Chapter overview

The purpose of this introduction is to position my research in a broader context. To this end, an overview of the HIV-1 pandemic and current progress in vaccine development will be provided. Detailed and more specific discussions will be reserved for the individual chapter introductions, so that relevant aspects will be outlined alongside my data.

1.2. The AIDS pandemic

The AIDS pandemic is considered one of the greatest global infection scourges in history and is, despite prevention and treatment measurements, still spreading, making HIV-1 the fourth biggest killer in the world (UNAIDS report 2009; Fauci, 2003). The start of the pandemic can be marked at 1981, when a range of unusual opportunistic infections and tumours were observed in previously healthy gay men in California (Nurnberg et al., 1984; 1981a; 1981b). Investigation soon showed that the men had seriously reduced $CD4^+$ T cell levels and were immunodeficient (Gottlieb et al., 1981; Masur et al., 1981). Despite treatment for the opportunistic infections and tumours, they died within weeks after the first signs of illness. In the following months, many more new instances of the immunodeficiency syndrome presented. After initially being called gay-related immunodeficiency syndrome (GRIDS) the syndrome received its final name in July 1982: acquired immunodeficiency syndrome (AIDS). The new disease spread in an epidemic manner also affecting heterosexual populations and risk factors were found to include anal intercourse, blood donation, and sharing needles for intravenous drug use (Fauci, 2003).

The causative agent of the new pandemic was unknown, but retroviral aetiology was suspected since the recently discovered Human T cell Leukaemia Virus type 1 (HTLV-1) manifested through similar modes of transmission and was the only virus identified to preferentially infect $CD4^+$ T cells (Fauci, 2003; Montagnier,

2002). An intensive search for the causative agent resulted, two years later, in the identification of a new T cell tropic retrovirus that was found to be associated with the syndrome. This retrovirus was first identified by Montagnier's group in 1982 who titled it Lymphoadenopathy-Associated Virus (LAV) and later in the same year by Gallo's group who called it Human T cell Leukaemia Virus type III (HTLV-III) (Gallo, 2002; Montagnier, 2002). A year later the new retrovirus was causally linked to AIDS (Barre-Sinoussi et al., 1983). HTLV-III and LAV were subsequently found to be identical and were renamed Human Immunodeficiency Virus (HIV) in 1986 (Coffin et al., 1986). Around the same time, the existence of a second type of HIV, called HIV-2, was discovered that caused a similar AIDS-like syndrome as the initially isolated HIV-1, but was only present in West-Africa (Clavel et al., 1986). Since HIV-2 is less pathogenic and does not significantly contribute to the AIDS pandemic (Pepin et al., 1991), I will mostly focus on HIV-1 for the remainder of this thesis.

Based on the epidemiology and incubation period of HIV-1 it was calculated that the new retrovirus had been spreading in the African continent for many decades and in the Western world for a few years before the first medically recognised cases in 1981. Archived blood samples dating back to the 1970s and 1960s were tested and found to be seropositive with the earliest sample dating back to 1959 in Kinshasa, Democratic Republic of Congo (Zhu et al., 1998). HIV-1 was characterised and shown to be phylogenetically related to a retrovirus that had caused infections in the *Pan troglodytes troglodytes* species of chimpanzee, so-called simian immunodeficiency virus (SIV_{CPZ}) (Corbet et al., 2000; Hahn et al., 2000), while HIV-2 was characterised and found to be related to a form of SIV present in sooty mangabeys (*Cercocebus atys*), so-called SIV_{SM} (Clavel et al., 1986). Surprisingly, the three main groups of HIV-1: M (major), O (outlier), and N (non-M, non-O) were found to be more similar to specific SIV_{CPZ} isolates than to each other, implying that multiple independent zoonotic transmission events had passed the virus to humans (Gurtler et al., 1994; Keele et al., 2006; Simon et al., 1998). Based on a wide collection of sequence data from HIV-1 isolates the founder event that transferred

the HIV-1 M group viruses to humans was initially estimated to have taken place in 1931 [1915-1941] (Korber et al., 2000), while a more recent estimate pointed at year 1933 [1919-1945] (Worobey et al., 2008).

The group O and N viruses are relatively rare and are only present in central Africa while the group M viruses have spread rapidly, undergone countless recombination events and have diversified substantially. Group M viruses evolved into nine distinct subgroups called clades: A-D, F-H, J and K (Figure 1.1) (Arien et al., 2007). Studies have dated the divergent evolution of HIV-1 group M into clades to 1956–1976 (Salemi et al., 2001; Travers et al., 2004). Human emigration and the seeding of new regional epidemics in central Africa might have been driving factors for the diversification (Salemi et al., 2001; Travers et al., 2004). Moreover, in some geographic regions recombinant forms between at least two clades are common (Hemelaar et al., 2006).

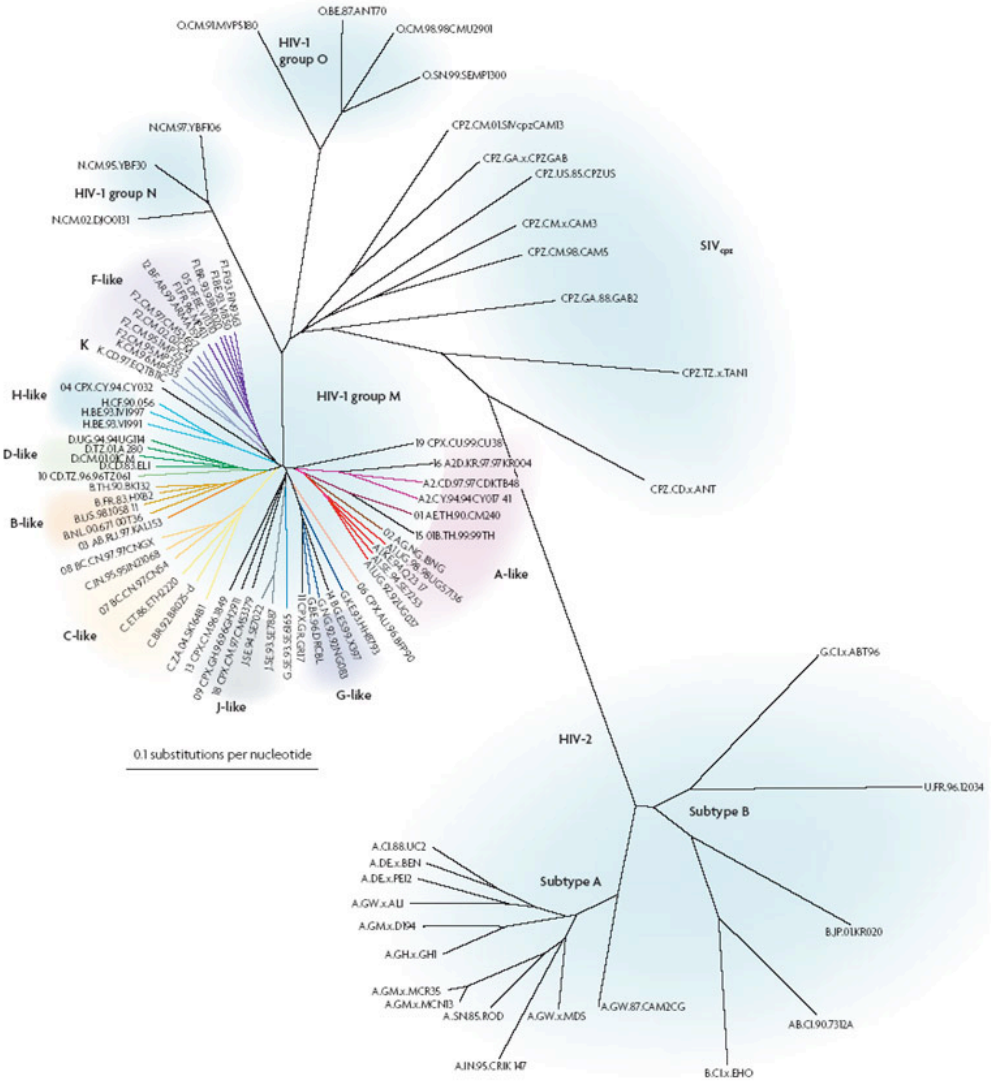


Figure 1.1 Phylogenetic tree of human and simian lentiviruses. HIV-1 and HIV-2 sequences are only 50–60% identical and cluster at distinct locations on the tree while SIVcpz branches out from HIV-1. HIV-1 has evolved into three groups: group M (major), O (outlier), and N (non-M, non-O). Group M viruses have further diversified into nine distinct clades: A-D, F-H, J and K. Furthermore, recombinant forms have evolved. Taken from: (Arien et al., 2007)**.**

The latest estimates from UNAIDS suggest that the number of global HIV infections is still rising with approximately 2.7 million (m) new infections per year and an annual number of approximately 2 m AIDS-related deaths (UNAIDS report 2009). At the end of 2010 the total estimated number of worldwide infections was 34 m [30.9 m–36.9 m] (UNAIDS report 2009) (Figure 1.2).

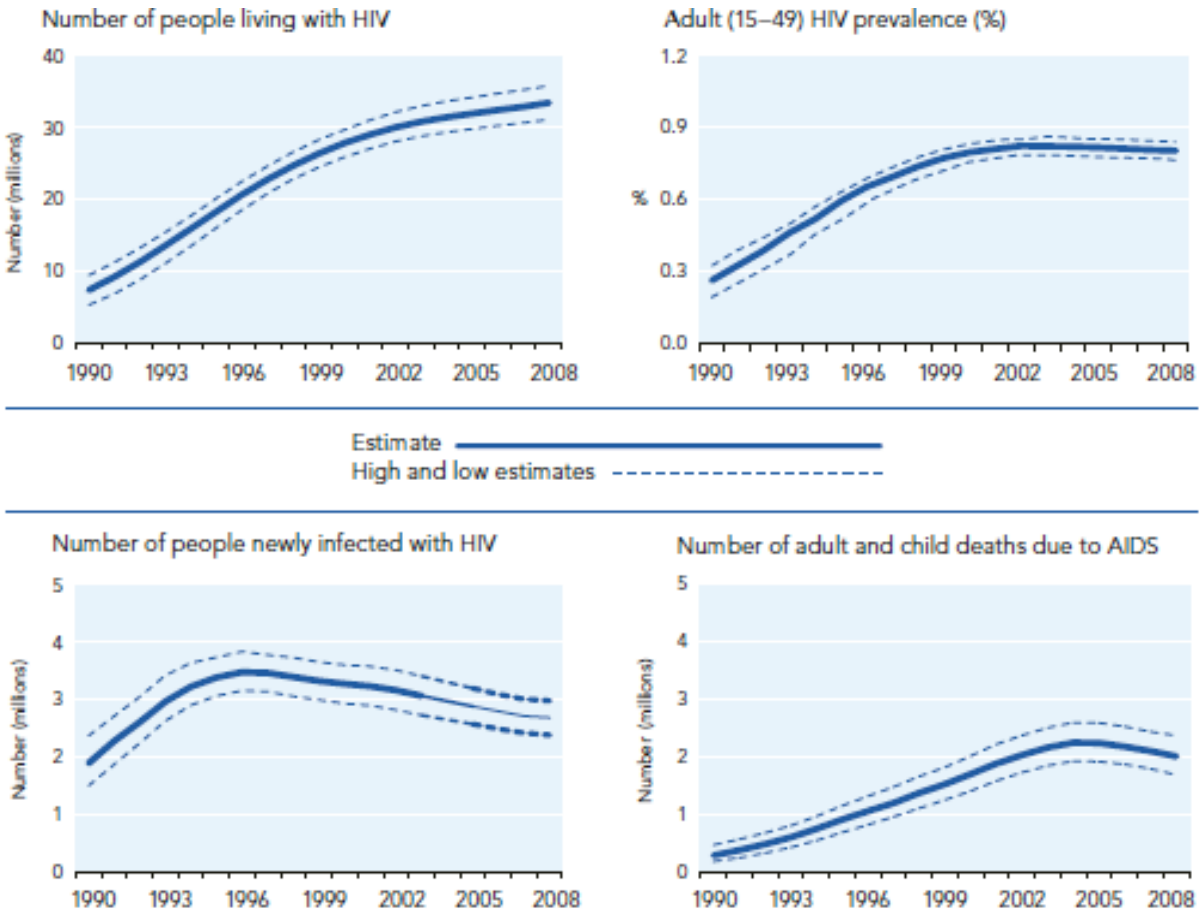
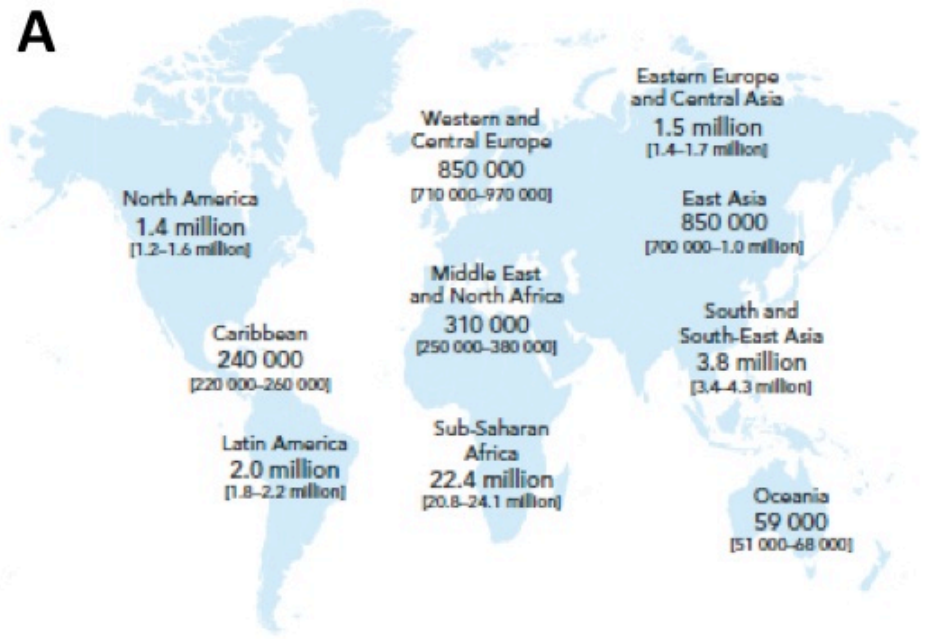


Figure 1.2 Global estimates between 1990 and 2008. Taken from: (UNAIDS report 2009).

An estimated 95% of new infections occur in low to middle income countries. Africa is, with approximately 22.5 m HIV-1 infected individuals, the most affected region, where prevalence rates can be above 10% in adult populations (Girard et al., 2011). Other highly affected regions are Asia and the Pacific region with approximately 4.7 m infections, and Latin America and the Caribbean with an estimated 1.6 m, but also approximately 2.3 m individuals are living with HIV-1 in the western world (UNAIDS report 2009). In the latter, therapy developments have transformed AIDS from a terminal disease to a chronic disease (see Section 1.7), but increasing infection rates in marginalised communities suggest that AIDS is also still a major health threat in the western world (El-Sadr et al., 2010; Girard et al., 2011; Hall et al., 2008; Magnus et al., 2009).

Clade C is the dominant HIV-1 strain in the most affected regions (Figure 1.3). In 2007 the estimated relative contributions of the clades and the main circulating recombinant forms (CRF's) to the global pandemic were: clade C: 52%, A1: 12%, B: 10%, O2_AG: 6.7%, G: 5%, unique recombinant forms (URF's): 4.2%, D: 3.6%, O1_AE: 3.1% (Arien et al., 2007). Clade B is best characterised since it is responsible for most western world infections and was isolated first. Relatively few characterisation studies have been conducted on non-clade B isolates.

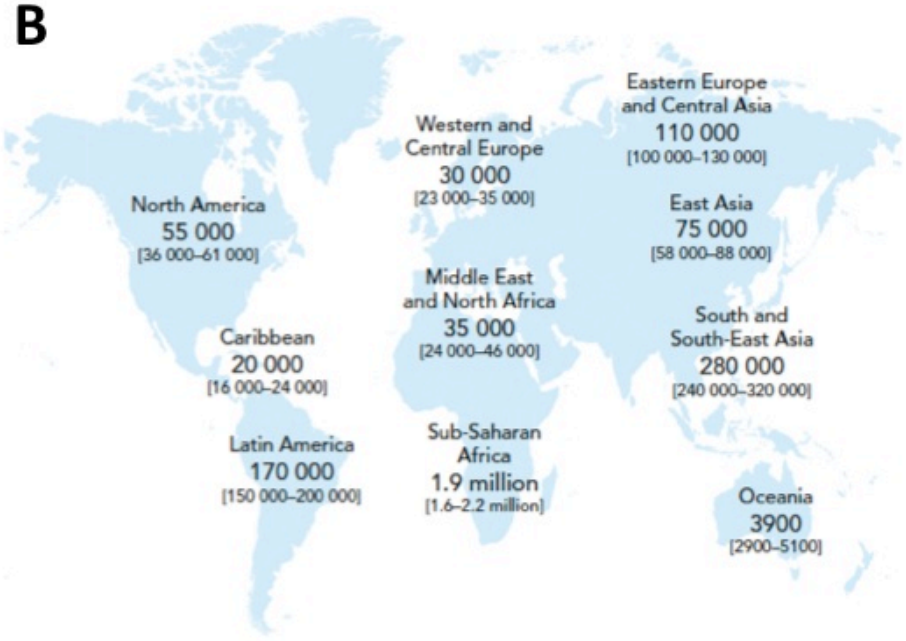
A



Adults and children estimated to be living with HIV, 2008

Total: approx. 33.4 million

B



Estimated number of children and adults newly infected with HIV, 2008

Total: approx. 2.7 million

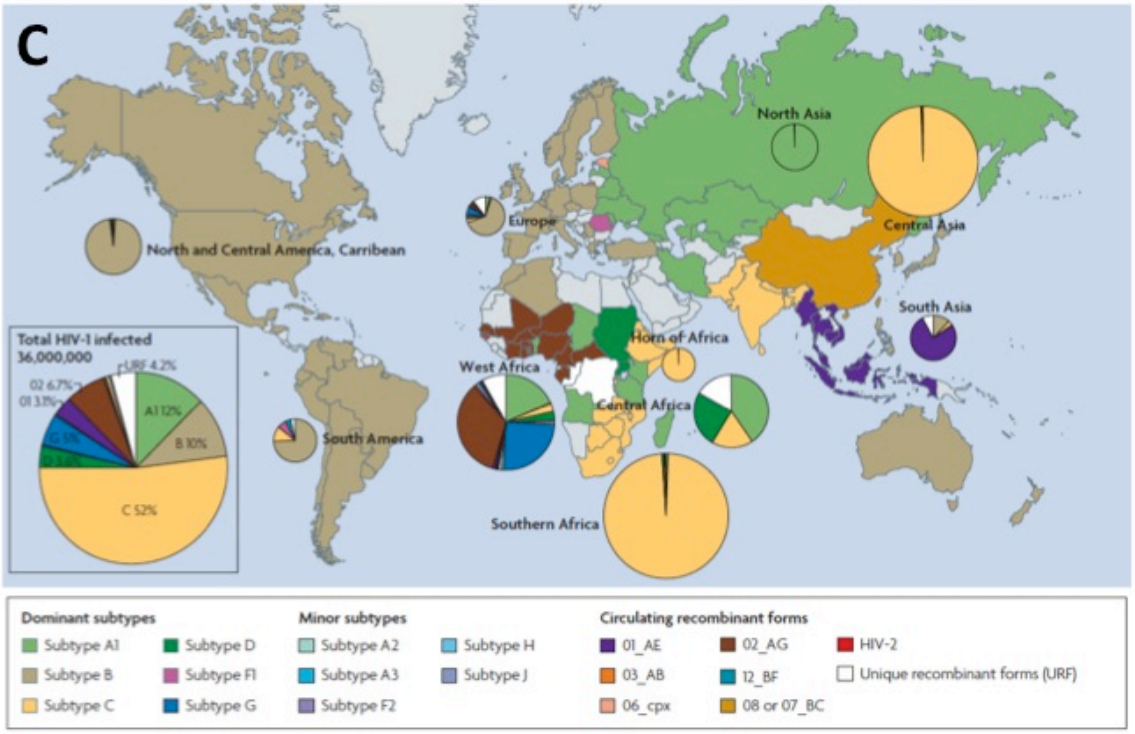


Figure 1.3 Global HIV-1 incidence figures and global distribution of HIV-1 subtypes. A. Adults and children estimated to be living with HIV, 2008. B. Estimated number of adults and children newly infected with HIV, 2008. C. The estimated frequency of each HIV-1 subtype and recombinant in each geographical region. Countries are colour-coded for dominant HIV-1 subtype. The pie charts show the relative contribution of each subtype, the size of the pie charts is proportional to the total number of HIV-1 infections in that region. Adapted from: (UNAIDS report 2009) and (Arien et al., 2007).

1.3. HIV-1 molecular organisation in a nutshell

HIV-1 is part of the genus Lentivirus and belongs to the family *Retroviridae*, which is a collection of enveloped RNA viruses that generally cause slow, progressive infections (Girard et al., 2011). HIV-1 has a genome that consists of two single-stranded positive sense RNA copies containing the *gag*, *pol,* and *env* genes, which code for structural proteins. *Gag* codes for the matrix (MA), capsid (CA), and nucleocapsid (NC) proteins (Figure 1.4) (Girard et al., 2011). *Pol* codes for the protease (PR), reverse transcriptase (RT), and integrase (IN) proteins that are required for viral replication and processing. *Env* codes for the envelope glycoproteins that will be discussed in the next Section 1.4. Furthermore, the HIV-1 genome encodes the regulatory proteins Tat and Rev and the accessory proteins Nef, Vif, Vpr, and Vpu.

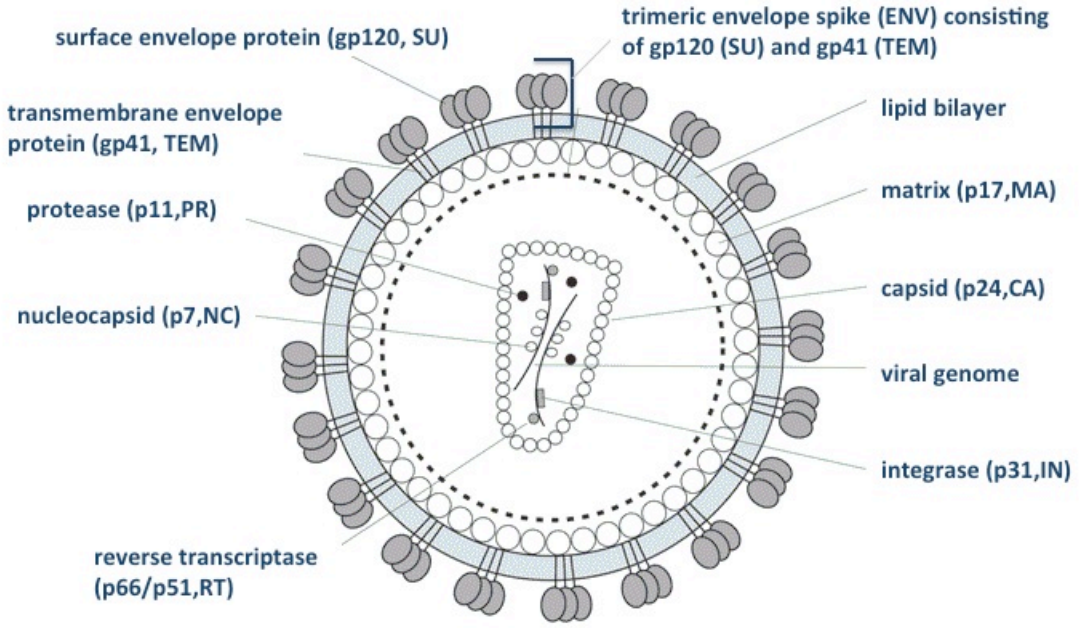


Figure 1.4 Schematic diagram of a HIV-1 virion. Adapted from Buchsacher 2003.

1.4. HIV-1 envelope glycoprotein: synthesis, function, and structure

On the surface of an HIV-1 virion are approximately 15 trimeric envelope (Env) spikes present (Liu et al., 2008; Zhu et al., 2006). The Env spikes are involved in HIV-1 entry into host cells and form the primary targets of antibody (Ab)-mediated immunity. Env is therefore the most attractive candidate antigen for use in Ab-based vaccination strategies, and understanding its structural and antigenic properties is of utmost importance for HIV-1 vaccine development. Here its synthesis, function, and structure will be briefly discussed.

Each monomer consists of the surface gp120 glycoprotein (SU) and the transmembrane gp41 glycoprotein (TM), which are both encoded for by the e*nv* gene (Figure 1.4) (Wyatt and Sodroski, 1998). The protein, initially consisting of single 160 kDa polypeptide precursor, is synthesised and folded into its native structure in the endoplasmatic reticulum (ER) (Freed, 2001; Wyatt and Sodroski, 1998). In the Golgi the precursor polypeptide assembles into trimers, a process that is probably driven by favourable interactions between helices present in the gp41 subunit (Freed, 2001), and undergoes intense glycosylation (Fenouillet et al., 1994). With 25-35 glycosylation sites and carbohydrates accounting for approximately 50% of its molecular weight (MW), Env is a heavily glycosylated structure (Li et al., 2005). The glycosylation steps are essential for correct folding into a stable conformation and mainly involve addition of N-linked oligosaccharides (Wyatt and Sodroski, 1998). A portion of the added glycans remain as high-mannose type oligosaccharides, while another portion is further processed by enzymes in the Golgi into complex-type oligosaccharides (Fenouillet et al., 1994). The resulting glycoprotein is subsequently cleaved into the gp120 and gp41 subunits that remain connected via weak non-covalent interactions and form functional Env spikes (McCune et al., 1988; York and Nunberg, 2004). After initially being expressed on the host cell surface, the Env spikes may mediate cell-cell fusion or are recruited to the budding virions upon binding of the MA region of Gag and the cytoplasmic tail of gp41 to the 47kDa tail interacting host protein (TIP47) (Lopez-Verges et al., 2006; Wyatt and Sodroski, 1998).

Both gp41 and gp120 subunits of the Env spike are crucially involved in virion binding to the target cell and subsequent membrane fusion. Upon encounter with a host cell, gp120 binds to the CD4 receptor and chemokine co-receptor CCR5 or CXCR4 on the target cell, which induces conformational changes that result in the rearrangement of gp41 into a trimeric coiled-coil intermediate and fusion of virus to host cell (Chan and Kim, 1998; Dalgleish et al., 1984; Deng et al., 1996; Dragic et al., 1996; Feng et al., 1996; Klatzmann et al., 1984; Weissenhorn et al., 1999).

Attempts have been made to understand the structure and function of gp120 and gp41 at the atomic level, in the hope that structural or functional interference could be used as a tool in the development of Ab-based vaccines (Burton et al., 2005). Most information has been provided by X-ray crystallography, and to a lesser extent cryo-electron tomography, of the Env components (Bennett et al., 2007; Chen et al., 2009; Huang et al., 2007; Huang et al., 2005a; Huang et al., 2005b; Koch et al., 2003; Kwong et al., 2000a; Kwong et al., 1998; Kwong et al., 2000b; Myszka et al., 2000; Pancera et al., 2010; Zanetti et al., 2006; Zhou et al., 2007; Zhu et al., 2006). The conformational flexibility of Env poses a challenge to the technique of crystallography and only after numerous trials involving complexing with ligands, deglycosylation, and truncation of the flexible variable loops, a gp120 core construct was successfully crystallised in complex with CD4 and the fab fragment of the mAb 17B in 1998 by the Kwong group (Figure 1.5). Later successful crystallisation of an unliganded gp120 core of SIV was achieved (Chen et al., 2005a). Crystallisation results have shown that the gp120 subunit falls into three tertiary structure components: the outer domain, the inner domain, and the domain that links these two: the bridging sheet (Figure 1.5) (Wyatt and Sodroski, 1998). The gp120 outer domain comprises most of the glycosylation sequons and is structurally similar for all crystallisation studies. The inner domain contains a relatively invariable beta-sheet, but other parts of it are structurally diverse. In trimeric formation most of this surface would be buried in the trimer centre. HIV-1 gp120 is composed of five conserved regions (C1-C5) and five variable loops (V1-V5) (Wyatt and Sodroski, 1998). The variable loops overlay the conserved regions, which has implications for the sensitivity of neutralising antibodies (nAbs). The

conformational flexibility of gp120 is due to the constant movement of the flexible V-loops, glycan structures and to rotation of the gp120 core parts around each other (Yuan et al., 2006). The CD4 binding site (CD4bs) is mainly composed of regions in the outer domain, and a so-called CD4 binding loop, but also consists of some parts of the inner domain, outer domain, and bridging sheet that approach each other during a conformational change that occurs upon CD4 binding (Chen et al., 2005b; Kwong et al., 2000a; Kwong et al., 1998). Comparison of unliganded SIV gp120 and CD4-liganded HIV-1 gp120 core crystal structures indicates that substantial conformational changes in the inner domain and bridging sheet occur upon CD4 binding (Dey et al., 2009; Huang et al., 2005a). The chemokine receptor binding site is well conserved between HIV-1, HIV-2 and SIV and probably evolved before the CD4 binding site (CD4bs) (Wyatt and Sodroski, 1998). It consists of cavities at the bridging sheet where the sulphonated tyrosines on the co-receptor N-termini are inserted on the co-receptors (Huang et al., 2007) and part of the V3-loop, that only come together to form the binding site after CD4 binding has occurred. This results in an entropy change that can allow the energetically unfavourable conformation in which chemokine receptor binding is possible (Figure 1.5) (Myszka et al., 2000). Differences in charge of the V3-loop between R5 (CCR5-tropic) viruses and X4 (CXCR4-tropic) and dual-tropic viruses are thought to account for the preferential binding of gp120 to CCR5 or CXCR4 respectively (Hartley et al., 2005). CCR5 tropism (R5 virus) allows the infection of $CD4^+CCR5^+$ cells, including memory $CD4^+$ T cells and macrophages, while CXCR4 tropism (X4 virus) enables HIV-1 infection of naïve $CD4^+$ T cells (Berger et al., 1998). New infections are R5- or dual-tropic and X4-tropism only occurs later in infection being both a cause and result of low $CD4^+$ T cell counts (Ribeiro et al., 2006). However, the switch in tropism is not required for disease progression (Ribeiro et al., 2006).

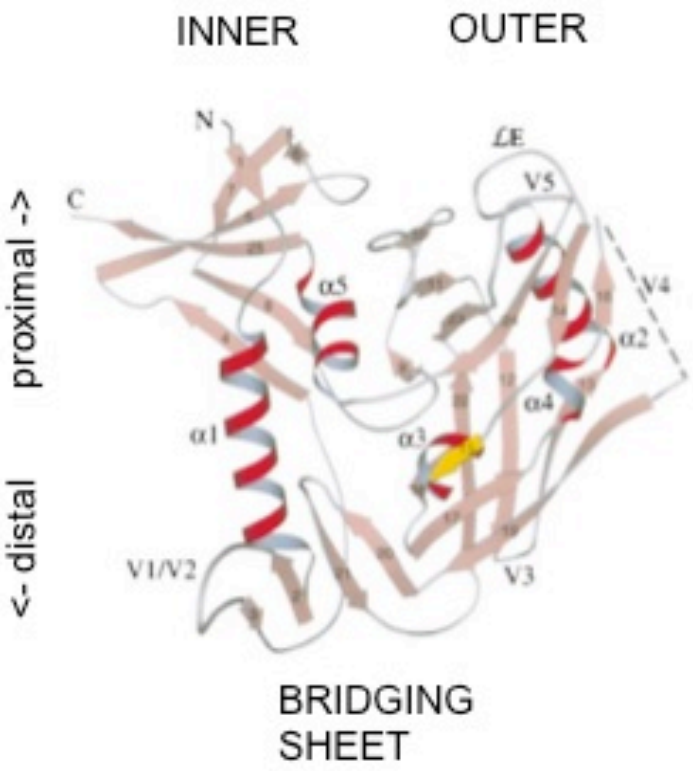


Figure 1.5 Structure of a HIV-1 gp120 core. This cartoon represents the crystal structure obtained of the gp120 core in complex with CD4 and the fab fragment of 17B. All major regions are depicted in this cartoon including the inner domain, outer domain, and bridging sheet. The CD4-binding loop is depicted in yellow. Adapted from (Kwong et al., 1998).

HIV-1 gp41 is also structurally flexible and takes on an alternative conformation upon CD4 and coreceptor binding to the gp120 subunit (Kwong et al., 1998). This conformational change drives gp41 dissociation from gp120 allowing the translocation of a gp41 N–terminus hydrophobic fusion peptide into the target cell membrane and the formation of a stable six-helix bundle in a pre-hairpin intermediate conformational state (Chan and Kim, 1998; Freed, 2001) (Figure 1.6). A hairpin is then formed that drives membrane fusion of the virus with the target cell (Chan and Kim, 1998). This fusion enables the entrance of the viral core into the target cell and signals the initiation of another round of viral replication (Chan and Kim, 1998).

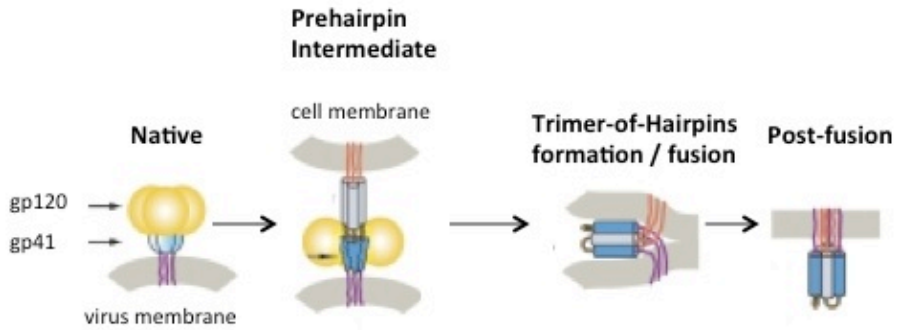


Figure 1.6 Gp41 conformational changes that induce membrane fusion. Binding of gp120 to CD4 and co-receptor induces a conformational change in gp41 that drives the formation of a pre-hairpin intermediate followed by a six-helix bundle (trimer-of-hairpins formation). The six-helix bundle is crucial for the process of gp41-induced membrane fusion of virus and target cell. Adapted from: (Eckert et al., 2008).

1.5. HIV-1 transmission and pathogenesis: a crucial role for the mucosa

HIV-1 acquisition can occur via multiple routes, but sexual transmission across the genital mucosa is the major contributor to the HIV-1 pandemic (UNAIDS report 2009). The mucosa, in particular the genital mucosa, thus plays a crucial role in HIV-1 transmission and pathogenesis, as will be outlined in this section. An understanding of the role of mucosal compartments in HIV-1 transmission and susceptibility as well as the capacity of mucosal immunity to protect against HIV-1 acquisition is therefore important in the development of a successful HIV-1 vaccination strategy.

Possible routes of HIV-1 transmission between individuals include vaginal/oral/anal sexual intercourse, direct blood contact in circumstances like needle sharing between intravenous drug users (IDU) or blood transfusion, and mother-to-child transmission (MTCT), which can occur during pregnancy, at birth, or during breast feeding (Rothenberg et al., 1998; Royce et al., 1997). Around 90% of the global new infections result from sexual transmission. Despite the probability of HIV-1 transmission per exposure event being relatively low for heterosexual contact compared to other transmission modes, approximately 60-70% of sexual transmission

events are acquired through heterosexual intercourse (Table 1.1) (UNAIDS report 2009). Also, in the most affected African regions heterosexual intercourse is the primary mode of HIV-1 transmission (Figure 1.7). The probability of male to female transmission is substantially higher than vice versa during heterosexual intercourse (Table 1.2) (Mehandru et al., 2004; Shattock and Moore, 2003).

Type of exposure to HIV-positive source	Risk of transmission per exposure	
Blood transfusion	90-100%	
IDU needle sharing	0.7%	
Receptive anal sex	≤ 0.3%	
Insertive anal sex	≤ 0.1%	
Receptive vaginal sex	**0.01-0.15%**	Heterosexual intercourse
Insertive vaginal sex	**≤ 0.1%**	

Table 1.1 Probability of HIV-1 transmission per exposure mode. Heterosexual intercourse has the lowest probability of transmission. Adapted from: www.Eurosurveillance.org

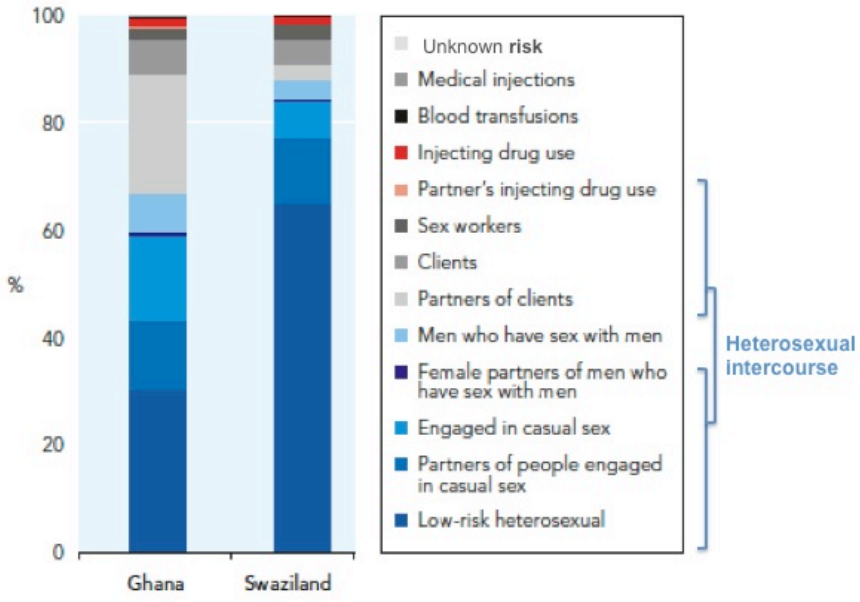


Table 1.2 Probability of transmission from male to female or vice versa.
Male to Female transmission accounts for the highest probability of HIV-1 acquisition following heterosexual intercourse. Adapted from: (Shattock and Moore, 2003).

Route of sexual transmission	Probability per exposure event
Male to Female	1/200 – 1/2000
Female to Male	1/200 – 1/10000

Figure 1.7. Distribution of new infections by mode of exposure in two heavily affected countries: Ghana and Swaziland, 2008. The majority of new infections in these countries results from heterosexual transmission. Data based on studies from: (Bosu et al., 2009; Mngadi et al 2009). Adapted from: (UNAIDS report 2009).

Since male-to-female transmission is the main contributor to the HIV-1 pandemic most studies have focused on understanding the early events of HIV-1 acquisition via the vaginal route. HIV-1 can be present in seminal fluid in either cell-associated or cell-free form. It is uncertain whether HIV-1 can be transmitted in both forms, but both cell-free and cell-associated SIV can initiate productive infection (Kaizu et al., 2006; Khanna et al., 2002; Sodora et al., 1998; Tachet et al., 1999).

To infect the new host via sexual transmission infectious HIV-1 virions must first traverse the CD4-negative mucosal epithelium. A physical barrier for the incoming virus is formed by the stratified squamous epithelium and single columnar epithelium that line the genital mucosa. However, the endocervix and the zone between the ecto- and endocervix are only lined by a single epithelial layer. This may facilitate viral entry, as mapping studies of infected founder viruses have confirmed these vaginal sites as the preferential sites of HIV-1 entry (Haase, 2010; Li et al., 2009).

Various cells including $CD4^+$ T cells, macrophages, and dendritic cells (DCs) have been implicated in HIV-1 transmission via the vaginal mucosa. DCs located in skin and epithelium that overlays the mucosa, called Langerhans cells (LCs) are located within the subepithelial lining and may express a surface adhesion molecule called Dendritic Cell–Specific Intracellular adhesion molecule–3 Grabbing Non-integrin (DC-SIGN, also called CD209), which binds and internalises HIV-1 virions (Geijtenbeek et al., 2000; Jameson et al., 2002). Furthermore, LCs express Langerin, another C-type lectin that internalises HIV-1 virions and induces HIV-1 degradation. However, it was suggested that a proportion of HIV-1 can escape the Langerin-induced degradation (de Witte et al., 2007; Wu, 2008). Hladik *et al* recently developed an *ex vivo* human organ culture system of epithelial sheets and used it to show that intra-epithelial $CD4^+$ T cells can be infected both directly and via LCs (Hladik et al., 2007). In the direct way, which probably accounts for initial infection, HIV-1 virions enter the cells via CD4 and CCR5-mediated fusion resulting in productive infection (Wu, 2008). In the LC-dependent way HIV-1 first enters LC up to 3 days after which it can be submitted to T cells through

trans infection (Hladik et al., 2007; Wu, 2008). The exact role of epithelial cells in the HIV-1 vaginal transmission and the possibility of productive infection of these cells is controversial (Morrow et al., 2007). Some studies suggest endocytosis and *trans* infection by epithelial cells might play a role (Bobardt et al., 2007; Wu, 2008). Transcytosis provides another route through the epithelial layer, but the efficiency is very low with less than 0.02% of the original HIV-1 inoculum transmigrating successfully (Bobardt et al., 2007). Finally, it is possible that direct exposure of susceptible cells following micro-abrasions that impair the epithelium integrity during intercourse, cervical ectopy, the menstrual cycle, or sexually transmitted diseases (STDs), play an important role, since HIV-1 transmissibility was shown to be enhanced in these conditions (Morrow et al., 2007; Pope and Haase, 2003). Once the epithelial barrier is breached the virus can access a multiplicity of cells including DCs, LCs, T cells, and macrophages in the underlying lamina propria (Morrow et al., 2007).

If the first susceptible cells are successfully infected, this small infected founder population expands locally in the lamina propria that underlies the cervicovaginal epithelium (Miller et al., 2005; Zhang et al., 1999). While $CD8^+$ T cells are dominant intraepithelially, the submucosa contains many $CD4^+$ T cells and the initial founder population consists mostly of 'resting' and 'activated' $CD4^+$ T cells (Haase 2010, Iqbal and Kaul 2007). The infection then spreads, initially to local lymph nodes (LNs), which drives the development of latent HIV-1 reservoirs, and then to systemic lymphoid tissue, particularly the gut-associated-lymphoid tissue (GALT) where the largest group of $CD4^+$ $CCR5^+$ T cells resides (Figure 1.8) (Schacker et al., 2000). The local expansion is a prerequisite for dissemination and self-propagating systemic infection (Miller et al., 2005; Zhang et al., 1999). HIV-1 gp120 has a particular specific affinity for the gut mucosal homing receptor integrin **α**4**β**7 that is expressed on activated $CD4^+$ T cells (Arthos et al., 2008). In addition to DCs, virus, and/or virus-infected T cells, B cells may be implicated in the early infection spread by binding virus through complement receptor CD21 (also referred to as CR2)(Moir et al., 2000). The GALT generally develops as the main site of HIV-1 infection, regardless of the site of viral entry and rapid, massive depletion of GALT lamina propria $CD4^+$ T cells occurs in which up to 80% of present cells are killed either through infection or by Fas-mediated

bystander killing (Figure 1.9) (Brenchley et al., 2006a; Brenchley et al., 2006b; Brenchley et al., 2004; Guadalupe et al., 2003; Haase, 2010; Mattapallil et al., 2005; Mehandru et al., 2004; Neutra and Kozlowski, 2006; Veazey et al., 1998).

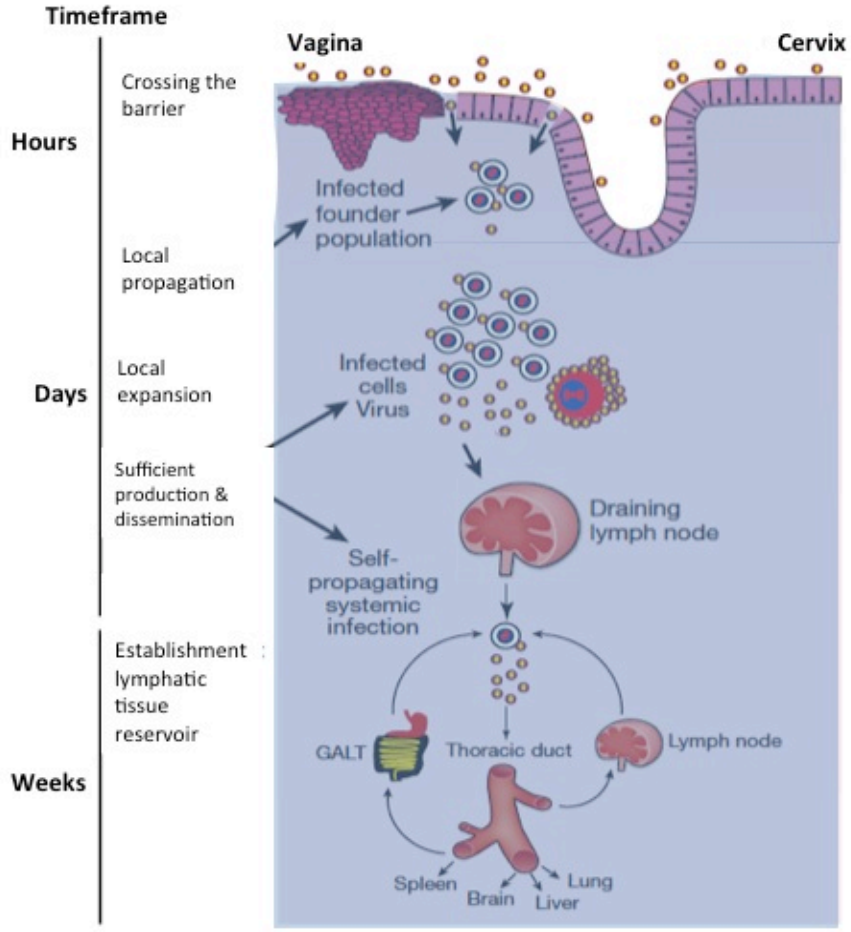


Figure 1.8. Time frame, sites and major events in vaginal HIV-1 transmission. In the first h HIV-1 founder virus gains access to local susceptible target cells. The small focal founder population consists initially of 'resting' $CD4^+$ T cells that lack conventional activation markers. The HIV-1 infection then proliferates locally in these 'resting' as well as activated $CD4^+$ T cells, which is a prerequisite for dissemination of a self-propagating infection that spreads to local draining LNs and via the bloodstream to systemic lymphoid structures, including the GALT. In the GALT rapid and massive $CD4^+$ T cell depletions occurs as the GALT develops into the main site of HIV-1 infection. Adapted from: (Haase, 2010).

The first clinical symptoms of HIV-1 infection often occur around 21 days after infection, when viraemia peaks and acute seroconversion syndrome can present, which is characterised by fever, lymphoadenopathy, rash and oral ulcers (Cooper et al., 1985). At this time serum viral loads may surpass 10^7 copies/mL (Cooper et al., 1985; McMichael et al., 2010). $CD4^+$ T cell numbers are low at the time of peak viraemia, but recover afterwards to near-normal levels in the blood, whereas numbers remain low in the GALT (Figure 1.9)(Brenchley et al., 2004; Veazey et al., 1998). B cell responses are also impaired in acute infection, although B cells are not depleted, through the destruction of cells that are necessary for the construction of germinal centres and the resulting loss of the structures (Levesque et al., 2009). Between twelve to twenty weeks following initial infection serum viral loads can drop 10- to 100-fold to a level that is called the *viral set point* (McMichael et al., 2010). This decline could be the result of either immune induction, for instance of HIV-1 specific $CD8^+$ cytotoxic T cell (CTL) responses or the exhaustion of susceptible target cells or both (Borrow et al., 1994; Goonetilleke et al., 2009; Huber et al., 2006; Ioannidis et al., 1998; Phillips et al., 1991). The height of the viral set point that will remain until late stage disease inversely correlates with the time to AIDS (Ioannidis et al., 1998). In addition, apoptosis of the intestinal epithelium, chronic immune activation, and to a lesser extent $CD4^+$ T cell loss, have been found to predict disease progression (Giorgi et al., 1999; Goujard et al., 2006; Li et al., 2008). Possible factors underlying this chronic immune activation are a decline in regulatory T cells when this T cell population gets infected by HIV-1, impairment of T cell homeostatic mechanisms, and enhanced microbial translocation from the intestine (Brenchley et al., 2006b; Eggena et al., 2005; Oswald-Richter et al., 2004).

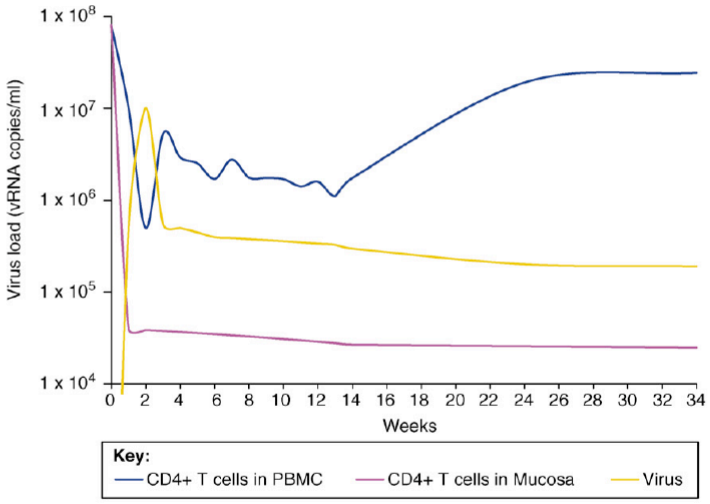


Figure 1.9. Mucosal CD4⁺ T cells in the intestines are selectively and rapidly depleted during HIV-1 infection. Most of the mucosal CD4⁺ T cells get infected early during infection and the majority of these cells get depleted in the first two weeks of infection. Adapted from: (Belyakov and Ahlers, 2008).

Studies have consistently reported the existence of a genetic bottleneck at HIV-1 transmission (Haase, 2010). Through sequencing, it has been shown that in 80% of individuals infected through mucosal transmission a single virus (or infected cell) initiated the productive infection and two to five viruses in the remaining 20% of individuals (Abrahams et al., 2009; Derdeyn et al., 2004; Keele et al., 2008; Salazar-Gonzalez et al., 2009), which is in line with studies on SIV infection (Keele et al., 2009). Also, it has been shown that in MTCT as well as in heterosexual transmission, the virus isolates present in the infected individual are genetically much less diverse than the isolates present in the transmitting individual (Wolinsky et al., 1992; Zhu et al., 1996). The genetic bottleneck plus the delay between local replication of the small founder virus population and infection dissemination suggest that there is a unique window of opportunity at the mucosa during early infection, when a less diverse and small virus population that is probably more susceptible to attack by our immune system is present (Derdeyn et al., 2004; Haase, 2010).

1.6. Natural innate and adaptive immune responses against HIV-1 in acute infection provide inadequate protection

The early immune responses to HIV-1 infection are thought to play an important role in clinical disease progression. The first adaptive responses are detectable just before peak viraemia and are able to partially control infection and drive virus diversification through the selection of escape mutations (Figure 1.10)(Borrow et al., 1994; McMichael et al., 2010; Wilson et al., 2000). Shortly after, innate and adaptive responses increase in strength, but they are 'too little, too late' to eliminate the virus (Haase, 2010; McMichael et al., 2010).

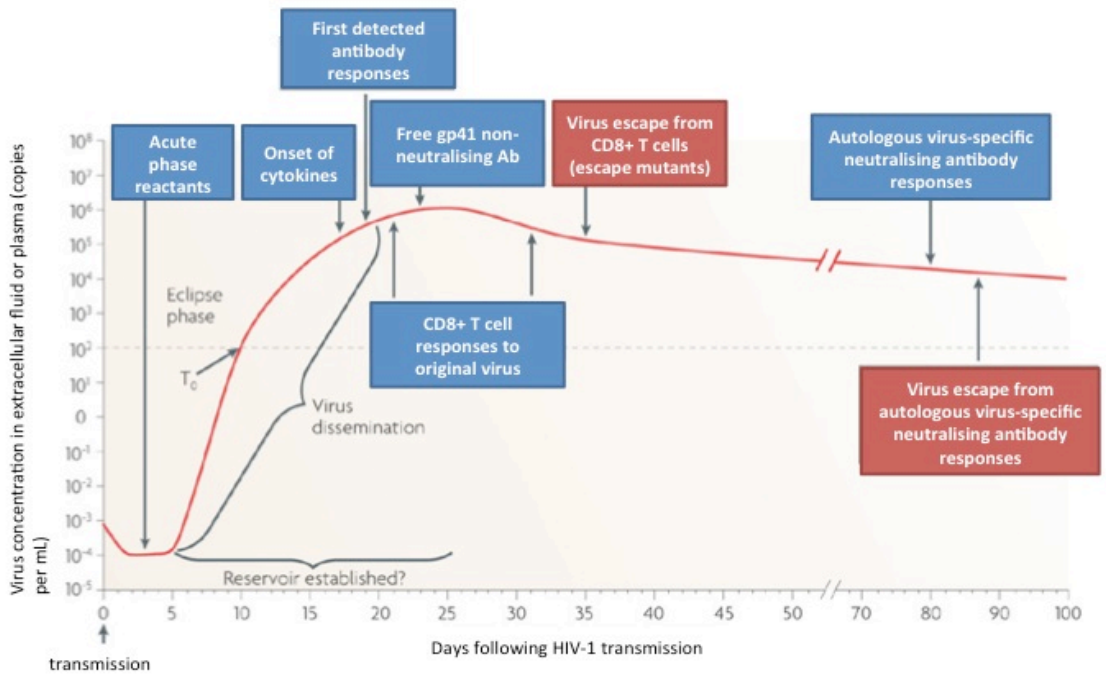


Figure 1.10 Schematic diagram of the earliest detected innate and adaptive immune responses in HIV-1 infection. The earliest systemically observed immune responses to HIV-1 are the enhanced plasma levels of acute phase proteins, which are detected when replication of a small founder virus population is still restricted to the local mucosal tissues and draining LN. Increased cytokine levels in the plasma are observed from around the time when HIV-1 virus is first detected in the plasma (T_0). The first antibody responses to HIV-1 are observed around 2-3 weeks following transmission, when plasma viral load is still rapidly increasing. Around this time also the first detectable $CD8^+$ T cell responses occur, but complete virus escape from these responses can develop rapidly, within 10 days of T cell expansion. Concurrently, viral reservoirs exist, which probably develop within days of infection. The first homologous-neutralising responses occur around day 80 following HIV-1 acquisition, when plasma viral load is still declining towards the viral set point. Within a week after the occurrence of these nAb responses, antibody escape mutants are detected in the plasma. Adapted from: (McMichael et al., 2010).

1.6.1 General early innate immune responses to HIV-1

Early innate immune responses to HIV-1 are characterised by increased levels of acute-phase proteins, probably triggered by the release of pro-inflammatory cytokines and possibly by extrinsic factors such as LPS from translocated gut bacteria (as a result of gut T helper 17 (T_h17) cell depletion), cytokines and chemokines, including IL-15, type I interferons (IFNs), CXC chemokine ligand 10 (CXCL10), interleukin (IL)-18, IL-10, TNF, and IL-22 (McMichael et al., 2010). These soluble factors enhance innate and adaptive immune responses and some of them have antiviral activity, but paradoxically they can also contribute to enhancement of infection (Section 1.1.2). The source of these cytokines and chemokines has not been determined, but probably includes monocytes, macrophages, DCs, NK, NKT cells, and later HIV-specific T cells (Brenchley et al., 2006b; Li et al., 2009; McMichael et al., 2010). Furthermore, DCs, mostly plasmacytoid DCs (pDCs), become activated and decline in numbers. Natural Killer (NK) cells and Natural Killer T cells (NKT) cells are activated in early infection, and probably help control viraemia through cytolysis of infected cells, release of cytokines and chemokines and influencing T cell responses through interaction with DCs (Martin et al., 2007; McMichael et al., 2010; Qi et al., 2006).

1.6.2 Mucosal early innate immune responses to HIV-1

Despite the unique window of opportunity, and many mucosal innate immune effectors being lined up when HIV-1 enters the body, the natural mucosal immunity usually falls short. In contrast with the traditional portrayal of mucosal epithelium as a passive physical barrier, many innate and adaptive immune factors contribute to an active immune system at the mucosa (Belyakov and Ahlers, 2008). In the context of HIV-1 infection, innate immune proteins, cationic proteins, cytokines and chemokines have been found to play a role. For example, the anti-leukoprotease Secretory Leukocyte Protease Inhibitor (SLPI) can protect activated $CD4^+$

T cells against HIV-1 infection at concentrations that equal the concentrations found in semen and saliva (McNeely et al., 1995; Shugars, 1999). It is mainly active against R5-tropic HIV-1 viruses that are the initial founder viruses in heterosexual transmission (McNeely et al., 1995). Furthermore, mucosally present Lactoferrin can inhibit the initial stages of HIV-1 transmission *in vitro* probably through inhibition of HIV-1 uptake (Groot et al., 2005; Harmsen et al., 1995; Lee-Huang et al., 1999; Moriuchi and Moriuchi, 2001; Saidi et al., 2006; Viani et al., 1999). Cationic small molecules called defensins, as well as the innate mucosal interferon response have also been found to play a role in early protection against HIV-1 and infection (Biragyn et al., 2002; Chen et al., 2006; Fleuridor et al., 2003; Haase, 2010; Iqbal and Kaul, 2008; Le Bon et al., 2003; Milush et al., 2007; Novak et al., 2007; Stark et al., 1998; Weinberg et al., 2006). Finally, a range of other cytokines and chemokines secreted in the genital tract may help protect against HIV-1 transmission for instance through competitive antagonism for chemokine receptors (Agace et al., 2000; Dragic et al., 1996; Iqbal and Kaul, 2008).

It is possible that these innate mucosal immune factors account for the low probability of transmission per exposure event, but the relevance of most of these factors *in vivo* remains disputable and some of them have paradoxically been shown to facilitate transmission by increasing target cell availability (Haase, 2010; Li et al., 2009). In addition, underlying inflammation may help breach the mucosal barrier, drive recruitment of susceptible target cells to the genital mucosa or stimulate the production of other immune proteins that facilitate HIV-1 transmission (Iqbal and Kaul, 2008). Even in the absence of pre-existing inflammation, heterosexual intercourse initiates local processes that serve to prepare the female genital mucosa for tolerance of the allogeneic sperm, implantation and potential fetus development, which may facilitate HIV-1 transmission (Haase, 2010; Robertson, 2005; Robertson et al., 2002).

1.6.3. Early T cell responses in HIV-1 infection

$CD8^+$ T cell responses probably play a central role in the reduction of acute viraemia (Koup et al., 1994; Kuroda et al., 1999). The first T cell responses develop during peak viraemia and are highest approx. 1 to 2 weeks later when virus levels drop to the set point. The exact identity of $CD4^+$ T cell responses in early infection has not been well defined and studies are complicated by the substantial depletion of these cells during infection. Nevertheless, expansion of HIV-1 specific $CD4^+$ T cells occurs, particularly to Gag, and the ability of these cells in HIV^+ individuals to respond to Gag epitopes by releasing IL-2 or IFN-**γ** is negatively correlated with plasma viral load (Boaz et al., 2003; Harari et al., 2004). Although CD4+ T cell responses decline rapidly, which probably negatively impacts on $CD8^+$ T cell memory, it is sufficient for strong $CD8^+$ T cell responses to develop (Gloster et al., 2004; Oxenius et al., 2001; Sun et al., 2004). The earliest $CD8^+$ T cell responses are directed at Env and Nef and probably play an important role in the control of viraemia (Goonetilleke et al., 2009), while later waves include specificity for Gag p24 and Pol proteins, which are probably important for the maintenance of the viral set point (Goonetilleke et al., 2009; Turnbull et al., 2009). However, these T cell responses often decrease rapidly through exhaustion or through the rise of virus escape mutants, which eventually renders them ineffective in controlling infection (Goonetilleke et al., 2009; Turnbull et al., 2009; Wang et al., 2009).

1.6.3.1. Immunoevasive HIV-1 strategies to escape T cell responses

HIV-1 can escape T cell responses through a variety of mechanisms, of which mutation is probably most important. HIV-1 RT is error-prone and combined with the high propensity for recombination and the constant pressure placed on the virus by the immune system this results in the constant generation of HIV-1 variants that can escape cellular and humoral immune responses and fuel ongoing infection (Korber et al., 2001). The high HIV-1 mutation rate allows epitopes to change into a form that is not recognised by present

CTL clones, and allows the replacement of virus isolates that are sensitive to the present CTL responses with a new population that is insensitive (Klenerman et al., 1994; Lill et al., 1992; Sheppard and Sattentau, 2005). Furthermore, HIV-1 can weaken the T cell response through bystander apoptosis of $CD4^+$ and $CD8^+$ cells, which can be induced through a range of HIV-1-mediated actions, including release of soluble factors by infected CD4+ T cells, Fas-mediated mechanisms or directly in a CXCR4-Env dependent manner (Mattapallil et al., 2005; Sheppard and Sattentau, 2005). Another example is the HIV-1 accessory protein Nef which can down-regulate Major Histocompatibility Complex I (MHC I) molecules on the surface of the infected cell that are needed for the presentation of HIV-1 peptides to CTL (Le Gall et al., 1998; Yang et al., 2002). Finally, HIV-1 can dysregulate T cell migration, for example through shielding of T cells by V2 and V3 loops (Sheppard and Sattentau, 2005).

1.6.4. Antibody responses during HIV-1 infection

HIV-1 specific antibody responses develop slowly and present approximately 3 weeks following transmission, initially being directed to Env gp41 and shortly after to gp120 (McMichael et al., 2010). Eventually antibody responses are made to all viral proteins, since B cells get exposed to material from defective and lysed virons, or cells, but Env remains the principal target due to its presence on the viral surface. HIV-1 specific antibodies can have a range of antiviral functions, including neutralisation, antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent complement-mediated inactivation (ADCMI), and antibody-dependent cell-mediated phagocytosis (ADCP), the most important of which will be briefly discussed below.

1.6.4.1. Antibody effector functions

ADCMI is mostly achieved by non-neutralising antibodies and does not require binding to functional trimeric Envs. It has been suggested to play a role in the resolution of acute viraemia, when nAb responses have not yet

developed. However, antigenic variation and the production of complement protection factors, including factor H, CD40 (MCP), CD55 (DAF), and CD59 (protectin), allow HIV-1 virions to avoid complement-mediated lysis (Banapour et al., 1986; Marschang et al., 1995; Montefiori et al., 1994; Saifuddin et al., 1997; Saifuddin et al., 1995).

IgG antibodies can mediate ADCC through interaction of the Fc domain with Fc receptors (FcRs) expressed by NK cells. ADCC responses have been found to target both homologous and heterologous HIV-1 isolates in acute and chronic infection and have been shown to correlate with lower plasma viral load and higher CD4+ T cell numbers. However, resistance through this immune function can again be achieved through HIV-1 diversification, as well as through induction of HLA-C and HLA-E on infected cells, which can negatively regulate NK cells and inhibit target lysis (Ward et al., 2004).

The most studied function of HIV-1 specific antibodies is virus neutralisation, which impedes infection of target cells and is achieved through blockage of the interaction between CD4 and gp120 or subsequent fusion (Klasse and Sattentau, 2001; 2002). nAb responses only develop around 12 weeks following the transmission event. The earliest occurring nAbs bind only a small range of exposed epitopes on Env of the infecting HIV-1 strain, which are usually variable and can be escaped by the virus, while the majority of conserved HIV-1 Env epitopes that correlate with broad neutralisation are hidden, recessed, or covered by glycans and therefore cannot be easily accessed by antibodies (Burton and Moore, 1998). bNAbs are therefore rarely detected in acutely infected individuals and if they develop then this is generally only after approximately 20-30 months of infection (Goonetilleke et al., 2009; Gray et al., 2009; Stamatatos et al., 2009). At this time, they do not seem to have clinical importance in controlling infection and disease progression (Johnston and Fauci, 2011). The clinical relevance of bNAbs in natural infection thus remains unclear. However, the rapid mutation of Env is mainly a result of nAb responses and CTL responses only make a minor contribution (Richman et al., 2003). Also, a recent study demonstrated that B cell depletion in an HIV-1-infected patient resulted in a 1.7 $\log_{10}$ rise

in HIV-1 plasma viral load, which could be partially restored upon administration of nAbs (Huang et al., 2010). This result suggest that B cells and nAbs might contribute to control of acute HIV-1 infection (Huang et al., 2010).

1.6.4.2. Immunoevasive strategies used by HIV-1 to escape antibody responses

In addition to antigenic diversification through mutation and sequence variability, for example of the V loops, HIV-1 has evolved other strategies to evade the humoral arm of the immune system. Conserved regions of Env that provide epitopes for bnAbs are recessed, hidden away from the immune system by glycans, or partially embedded in the HIV-1 membrane. The conserved CD4bs is, for example, partially masked by the V1/V2 loop, CD4b loop, and glycans, while the conserved co-receptor binding site is also masked by the V1/V2 loop and only exposed after CD4 binding. Even when the CD4I nAb epitope is exposed, the small distance between the virus and target cell ensures that the respective region cannot be reached by bulky nAbs. Furthermore, the presence of exposed non-neutralising epitopes helps to direct the immune response away from neutralisation relevant regions. Also, Env is covered by host-derived glycans suggesting that a major part of the Env surface might be recognised as 'self' and herewith evade the host immune response. A schematic overview of this, including references, is presented in Table 1.3.

Strategy	Mechanism	References
Conformational masking	Antibodies pay a significant entropy penalty to bind the receptor-binding sites	(Burrer et al., 2005; Moore and Sodroski, 1996; Moore et al., 2006; Wyatt et al., 1997)
Decoy	Irrelevant epitiope exposure on monomeric gp120, gp41 that has shed gp120, and immature gp160	(Kwong et al., 2002; Sheppard and Sattentau, 2005)
Glycan shield	Shifting glycans can sterically block antibodies reaching their target epitopes and may be recognised as 'self' by the immune system	(Wei et al., 2003; Wyatt and Sodroski, 1998)
Protection of CD4bs	The CD4bs is recessed in a pocket that limits access by antibodies, is partially obscured by the V1/V2 loop and CD4b loop, and tolerates greater sequence divergence because it contacts CD4 through many main-chain atoms of amino acids	(Chen et al., 2005b; Kwong et al., 2000a; Kwong et al., 1998; Wyatt and Sodroski, 1998; Kolchinsky et al., 2001; Wu et al., 1996; Wyatt et al., 1993)
Protection of coreceptor-binding site	The coreceptor-binding site is occluded by the V1/V2 loop prior to ligation and afterwards the site lies in close apposition to the target cell, restricting access of bulky IgG molecules	Chen et al., 2005b; Kwong et al., 2000a(Kolchinsky et al., 2001; Wu et al., 1996; Wyatt et al., 1993; Decker et al., 2005; Labrijn et al., 2003; Myszka et al., 2000)
Sequence variability	Five V loops that are highly tolerant of amino acid substitutions, additions and deletions induce strain-specific antibodies*	(Leonard et al., 1990; Pinter et al., 2004; Starcich et al., 1986)
'Silent' face	A hyperglycosylated region of gp120 has low immunogenicity	(McCaffrey et al., 2004; Reitter et al., 1998; Wyatt et al., 1998)

Table 1.3. Immunoevasion strategies used by HIV-1 to escape humoral responses. * The V3 loop is an exception as its length is highly conserved. Taken from (Sheppard and Sattentau, 2005).

1.7. Therapeutic approached and shortcomings

Therapy options for HIV-infected individuals have significantly evolved since AIDS was first recognised in 1981, and a form of triple therapy called Highly Active Anti-Retroviral Therapy (HAART) has revolutionised HIV-1 treatment and has become the standard treatment for HIV-infected individuals in the developed world. However, significant HAART-inherent problems and obstacles in worldwide HAART coverage remain, and since no other therapeutic intervention has been successful, an effective prevention strategy will be needed to halt the pandemic (Dieffenbach and Fauci, 2011).

For several years after the recognition of AIDS there was no effective therapy to moderate the disease course. The first antiretroviral drug found to have an effect on HIV-1 replication was Zidovudine or Zidothymidine (AZT), a Nucleoside Reverse Transcriptase Inhibitor (NRTI) (Ezzell, 1987). Despite delaying the decline in $CD4^+$ T cell counts, AZT however failed to improve long-term disease progression (1994; Volberding et al., 1995). The identification of more NRTIs and Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs) allowed the introduction of more effective dual therapy that had a greater and longer-lasting impact on HIV-1 replication. However, the real breakthrough in AIDS therapy came with the introduction of a new class of antiretroviral drugs: the protease inhibitors (PIs) in the mid-1990s. Application of a combination of three drugs from at least two drug classes in a triple therapy form called HAART has revolutionised HIV-1 therapy in the developed world. In recent years more classes of ART drugs have been introduced including fusion inhibitors, CCR5 antagonists, maturation inhibitors and integrase inhibitors (Reeves and Piefer, 2005). As a result a still growing armamentarium of antiretroviral therapy (ART) is available that includes nearly 30 different single or multiple drug products (Lori, 2011). Successful management and compliant HAART can, despite not being able to eliminate the virus, reduce viral load below detection levels (< 50 copies/mL) indefinitely, improve

$CD4^+$ T cell numbers and herewith delay disease progression (Peters and Conway, 2011; Pomerantz and Horn, 2003). An increase in the global implementation of HAART from 7% in 2003 to 42% in 2008 has successfully reduced the number of AIDS related deaths (UNAIDS report 2009).

Nevertheless, HAART has serious disadvantages, including short and long-term side effects (Carr and Cooper, 2000; Cazanave et al., 2008; Peeters et al., 2010; Peters et al., 2011). While recent HAART improvements include the introduction of a once-daily drug combination that improves adherence and new drugs that lack many of the side effects, a still increasing number of new adverse conditions are being discovered (Aboud et al., 2007; Peeters et al., 2010; Peters et al., 2011; Vogel and Rockstroh, 2009). Furthermore, HAART does not eradicate latent HIV-1 reservoirs and the resulting need for strict adherence and lifelong costly treatment prevents widespread application of this therapy (Reeves and Piefer, 2005). Additionally, the rise of drug-resistant HIV-1 stains can eventually result in HAART failure (Reeves and Piefer, 2005). Finally, population analyses reported that life expectancy is not fully repaired even with optimal management and well controlled HIV-1 infection (Lohse et al., 2007; Losina et al., 2009).

In addition to HAART, the development of therapeutic vaccination has received much attention. Strategies explored include protein-based therapy, *ex vivo* DC vaccines, and DNA vaccines (Jones, 2010; Routy and Nicolette, 2010). The goal of therapeutic vaccine development is the induction of immune control in order to make HIV-1 infected individuals less dependent on HAART. In addition to challenges that are also encountered in the development of a prophylactic HIV-1 vaccine, other factors, such as the impaired immune system and the damaged LN structures in HIV-1-infected individuals have limited the success of therapeutic vaccination (Letvin and Walker, 2003).

1.8. HIV-1 prevention in the absence of a vaccine

In the absence of a prophylactic HIV-1 vaccine, various strategies have been proposed and implemented to reduce the spread of HIV-1 infection. These prevention strategies can be divided in four groups: pre-exposure, during exposure, immediately after exposure, or the prevention of secondary infection that is focused on infected individuals (see Figure 1.11) (Cohen et al., 2008).

Pre-exposure measures that are aimed at reducing the chance of exposure include behavioural measures such as education about prevention, reduction of sexual partner numbers, reduction of needle sharing and the promotion of abstinence, monogamy, and condoms (Cohen et al., 2008).

For the prevention during or shortly before exposure most options of intervention are available, including the creation of a physical barrier through condom use or circumcision, the treatment of STDs that facilitate HIV-1 transmission, management of MTCT, and the use of ART, for instance formulated in microbicides (Cohen et al., 2008). Studies in NHP and other animal models have indicated that oral or systemic use of ART before exposure in so-called pre-exposure prophylaxis (PreP) can prevent HIV-1 transmission (Cohen and Kashuba, 2008; Van Rompay et al., 2001; Youle and Wainberg, 2003). Based on these results clinical trials are currently ongoing to further investigate the efficacy of PreP (Garcia-Lerma et al., 2008). The prevention of MTCT that can occur during pregnancy, delivery, or breast-feeding, involves a multitude of strategies that are applied to work synergistically (UNAIDS report 2009), including reducing overall HIV-1 infections in young individuals, reducing unwanted pregnancies and applying ART to young HIV-1-infected mothers (UNAIDS report 2009). Topical microbicides are protective agents formulated in gels, films or creams for vaginal or rectal application prior to sexual intercourse to prevent HIV-1 transmission (Stone, 2002). Microbicides can block HIV-1 transmission in various ways. A variety of agents have been explored in past and on-going trials for varying

HIV-1 inhibiting effects, including disruption of the HIV-1 Env glycoproteins, enhancement of the acidic vaginal pH (3.8-4.5) and lactobacilli microenvironment, polyanions that inhibit viral attachment and entry, CCR5 inhibitors, fusion inhibitors, RT inhibitors, other ART agents, and combinations of these [reviewed in detail: (Klasse et al., 2008)].

An example of an immediate post-exposure intervention is the use of antiretroviral treatment (ART) in so-called post-exposure prophylaxis (PeP). Guidelines currently advise the use of three ART medicines for 4 weeks following high-risk exposure (2001). Several studies have shown the effectiveness of such strategies (Cohen et al., 2008; Hosseinipour et al., 2002). The only prevention option for secondary infections is also the use of ART in so-called 'prevention for positives' (Cohen et al., 2008). During unprotected sexual exposure the transmission event is dependent both on the infectiousness of the 'index case' and the susceptibility of the 'host'. As such, the viral load of the infected person is positively correlated with the risk of transmission, and HAART treatment of HIV^+ individuals was reported to have a beneficial effect on further infection, although other studies place a doubt on this (Cohen et al., 2008; Fang et al., 2004; Keele et al., 2008; Quinn et al., 2000). Furthermore, relatively recently, male circumcision has been investigated as a possible intervention measure. The mucosal foreskin glans of the penis is rich in HIV-1 susceptible cells and removal of this structure has been shown in three randomized trials to induce at least 60% reduction in HIV-1 acquisition (Auvert et al., 2005; Bailey et al., 2007; Gray et al., 2007).

Altogether successes with these different prevention efforts have contributed to HIV-1 infection decline and the stabilisation of the epidemic in some areas. The recent CAPRISA tenofovir gel microbicide trial showed ~39% protection (Abdool Karim et al., 2010), condom use can reduce risk of infection by ~80% (Pinkerton and Abramson, 1997), ART can reduce MTCT from ~30-35% without intervention to ~1 to 2% (UNAIDS report 2009) and globally approximately 70, 000 infant infections have been averted (UNAIDS report 2009).

Nevertheless, effective implication of all non-vaccine prevention strategies, except for male circumcision, is complicated by the need for prolonged drug administration and adherence, poor health infrastructure, side effects that require continuous monitoring and potential drug resistance (Mofenson, 2011). Effective prophylactic vaccination is therefore still generally considered the 'holy grail' to HIV-1 prevention (Belyakov and Ahlers, 2008; Johnston and Fauci, 2011; Mofenson, 2011).

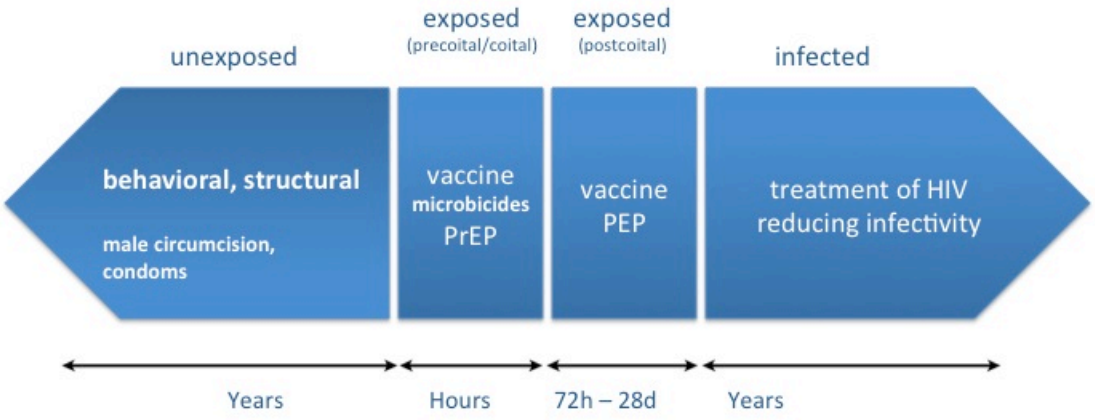


Figure 1.11. HIV-1 prevention opportunities. HIV prevention opportunities can be divided into four groups: pre-exposure, during exposure, immediately after exposure or prevention directed at infected individuals to prevent secondary infection. Adapted from: (Cohen et al., 2008).

1.9. Challenges in HIV-1 vaccine development presented by HIV-1 virus

Vaccines continue to be the most effective means to control and eradicate human infections and an effective prophylactic HIV-1 vaccine is the only means to achieve the necessary population coverage to end the HIV pandemic (Mbow et al., 2011; Dieffenbach at AIDS VACCINE 2011). Modelling has indicated that a vaccine with 50% efficacy directed at 30% or 60% of the highest risk populations could have a major public health impact and could reduce new infections by 5-15% over 10 years (Roehr, 2011b). The concept of training the immune system through vaccination can be dated back to the 18th century when Edward Jenner started the inoculation of cowpox pus and showed that his subjects became immune to smallpox (Lori, 2011; Riedel, 2005). Since then effective vaccines have been developed for a range of pathogens including smallpox, measles, and poliovirus that have allowed the eradication (smallpox) or near eradication (polio) of widespread epidemics (Lori, 2011). Unfortunately, HIV-1 is unique in many ways compared to these pathogens and its characteristics place some important challenges and obstacles to the development of an effective prophylactic HIV-1 vaccine. HIV-1 evasion strategies to escape humoral and cellular responses were described before (Section 1.6.3.1 & 1.6.4.2.). HIV-1 also has more general mechanisms for evasion of the adaptive immune response.

For example, HIV-1 infection results in reservoirs of latent proviral DNA, which are undetectable by the immune system and therefore ensure that long-term infection is maintained (Chun et al., 1997; Chun et al., 2008).

Another difficulty in HIV-1 vaccine development is that HIV-1 targets CD4$^+$ T cells, which are vital for orchestration of an adaptive B cell or CTL immune response required to combat the infection. The before mentioned loss of germinal centres also adds to weakening of the immune system (McMichael et al., 2010).

This rapid deletion of the key immune cells and the impairment of the immune system complicate the identification of HIV-1-specific immune responses in HIV^+ individuals (Lichterfeld et al., 2004; Schoenberger et al., 1998; Sun and Bevan, 2003; Wan and Flavell, 2009).

Furthermore, and perhaps the greatest challenge, the vast diversity of HIV-1 isolates and the high rate of mutation creates a major hurdle for HIV-1 vaccine development. The enormous diversity of HIV-1 isolates, means that an effective vaccine candidate requires induction of immunity that is effective not just against a limited range of HIV-1 isolates, but against the wide array of distinct HIV-1 virus isolates that are currently circulating in the human population.

Finally, for almost all pathogens for which effective vaccination has been introduced there is a natural model of protection. In these cases infected individuals develop an immune response that ultimately clears the pathogen from the body and establishes durable protection, providing an example for vaccine development. The absence of such a natural model of protection for HIV-1 infection makes the search for correlates of protection uniquely challenging and means that vaccine development needs to aim at inducing a response that is qualitatively and/or quantitatively different from that induced by natural infection (Nabel and Fauci, 2010).

1.10. Immune correlates of protection against HIV-1 infection

Identifying which of the host immunological factors, if any, are correlated with protection from HIV-1 infection is a major challenge in HIV-1 vaccine development as was briefly described in the previous section. This is because of the lack of a 'natural' protection model, and is mainly based on understanding the immune activation in long-term nonprogressors[1] (LTNPs), exposed seronegatives [2](ESNs), and non-human primate (NHP) models (Deeks and Walker, 2007; Hladik et al., 2003; Rowland-Jones et al., 1995). Also, and probably even more importantly, a recent success in HIV-1 vaccine trials with the so-called RV144 vaccine candidate, a combination of pox virus prime (ALVAC) with gp120 boost (AIDSVAX B/E) that provided 31% protection against infection in treatment versus control groups has provided researchers with another pool of sample material to try and determine possible immune correlates of protection from this trial (Rerks-Ngarm et al., 2009; Roehr, 2011a).

1.10.1. Broadly neutralising antibodies (bNAbs)

The hallmark of many historically successful human vaccines is their ability to induce nAbs (Amanna et al., 2007). In the context of HIV-1 infection, passive infusion studies in NHP models of infection have demonstrated that the infusion of several bnAbs can completely prevent acquisition of Simian-Human Immunodeficiency Virus (SHIV) (Baba et al., 2000; Bogers et al., 2008; Hessell et al., 2009; Hessell et al., 2010; Johnston and Fauci, 2011; Mascola et al., 2000; Pal et al., 2006; Parren et al., 2001; Walker and Burton, 2010). BnAbs might also play a role in the slower progression towards disease in LTNPs (Baker et al., 2009). Therefore, despite the observation that non-neutralising antibody functions might have contributed to the

[1] In the 1990s it was reported that some (5-15%) of HIV-1 infected individuals were identified in the 1990's who successfully control their infection in the absence of treatment. They were called named long-term nonprogressors.

[2] Individuals have been identified who despite repeated exposure to HIV-1 virus remain uninfected. These individuals are called exposed seronegatives.

RV144-induced modest protection, which will be discussed later, an HIV-1 vaccine strategy that induces nAb prior to or shortly after HIV-1 exposure will probably be highly effective (Johnston and Fauci, 2011). Recent findings have indicated that polyreactive antibodies have a stronger neutralisation capacity, which has lead to the idea that vaccination strategies should explore ways to enhance antibody polyreactivity (Dimitrov et al., 2011).

Despite the multiple immunoevasion techniques employed by HIV-1 to escape the humoral responses, bnAbs are occasionally found in HIV-1 infected individuals (Table 1.3). Because of the rarity of bnAbs and due to 'noise' in isolation techniques, created by the vast number of Env antibodies produced that do not recognise the native conformation and hence are non-neutralising, the isolation of bnAbs is a challenging task and only relatively few bnAbs have been identified (Burton and Weiss, 2010). Yet, the future search for more bnAbs might be facilitated by a recent novel selection technique to isolate bnAbs (Wu et al., 2010) and by information obtained from determined crystal structures of already identified bnAbs (Burton and Weiss, 2010; Zhou et al., 2010).

A few regions on gp120 have been implicated in broad antibody neutralisation and occasionally monoclonal antibodies (mAbs), obtained from individual B cells, are identified with specificity for any of these regions (see Figure 1.12). For a long time IgGb12 (B12) that binds to the **highly conserved CD4bs**, was the most broad and potent bnAb that was identified (Burton and Weiss, 2010; Zhou et al., 2007). Another nAb that was identified a long time ago, 2G12, also binds to the outer domain of gp120, but recognises a conserved collection of **oligomannose residues** on Env gp120 (Trkola et al., 1996). mAbs 19b and 447-52d were found to bind to the **V3 loops** on Env, a region that was originally associated with non-broad HIV-1 neutralisation, but does show some limited conservation between viral isolates (Cardoso et al., 2005; Ofek et al., 2004; Pejchal et al., 2009). Furthermore, an epitope region was identified on gp120 that overlaps the coreceptor-binding site

and is only exposed upon CD4-binding, this epitope is therefore called the **CD4-induced** (CD4i) binding site and antibodies known to be specific for this region include mAb X5, 412D, 4.8D, and 17B (Choe et al., 2003; Moulard et al., 2002; Thali et al., 1993). However, these mAbs are weakly or non-neutralising as they have limited access to the native Env spike on the virion. Three other early-identified bnAbs 2F5, 4E10, and Z13e1 in contrast bind to the membrane-proximal external region (**MPER) on Env gp41.** Reported crystal structures of these MPER-binding bnABs showed that 2F5 binds to an extended loop structure, while 4E10 targets a helical conformation, and Z13e1 binds to an elbow in MPER (Cardoso et al., 2005; Ofek et al., 2004; Pejchal et al., 2009). Since 2009 a range of new nmAbs has been identified, of which a selection will be discussed here. Firstly, bnAbs PG9 and PG16 that bind to the gp120 **V loops** were identified by selection of an individual with high cross-neutralising antibody responses and a functional screen of his B cells (Walker et al., 2009). The finding of these Abs suggests that despite the structural variability of these loops, conserved regions are present, that, when targeted by Abs could allow broad neutralisation. PG9 for example avoids the problems of V loop structural variability by binding to the atomic backbone of the protein instead of to the variable side chains, which allows neutralisation of around 80% of HIV-1 strains (Sattentau, 2011). Subsequently, CH01-04 and PGT141 mAbs were isolated and demonstrated to also bind to the V2/V3 loops and PGT121, PGT125, and PGT135 antibodies were demonstrated to have neutralising activity and have V3 loop specificicty (Bonsignori et al., 2011; Walker et al., 2011). Later Corti *et al* identified the new nAb HJ16, while in 2011 nAbs VRC-PG04 and 3BNC60 were isolated that, similar to b12, binds the CD4bs (Corti et al., 2010; Scheid et al., 2011; Wu et al., 2011). The most potent and uniquely broad identified nmAb to date is VRCO1 that also targets the CD4bs and neutralised ~90% of the 200 HIV-1 isolates investigated (Walker et al., 2009; Wu et al., 2010; Zhou et al., 2010).

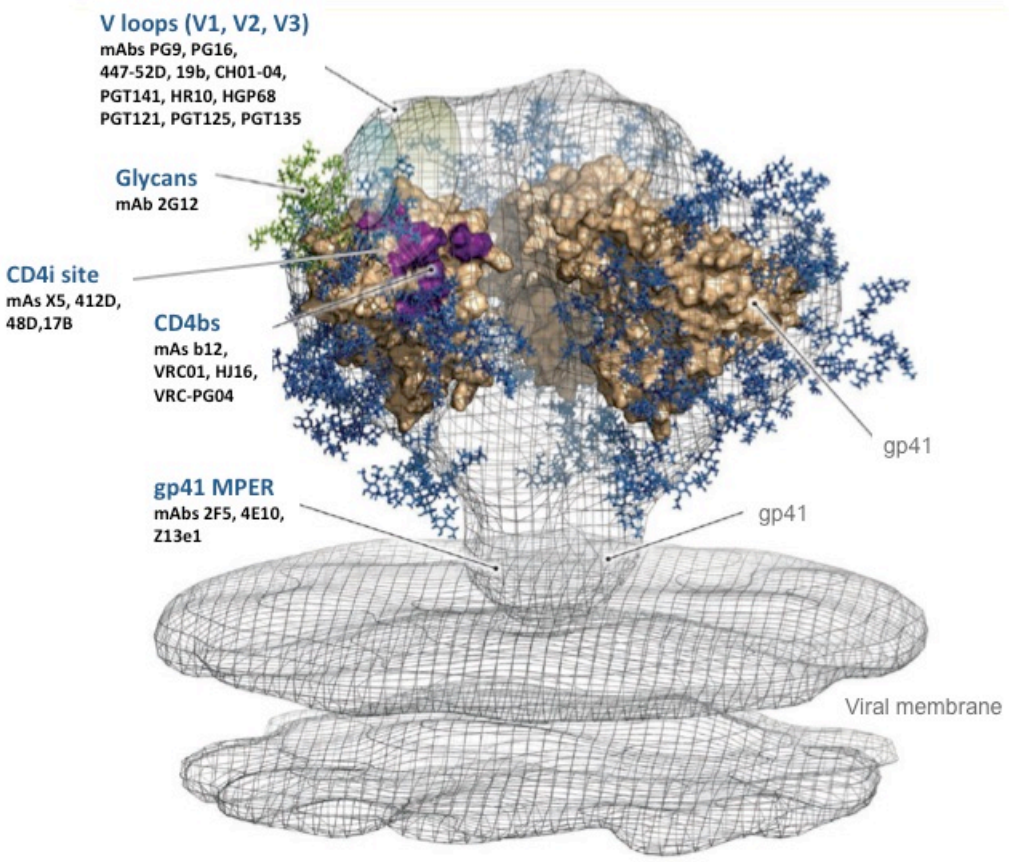


Figure 1.12. Env epitope regions involved in nAb responses to HIV-1 and a selection of nAbs that binds these regions. Adapted from (Burton and Weiss, 2010).

In general, there are numerous challenges in the design of nAb-based HIV-1 vaccines. In addition to obstacles like mutation-driven viral evolution and the conformational flexibility of the HIV-1 Env gp120, the isolated bnmAbs are frequently structurally atypical (Dimitrov et al., 2011). 2G12 has, for example, swapped its heavy chains, leading to a single extended antigen binding surface (Calarese et al., 2003). Other isolated nmAbs were reported to have unusually long heavy chains, sulphated tyrosines, additional disulphide bridges, and N-linked glycosylation in the variable domains amongst other atypical properties (Choe et al., 2003; Pejchal et al., 2010; Saphire et al., 2001; Zhou et al., 2010). Also, a predominant characteristic of nmAbs is an extensive number of

somatic mutations from the germ-line sequences in their variable domains, which suggests a high level of affinity maturation and might explain why nAbs are generally found only at later stages of HIV-1 infection (Chhatbar et al., 2011). One of the attractive templates for vaccine design for example, the CD4bs, has intrinsic complexities that compound the elicitation of Abs with specificity for this site (Sattentau, 2011). A final challenge in the development of a nAb-based HIV-1 vaccine lies in the fact that none of the currently identified 'extremely' broad nAbs is yet capable of neutralising all circulating HIV-1 isolates (Mascola and Montefiori, 2010; Wu et al., 2010).

1.10.2. IgA and non-neutralising antibodies

The first immune responses that were found to correlate with protection in the RV144 trial were IgG and IgA immunoglobulins to the V1/V2 region on Env gp120 (US Military HIV Research Program Briefing 2010, IAVI report, 2011). The presence of systemic IgG antibodies to this region correlated with 43% reduction in infection and for subjects with high IgG antibody responses to the V1/V2 a 71% reduction in infection was observed. Only low neutralisation was observed for highly neutralisation sensitive Tier 1 and neutralisation sensitive Tier 2 viruses in neutralisation assays, suggesting that non-neutralising antibody functions are able to provide modest protection against HIV-1 acquisition (IAVI report, 2011). This finding is in line with recent suggestions and observations in LTNPs, ES cohorts, and NHP models, that ADCC, ADCMI, and other mechanisms of antibody-dependent viral inhibition, despite traditionally thought to be less relevant than neutralising responses, may mediate immune responses to HIV-1 and in this way help control or prevent HIV-1 infection (Baker et al., 2009; Florese et al., 2009; Hidajat et al., 2009; Johnston and Fauci, 2011; Peng et al., 2005; Pulendran and Ahmed, 2011; Rerks-Ngarm et al., 2009; Stratov et al., 2008). Furthermore, a recent study by Hessel *et* al. showed that mutation of the Fc region of mAb B12 disrupting FcR binding, before passive immunisation with this mAb in the NHP model, resulted in a marked decrease in the level of

protection (Hessell et al., 2007). These observations have recently shifted some of the focus of antibody-based vaccine development away from solely bnAb-based vaccination towards so-called broadly binding Ab vaccines (Figure 1.13) (Neutra and Kozlowski, 2006).

Surprisingly, IgA serum antibody responses to the V1/V2 region correlated positively with infection rate. The presence of systemic IgA antibodies to this region was correlated with 54% increase in infection, suggesting that these antibodies interfere with immune responses against HIV-1 infection. One hypothesis is that serum IgA antibodies might block IgG-mediated ADCC through competitive antagonism, similar to previous observations in cancer studies (IAVI report, 2011).

In the RV144 trial, no mucosal samples were collected so no information was provided as to the possible role of mucosal antibodies. IgA antibodies are in contrast with other antibodies, resistant to degradation by mucosal proteases, allowing them to be a key player in mucosal immunity (Neutra and Kozlowski, 2006). Furthermore, IgA antibodies at the mucosa are dimeric in contrast with monomeric serum IgA antibodies, and have been found to be involved in a multitude of immune effector mechanisms, including entrapment of pathogens in the mucous, agglutination, blocking epithelial attachment, and ADCC (Black et al., 1996; Lamm, 1997; Neutra and Kozlowski, 2006; van Egmond et al., 2001). IgG antibodies at the mucosa can also mediate neutralisation and blocking of pathogens, but are thought to be a minor player compared to IgA and most studies have focused on mucosal IgA. In ESN studies for example, the presence of mucosal IgA antibodies correlated with resistance to sexually transmitted HIV-1 infection (Hirbod et al., 2008; Kaul et al., 2001; Mazzoli et al., 1997). Furthermore, dimeric IgA and IgM antibodies to a conserved gp41 epitope, as well as purified IgA from cervicovaginal and saliva samples, have been reported to block HIV-1 transcytosis (Bomsel et al., 1998; Devito et al., 2000). Although mucosal immunisation and mucosal antibody responses have not been extensively investigated yet, initial results in mice and NHP studies suggested that mucosal antibody responses may be

important in protection against HIV-1/SIV acquisition (Bertley et al., 2004; Fuller et al., 2002; Malkevitch and Robert-Guroff, 2004; Veazey et al., 2003). An effective HIV-1 vaccine should therefore generate a rapidly responding resident mucosal population of B cells and neutralising and non-neutralising antibodies to quickly remove, or reduce infectivity of, the entering virus (Haase, 2010).

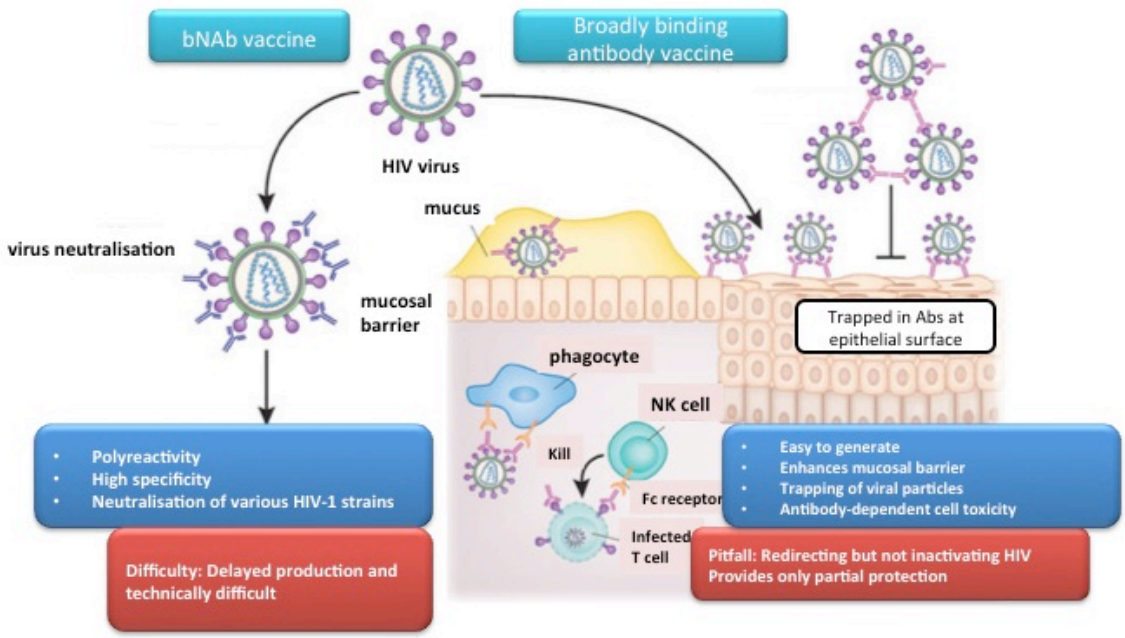


Figure 1.13. Neutralising and non-neutralising functions of (mucosal) antibodies. Recent studies have confirmed the importance of non-neutralising antibody mechanisms in viral inhibition and protection, which could be used in a broadly binding antibody vaccine approach. Adapted from (Neutra and Kozlowski, 2006).

1.10.3. Cellular immunity

In contrast to the Step trial, based upon elicitation of $CD8^+$ T cell-mediated immunity, that failed to show any protection (Table 1.4), the RV144 trial did not induce any substantial $CD8^+$ T cell responses (Buchbinder et al., 2008; Johnston and Fauci, 2011; McElrath et al., 2008; Rerks-Ngarm et al., 2009). In contrast, mainly $CD4^+$ T cell responses were found in the RV144 trial, re-emphasising the importance of humoral responses in the observed modest protection (IAVI report, 2011). HIV-1 specific $CD4^+$ T cell responses have also been detected in ES cohorts (Eyeson et al., 2003; Kebba et al., 2004). Nevertheless, it is generally assumed, based on various results from studies on ESNs, LTNPs and NHP models, that the induction HIV-1 specific $CD8^+$ T cell responses will be needed in an effective HIV-1 vaccination strategy (Belyakov et al., 1998a; Johnston and Fauci, 2011). In ESNs HIV-1 specific $CD8^+$ T cells have for example been associated with resistance to sexual HIV-1 transmission (Betts et al., 2006; Chun et al., 2001; Kaul et al., 2001; Mazzoli et al., 1997). Similarly, studies have suggested that $CD8^+$ T cells of LTNPs might be superior to those of HIV-infected progressors in proliferation, cytolytic ability, cytokine secreting capacity and/or ability to respond to varying HIV-1 isolates (Bailey et al., 2006; Betts et al., 2006; Migueles et al., 2002). Furthermore, NHP models have shown a role for $CD8^+$ T cells in the control of SIV replication (Acierno et al., 2006; McMichael, 2006). In such studies, mucosal presence, targeting of conserved epitopes, polyfunctionality and high avidity of $CD8^+$ T cell responses were found to play an important role (Barouch et al., 2002; Belyakov et al., 1998b; Belyakov et al., 2001; Belyakov et al., 2007; Bertley et al., 2004; Borrow et al., 1997; Fuller et al., 2002; Kuznetsov et al., 2004; Makitalo et al., 2004; Malkevitch and Robert-Guroff, 2004; McMichael, 2006; Veazey et al., 2003; Sankaran et al., 2005) . Therefore, even though cytotoxic T cells (CTLs) cannot prevent HIV-1 entry they might be crucial for the containment or clearance of HIV-1 infection and an ideal vaccine should therefore probably, in addition to potent antibody responses, induce (mucosal) high avidity $CD8^+$ T cell responses (Belyakov et al., 2007; Duerr, 2010).

1.10.4. Other factors

In addition to the above-mentioned possible correlates of protection, LTNP studies have found other factors that could potentially play a role in the slow progression of these HIV^+ individuals, such as host cellular restriction factors in innate immunity, including APOBEC3G, a member of the apoliprotein B editing complex (APOBEC) cellular deaminase family that inhibits HIV replication, and TRIM-5**α**, a member of the tripartite protein family that triggers destruction of the HIV-1 capsid (Strebel et al., 2009). Other factors in immune-mediated HIV-1 control in LTNPs include increased survival and functioning of $CD4^+$ T cells and a possible role for HLA molecules on NK cells that increases NK-mediated killing of infected cells (Baker et al., 2009). The role of complement in HIV-1 infection remains controversial, as some *in vitro* studies have found complement-mediated enhancing effects on HIV-1 infection and mechanisms by which HIV-1 uses complement activation to its benefit, while others reported complement-mediated inhibition (Banki et al., 2005; Belyakov et al., 2008; Huber and Trkola, 2007). Results in infected patients and vaccinees, including the observation that control of viraemia correlates with complement activity during acute infection, and that antiviral activity of antibodies can be enhanced in the presence of complement, possibly via ADCMI, have recently indicated that complement does probably contribute to control of and protection against HIV-1 infection (Aasa-Chapman et al., 2005; Huber et al., 2006; Sheppard et al., 2007).

1.10.5. Considerations about immune correlates of protection

When interpreting the immune factors observed in LTNPs, ES individuals, and human or NHP vaccine trials, it is important to take into account that the use of ES studies in the search for correlates of protection against HIV-1 infection, should be met with caution, since a proportion of these individuals do become infected and seroconvert over time, albeit at a slow rate. In addition, no clear correlate of protection can be defined in the ES group of individuals due to the differences between ES individuals and the weakness of the responses

(Hladik et al., 2003; Sheppard and Sattentau, 2005), the question sometimes arises as to whether these ES individuals are just representing a group of less susceptible individuals in a normally distributed susceptibility to infection instead of being a distinct group of 'protected' individuals. Immune responses in ES individuals might result from exposure to non-functioning virions or low-level infection. Because of this concern, vaccine trials and to a lesser extent LTNPs provide better models for the search for correlates of protection. Additionally, natural non-pathogenic SIV infection in NHP can be studied to better understand AIDS progression and protection against infection. These natural hosts of SIV infection, remain healthy after infection, have intact $CD4^+$ T cell numbers, and do not progress to present AIDS-like symptoms, despite a high and persistent viral load (Sheppard and Sattentau, 2005; Silvestri et al., 2003). Identified potential factors implicated in the lack of disease progression in these animals, include absence of gut bacteria translocation, $CD4^+$ T cell hyporesponsiveness, which is also observed in some ES cohorts (Jennes et al., 2006; Nicastri et al., 1999), and attenuated immune activation, which probably avoids the immune-mediated damage present in pathogenic SIV infection (Brenchley et al., 2006b; Sheppard and Sattentau, 2005; Silvestri et al., 2003; Staprans et al., 2004). In addition to providing information on possible factors involved in AIDS pathology, these findings therefore also caution the field of HIV-1 vaccine development that suboptimal immunisation might do more harm than good (Sheppard and Sattentau, 2005).

1.11. HIV-1 vaccination strategies

Vaccination strategies have successfully been developed for protection against a range of infections. While older vaccination methods were based on live attenuated or inactivated virus, more recently the use of subunit vaccines has become more popular because of its increased safety. Successful live-attenuated vaccines include chickenpox, oral polio, measles, mumps, rubella and varicella-zoster vaccines. Examples of inactivated vaccines

are hepatitis A and Salk polio vaccines. Hepatitis B and papilloma vaccines are based on subunit proteins (Ulmer et al., 2006).

Despite the enormous success that the above strategies have had in protection against a wide variety of human viral infections, the achievements in the context of HIV-1 vaccination have been very limited and only a handful of early trials have successfully progressed to Phase IIb/III (Table 1.4). Nevertheless, since the first phase-I clinical trial was conducted in 1987 with a gp160 subunit HIV-1 vaccine candidate, a multitude of Phase I/II trials has been conducted including more than 40 candidate vaccines based on various vaccination strategies that all have advantages and shortcomings (Table 1.5) (Mascola and Montefiori, 2010; Ross et al., 2010). Initially, prophylactic HIV-1 vaccine approaches attempted to elicit nAbs that should neutralise HIV-1 virions before infection of target cells can occur, but later vaccine candidates were developed to test the impact of CTL responses upon viral load and disease progression. Current approaches generally attempt to induce all three of the following: 1) neutralising humoral, 2) non-neutralising humoral, and 3) cellular immunity. The clade origin of the antigens in clinical trials does generally not well reflect their global prevalence. Clade B antigens were in 2006 for example tested in approximately 78% of clinical trials, either alone or in combination with antigens belonging to other clades, whereas clade B in that year only accounted for approximately 10% of global HIV-1 infections (Hemelaar et al., 2006; IAVI, 2006). In currently tested strategies, clade B is still relatively overrepresented. In the following paragraphs the major HIV-1 vaccination strategies will be outlined.

Vaccine type	**Clinical trial code (location)**	**Results**
Gp120 (B/B or B/E) + Alum	**VAX003** (USA) **VAX004** (Thailand)	No protective efficacy observed
Ad5-HIV-1 trivalent vaccine (Gag, Pol, Nef)	**Step** (USA, Australia) **Phambili** (South Africa)	No protective efficacy observed, enhancement of infection rate in uncircumcised Ad5-seropositive male volunteers
Canarypox (ALVAC)-Gag, Pol, Env E + gp120 B/E + Alum	**RV144** (Thailand)	31% protective efficacy against HIV-1 acquisition
DNA-Gag, Pol, Env priming + Ad5-Gag, Pol, Env (A,B,C) boost	**HVTN505** (USA)	On-going phase IIb trial

Table 1.4. Overview of Phase IIb/III HIV-1 vaccine efficacy trials. Adapted from (Girard et al., 2011)

Vaccination approach	**Features and limitations**
Live attenuated vaccines	Establish persistent viral infection with unknown long-term consequences.
Inactivated whole virus or virus-like particles (VLPs)	Low immunogenicity, limited nAb responses, no protection in NHP model.
Subunit Env vaccines + adjuvants	Improved but limited nAb responses, homologous protection in NHP model.
Subunit Tat and/or Nef vaccines + adjuvants	Protection in some NHP models.
DNA vaccines	Elicit mostly $CD4^+$ T cell responses, need at least three immunisations for full potency, limited potency if not combined with other strategy.
Live recombinant (vectored) vaccines	Elicit $CD8^+$ > $CD4^+$ T cell responses, limited immunogenicity on its own
Prime-boost regimens	Promising, live recombinant vectored vaccine combined with DNA priming induces protection in NHP model. Gp120 subunit vaccine with live recombinant (vectored) vaccine was able to induce some protection in humans.
Mucosal immunisation	Promising. Possibly providing the way to achieve efficacious vaccination through enhancement immunity and protection at the desired location: the site of HIV-1 entry. Mucosa provides window of opportunity when small, non-diverse founder HIV-1 population enters the body, but immunisation via this route has not yet been adequately investigated.

Table 1.5. Overview of the main HIV-1 vaccination strategies and their features. Adapted from (Girard et al., 2011).

1.11.1. Live attenuated vaccines

The most promising results with HIV-1 vaccination so far have been obtained by the immunisation of macaques with live-attenuated viruses (Koff et al., 2006; Nilsson et al., 1998; Tenner-Racz et al., 2004). Nevertheless, this approach has been abandoned because its safety cannot be guaranteed (Berkhout, 2011). Most studies on live attenuated HIV-1 vaccines have been carried out with macaques that are infected with SIV. Live attenuated SIV strains have been developed through deletion of individual or combinations of SIV genes or gene regions, and through administration of ART immediately following wild-type SIV vaccination (Berkhout et al., 2011; Cole et al., 2004; Johnson and Desrosiers, 1998; Wijesundara et al., 2011; Wodarz et al., 2000). Early studies in this NHP model demonstrated that *nef*-deleted SIV vaccination induces full protection against later SIV challenge (Girard et al., 2011). However, in most cases macaques were not protected against superinfection, which is infection with a second strain of virus (Girard et al., 2011). Also, the attenuated SIV was found to induce a persistent infection that in the context of spontaneous virus evolution through error-prone replication machinery resulting in virus mutations and virus diversity can result in regain of pathogenicity (Berkhout et al., 1999). In vaccinated macaques, the attenuated SIV virus indeed regained virulence and induced disease in vaccinated macaques (Baba et al., 1995; Baba et al., 1999; Chakrabarti et al., 2003; Hofmann-Lehmann et al., 2003; Whatmore et al., 1995). Also, a group of LTNPs infected with a naturally attenuated HIV-1 version eventually developed AIDS (Churchill et al., 2006). Furthermore, in long-term cell culture infections an HIV-1 Δ3 variant with deletions in the *vpr, nef*, and long terminal repeat (LTR) regions gained compensatory changes in the viral genome that eventually caused a recovery of replication capacity (Berkhout et al., 1999). These studies re-emphasise the genetic instability and evolutionary capacity of attenuated HIV/SIV variants, which pose a safety risk on the use of this strategy.

1.11.2. Inactivated vaccines and virus-like particles (VLPs)

Similar to live attenuated HIV-1 vaccination, the use of whole inactivated HIV-1 vaccination has predominantly been investigated in macaque SIV infection models. SIV vaccines were developed by virus inactivation through mild oxidation with aldithriol-2 to remove important Zn^{2+} ions from Gag and Int proteins, alkylation with N-ethyleneimine, UV inactivation of viral RNA, heat-inactivation, formalin-inactivation and/or detergent disruption (Desrosiers et al., 1989; Poon et al., 2005). Early studies showed that whole inactivated SIV vaccination can induce nAbs and can protect macaques against live SIV challenge (Desrosiers et al., 1989; Murphey-Corb et al., 1989). However, the whole inactivated HIV-1 vaccination approach was eventually abandoned when the observed SIV protection was found to result from immune responses against the HLA from the human cell lines in which SIV was grown instead of against SIV itself (Stott, 1991).

When co-expressed in cell cultures the HIV or SIV Gag and Env proteins can spontaneously form virus-like particles (VLPs) that are composed of only the viral core and Env proteins (Doan et al., 2005). VLPs have been studied as vaccine candidates in NHP models, but were found to be poorly immunogenic on their own, even when gp41 sequence or adjuvant was included (Buonaguro et al., 2007; Kim et al., 2007). Currently, variations of VLPs are under investigation for their potential use in HIV-1 vaccination, including consensus Env sequencing containing VLPs and hybrid VLPs, but so far none of them has shown promising immunogenicity (Phogat et al., 2008; Zhang et al., 2004).

1.11.3. DNA vaccines

DNA vaccination was first developed in the 1990s with its first application being a HIV-1 candidate vaccine (MacGregor et al., 1998). DNA vaccination is based on bacterial plasmids that express the antigen *in situ*, which induces T_h1 immune responses (Wang et al., 1995; Wang et al., 1993). The strategy rapidly gained popularity because of its favourable characteristics being relatively cheap, easy to manufacture, and enabling the development of a prolonged immune response against the encoded antigens (Girard et al., 2011). Nevertheless, prophylactic HIV-1 DNA vaccines have had limited success. HIV-1 DNA vaccine candidates have repeatedly failed to elicit strong protective immunity in humans and require multiple boosting, possibly due to inadequate uptake of the DNA vectors by host cells or due to insufficient amounts of antigen expressed from the vaccines (Ranasinghe and Ramshaw, 2009).

Improvement of DNA vaccination potency has been achieved through novel means of vaccine delivery, including electroporation, optimised codons, and co-expression of cytokines (Barouch et al., 2000; Giri et al., 2004; Graham et al., 2006; Hirao et al., 2008; Kutzler et al., 2005). Optimised plasmid DNAs that express most of the SIV proteins have been able to induce strong cellular and humoral immunity and reduced viraemia following SIV_{mac} challenge in macaques (van Drunen Littel-van den Hurk and Hannaman, 2010). Nevertheless, prophylactic DNA vaccination alone generally remains poorly immunogenic.

1.11.4. Live recombinant (vectored) vaccines

Live recombinant vaccines consist of a live viral or bacterial vector that is modified to express the SIV or HIV antigens (Girard et al., 2011). The antigens are expressed inside target cells where they are eventually degraded by the proteosome and expressed via the MHC class I pathway, resulting in a $CD8^+$ T cell response, similar to DNA vaccines (Girard et al., 2011). Alternatively, antigens can be presented by phagocytes to CTLs via cross-

presentation when apoptotic target cells are phagocytosed. The immunogenicity depends generally on the choice of HIV vaccine inserts, the best vaccines expressing numerous HIV-1 proteins including Env (Amara et al., 2002; Letvin, 2005).

A variety of live viral vectors have been studied in human HIV-1 vaccination, predominantly Poxvirus and Adenovirus vectors. The majority of these vectors have been converted into replication-defective vectors by deletion of essential genes, for example the E1A gene in Adenoviruses. Other vectors are intrinsically incapable of replication in primates, such as Canarypox (ALVAC) and Fowlpox, and the mammalian poxvirus strain 'Modified Vaccinia Ankara' (MVA), and adenovirus vectors (including adenovirus serotype 5 (Ad5). Replication defectiveness improves safety, but negatively affects immunogenicity, resulting in a need for higher doses of vector.

Most of the vectored vaccines have only been tested in animal models and have failed to show promising results (Girard et al., 2011). One candidate however, a replication incompetent recombinant Ad5 vector that encoded the Gag protein showed protection in a SHIV model (Shiver et al., 2002), but later investigation in a human clinical trial (Step trial, see Table 1.4) reminded the field that NHP models are not always good predictors of success in human vaccination and that HIV-1 T cell vaccines tend to generally not elicit the same level of immunity in humans as in NHP models (Graham et al., 2006).

The Step trial was conducted, following the above success in the SHIV model, to test the efficacy of a Merck HIV-1 vaccine candidate based on an Ad5 vector encoding HIV-1 Gag, pol and nef proteins (Buchbinder et al., 2008). Surprisingly, vaccine recipients in this human Phase IIb trial were found to be more susceptible to HIV-1 infection than placebo recipients. As a result, the Step trial as well as the equivalent Phambilii trial in

South Africa (Table 1.4) were suspended. Subsequent studies have indicated the role of male circumcision and pre-existing antibodies against Ad5 in the outcome of this trial (Gray et al., 2010).

The trial confirmed that immune responses to the vectors and potential pre-existing immunity to the vector provide a daunting challenge to vectored vaccine strategy. Similarly, repeated immunisations with the same vector can limit the efficacy of live recombinant vectored vaccines (McCoy et al., 2007; Sauter et al., 2005).

Adenovirus vectors to which fewer people have pre-existing immunity have been investigated to partially tackle this problem. Most promising are the Ad26 and Ad35 vectors that have been engineered to express mosaic antigens, which should broaden the induced immune response to account for the diversity in circulating HIV-1 strains (Barouch et al., 2010; Corey and McElrath, 2010; Kong et al., 2009; Santra et al., 2010), but both vectors have yet only been investigated in Phase I trials, where they were shown to be safe and immunogenic (IPCAVD 001 and IAVI B001 respectively) (Girard et al., 2011).

1.11.5. Subunit vaccines

As an answer to safety problems observed in classic live attenuated and whole inactivated virus vaccines, the vaccination field in general has increasingly moved to the use of subunit vaccination strategies. These are particularly good at eliciting antibody and $CD4^+$ T helper responses, but generally less good at generating $CD8^+$ T cell responses (Girard et al., 2011). In the context of HIV-1 vaccination, recombinant Env immunogens are the most attractive antigen candidates, because they are the only target of nAb responses, which are likely to account for the success of most human antiviral vaccines (Amanna et al., 2007). Env immunogens have therefore received most attention within the development of subunit vaccines as a safer

approach for HIV-1 vaccination, but other subunit vaccination approaches, including Tat-based vaccines, have also been investigated (Girard et al., 2011).

1.11.5.1. Native Env molecules

Initial HIV-1 protein subunit vaccines were based on soluble forms of monomeric Env (Mooij et al., 1998) gp160, gp140 or gp120 formulated in Alum (Belshe et al., 1993; Dolin et al., 1991; Keefer et al., 1994). In NHP models these subunit vaccines were reported to induce predominantly nAbs directed at the hypervariable V3 loop in Env gp120 resulting in protection against homologous SIV/SHIV, but failure to protect against heterologous SIV/SHIV challenge (Berman et al., 1990; Berman et al., 1996; Fultz et al., 1992; Girard et al., 1991; Girard et al., 1995; Stott et al., 1998). Despite this lack of success, a phase II trial of a gp120 candidate vaccine was conducted, followed by Phase III efficacy trial by VaxGen (VAX003, Table 1.4) involving more than 5000 volunteers at high risk of HIV-1 infection in the USA, Canada and the Netherlands. The vaccine, based on two clade B recombinant gp120s (MN and GNE8), induced homologous nAb responses to HIV-1 MN and SF162 but failed to trigger nAbs against other Tier 1 and Tier 2 HIV-1 isolates (Flynn et al., 2005; Gilbert et al., 2010; Gilbert et al., 2005b). No statistically significant reduction in HIV-1 acquisition was observed for the vaccinee group (Gilbert et al., 2005b). A similar trial in Thailand, VAX004, involving more than 2000 drug users and a clade B/E mixed recombinant gp120 candidate vaccine similarly failed to show a statistically significant reduction of HIV-1 infection in the vaccinee group (Table 1.4) (Flynn et al., 2005; Gilbert et al., 2005a; Gilbert et al., 2005b; Pitisuttithum et al., 2006).

1.11.5.2. Modified Env molecules

Studies have later confirmed that the neutralisation capacity of nAbs correlates with their ability to interact with trimeric Env spikes instead of with monomeric gp120 (Fouts et al., 1997; Parren and Burton, 2001; Sattentau and Moore, 1995). Subsequently, several strategies have been attempted to design HIV-1 vaccine immunogens that resemble the HIV-1 Env trimeric glycoprotein including gp140 stabilised in trimeric formation. Additional optimisation strategies have also been explored, including Env cocktails, mosaic antigens, Env-CD4 complexes, Env deglycosylation and Env hyperglycosylation. Despite technical challenges in production of HIV-1 Env trimers and the fact that none of the resulting immunogens has achieved bNAbs of sufficient breadth and potency, some of the above approaches have successfully created immunogens that are at least partially superior to monomeric gp120 at inducing nAb responses.

The formation of trimeric gp140 (that consist of gp120 plus the ectodomain of gp41) molecules can be achieved through various approaches. In some approaches, cleavage of the gp140 occurs, but the molecules can be stabilised through the addition of disulphide bonds between gp41 and gp120 (Schulke et al., 2002). These so-called SOS proteins elicited strong homologous, but weak heterologous neutralisation responses in rabbits that were immunised with a SOS-encoding DNA vaccine, followed by a soluble SOS glycoprotein subunit immunogen boost (Beddows et al., 2005). Others are naturally uncleaved or do not cleave as a result of modification. Although some of these forms are naturally stable in trimeric form (Beddows et al., 2005; Jeffs et al., 2004), others need to be stabilised, which can be also be achieved in various ways. Firstly, trimeric gp140 forms have been engineered that are stabilised through fusion with a C-terminal, trimeric GCN4 motif (Yang et al., 2000; Yang et al., 2002). Further optimisation of these gp140 trimers has been attempted by deletion of a 30 amino acid sequence from the V2 loop to better expose neutralisation epitopes (Barnett et al., 2010; Srivastava et al., 2003a; Srivastava et al., 2003b). These ΔV2 gp140 trimers induced strong Env-specific antibody responses in, for example, the NHP SHIV model when formulated in MF59 adjuvant (Section 1.11.)

and heterologous nAb responses were limited but improved compared to unmodified gp140 immunogen (Barnett et al., 2008; Li et al., 2006; Lian et al., 2005; Xu et al., 2006). Other approaches have attempted to stabilise Env in trimeric form through deletion of the furin cleavage site, or through the incorporation of trimerization motifs into the gp41 subunit (Phogat and Wyatt, 2007; Sanders et al., 2002). In general, modestly higher neutralisation responses have been achieved through these modifications and the formation of trimeric Env immunogens, but the desired breadth and neutralisation potency has not yet been achieved (Earl et al., 2001; Grundner et al., 2005; Kim et al., 2005). In contrast to these findings, Jeffs' group showed that oligomerisation is isolate dependent, but demonstrated that extensive modifications are not always necessary to produce stable trimeric HIV-1 gp140 (Jeffs et al., 2004). As such, trimeric CN54 gp140, used for the research discussed in this thesis, was shown to be stable in the absence of modifications (Jeffs et al., 2004).

Since CD4 binding to Env gp120 induces the formation and exposure of certain neutralisation epitopes that overlap the co-receptor binding site, another approach has attempted to trigger a similar conformation through cross-linking of CD4 to gp120/gp140 proteins (Devico et al., 1996; Fouts et al., 2000). In macaques immunisation with such a CD4-crosslinked Env immunogen was shown to elicit antibodies to CD4i epitopes that correlated with control of SHIV replication (DeVico et al., 2007). Deglycosylation and hyperglycosylation of Env immunogens has also been performed in attempts to improve Env immunogenicity. In the first case, glycosylation was removed to improve exposure of critical neutralising epitopes. Indeed, these deglycosylated viruses were found to be more susceptible to neutralisation by CD4bs antibodies (Malenbaum et al., 2000). In the second case, glycans were added to mask non-neutralising epitopes in an attempt to reduce the immunogenicity of these non-neutralising regions and to focus the antibody response to critical conserved epitopes. Pantophlet *et* al for example described the design of a hyperglycosylated Env gp120 immunogen that could still bind mAb B12, but was not recognised by various non-neutralising or weakly neutralising antibodies

(Pantophlet et al., 2003). However, the use of this hyperglycosylated gp120 immunogen did not improve the breadth of neutralisation response elicited in a rabbit immunisation model (Selvarajah et al., 2005).

Furthermore, cocktails of Env immunogens that belong to distinct HIV-1 clades, for example A, B, and C, have been prepared as a multivalent approach to improve the induction of bnAbs (Hurwitz et al., 2005; Kong et al., 2003; Lu et al., 2010; Nabel, 2005; Rollman et al., 2004; Seaman et al., 2005). Some improvement in the breadth of the neutralisation response was observed with these cocktail immunogens, but this was a result of the additive effect of the induced nAbs, while the breadth of individual nAbs was not improved (Chakrabarti et al., 2005). Moreover, synthetic genes have been derived from theoretically designed consensus sequences of HIV-1 gp120 and have been reported to be slightly more potent inducers of broad cross-subtype T and B cell responses and bnAbs (Gao et al., 2007; Gao et al., 2005; Liao et al., 2006; Thomson et al., 2005). Furthermore, mosaic antigens, which are genes that are biometrically designed based on in silico algorithms that optimise overlap between vaccine sequences and worldwide HIV-1 circulating isolate sequences have been found to improve the diversity of immune responses (Corey and McElrath, 2010; Fischer et al., 2007), and this approach is being applied to the design of Env-based antigens.

Identified bnmAbs provide information about 'vulnerable sites' on gp120 that need to be conserved and cannot undergo substantial mutational changes without negatively impacting on Env function. Ideally, a vaccine would target these sites since they correlate with broad neutralisation. In an approach called 'reverse vaccinology', minimal natural antigen identified bnmAb are mimicked and immunogens are designed based on these mimics (McGaughey et al., 2004; Rutz et al., 2004; Walker and Burton, 2010; Wang et al., 2004). Whether this approach can result in the design of potent immunogens capable of inducing broad neutralising immunity, still needs to be established.

In summary, Env immunogens have undergone multiple modifications that have successfully enhanced its resemblance of native HIV-1 Env spikes and increased its immunogenicity. Nevertheless, adequate heterologous protection has not been observed when Env subunit vaccination is used on its own, and the quest remains to find an optimal formulation, adjuvant, or delivery method to achieve the desired robust heterologous antibody responses and protection.

1.11.5.3. Tat-based vaccines

Another HIV-1 subunit vaccination strategy involves Tat protein in an attempt to contain HIV-1 infection by eliciting immunity to a protein that is expressed early in the replication cycle (Caputo et al., 2004; Goldstein, 1996). The usefulness of this strategy remains controversial with some NHP studies reporting little or no protection against SIV_{mac239} or SHIV challenge (Allen et al., 2002; Goldstein et al., 2000), while others showed containment of infection (Cafaro et al., 1999; Maggiorella et al., 2004; Pauza et al., 2000). A possible reason for this discordance could be uncontrolled innate immunity genetic factors in the host monkeys. Following a Phase-I trial that confirmed safety and immunogenicity in humans, a Phase II has been started in drug-treated HIV-infected volunteers. Furthermore, a candidate vaccine based on a Tat-Nef fusion protein mixed with gp120 subunit vaccine has been investigated in various adjuvants in NHP models (Voss et al., 2003) and was shown to induce strong and durable $CD4^+$ T cell immunity in human volunteers in a Phase I trial (Leroux-Roels et al., 2010) indicating that combining Tat vaccines with Env subunit vaccination might provide a way to improve vaccine potency (Caputo et al., 2008; Caputo et al., 2009).

1.11.6. Heterologous prime-boost immunisation regimens

Whilst HIV-1 vaccine development has shifted its focus away from stimulation of individual arms of the immune system towards the current goal to activate both humoral and cellular arms of immunity, the idea of heterologous prime-boost regimens has entered the scene. In general it is found that combination of different strategies in a prime-boost regimen increases overall immunogenicity and observed protection in various model systems. As such, DNA vaccines are generally more potent as priming strategies when combined with another approach and the immunogenicity of VLPs can be substantially improved when used in combination with a DNA prime (Buonaguro et al., 2007; Casimiro et al., 2005; Girard et al., 2011; Kawada et al., 2007; McDermott et al., 2005; Santra et al., 2008; Schell et al., 2009; Suh et al., 2006). Also the efficacy of live recombinant (vectored) vaccines and subunit vaccines can be optimised through heterologous prime-boost regimens, of which the best example are probably the clinical trials leading up to the Phase III RV144 trial. In the RV144 trial two doses of a non-replicating Canarypox vector, encoding clade B Gag/Pro and CRF01_AE Env antigens (ALVAC-HIV vCP1521 from Sanofi Pasteur), were administered and two doses of the same ALVAC component with subtype B and CRF_AE recombinant gp120s (AIDSVAX equivalent to the VAX004 trial, Table 1.4). The fact that 31% protection was observed, (and 61% protection after 1 year) while the separate components, ALVAC and AIDSVAX, had failed to show strong immunogenicity and efficacy when they were individually tested before, indicates the potential of heterologous prime-boost regimens. For this reason, additional clinical trials and extensions of the RV144 are currently planned (Girard et al., 2011). Also, a Phase IIb trial of DNA/Ad5 vaccine candidate is currently underway involving volunteers who do not have pre-existing immunity against Ad5 (Table 1.4).

1.11.7. Novel vaccination strategies

In addition to these well studied HIV-1 vaccination strategies new approaches are constantly being developed and tested, including so-called single-cycle SIV (scSIV) viruses and conditionally replicating SIV vaccines as a safer alternative for live attenuated and whole inactivated virus vaccination, novel lipopeptide vaccination strategies that were shown to be immunogenic and are in clinical development now, and an approach to prevent $CD4^+$ T cell loss that uses administration of a conserved peptide sequence of gp141 (SWSNKS) that induces NKp44L expression, which is found to negatively correlate with $CD4^+$ T cell loss, and is now investigated in a Phase I clinical trial (De Groot et al., 2005; Durier et al., 2006; Gahery et al., 2005; Kuate et al., 2003; Liu and Chen, 2004; Vieillard et al., 2008; Vieillard et al., 2005).

1.11.8. Mucosal HIV-1 immunisation

Throughout this introductory chapter the importance of local HIV-1 specific mucosal immune responses for effective vaccination to help prevent HIV-1 viral acquisition and/or dissemination of infection has been outlined (Azizi et al., 2010; Belyakov and Ahlers, 2008; Chen and Cerutti, 2010). Although systemic delivery of HIV-1 vaccines can induce mucosal responses to a certain extent (Baig et al., 2002; Fuller et al., 2002; Lamm, 1997; Levine, 2000; Stevceva et al., 2002), it is evident from many studies in mice and NHP that stronger mucosal immunity can be obtained via mucosal immunisation than through systemic administration with the same vaccine formulation (Belyakov et al., 2001; Belyakov et al., 2007; Duerr, 2010; Kuznetsov et al., 2004; McMichael, 2006; Neutra and Kozlowski, 2006). The underlying reason is probably that mucosal homing receptors, including $\boldsymbol{\alpha_4\beta_7}$-integrin and CCR10 are generally not expressed on B cells that are activated in peripheral LNs, causing a general failure to induce mucosal antibody responses following systemic immunisation, while a fraction of mucosally activated B cells generally express $\boldsymbol{\alpha_4\beta_1}$-integrin and leukocyte (L)-selectin, peripheral homing receptors (Kunkel and Butcher, 2003), which allows the induction of systemic IgG

and IgA responses following mucosal immunisation. Furthermore, mucosal DCs can travel to systemic induction sites, such as the spleen and LNs to present antigen at these locations (MacPherson and Liu, 1999). Similarly, $CD8^+$ T cells generally show a preference for residence at sites were antigen was originally encountered, despite relatively unrestricted initial migration patterns, although a few studies have shown that T cells can in some situations be imprinted with the mucosal $\alpha_4\beta_7$-integrin homing receptor outside mucosal inductive sites (Kunkel and Butcher, 2003; Lin et al., 2007; Qimron et al., 2004; Tatsis et al., 2007).

Despite the expected need for mucosal HIV-1 vaccination strategies, only few mucosal HIV-1 vaccine formulations have been tested and mucosal antibody responses have generally not been well studied (Neutra and Kozlowski, 2006). The handful of mucosal NHP studies is nicely reviewed in (Duerr, 2010). Generally, most of the HIV-1 vaccine candidates have been incapable of inducing mucosal IgA responses due to poor immunogenicity and the lack of an appropriate adjuvant (Chhatbar et al., 2011). Yet, mucosal live recombinant (vectored) vaccine priming and gp120 subunit boosting in monophosphoryl lipid A (MPL-A) in macaques was found to result in strong rectal IgA and IgG responses that protected against rectal SIV challenge (Pinczewski et al., 2005; Zhou et al., 2007). Similarly, i.m. priming with i.n. boosting of a gp41-based vaccine resulted in mucosal transcytosis-blocking IgA antibodies that correlated with protection against SHIV challenge in macaques (Bomsel et al., 2011). Furthermore, a combination of systemic and mucosal immunisation with the previously described ΔV2 $gp140_{SF162}$ trimer in MF59 was found to protect rhesus macaques against i.v. challenge with homologous, but not heterologous SHIV (Barnett et al., 2008; Xu et al., 2006). In human, an even more limited number of mucosal HIV-1 vaccines has been tested and none of these appeared effective in the induction of HIV-1 specific mucosal IgA antibodies (Neutra and Kozlowski, 2006). One study looked at the possibility of mucosal prime/systemic boost or the reverse, which is generally thought to be an attractive way for inducing both robust systemic and mucosal immunity (Belyakov and Ahlers, 2008). In this study, human volunteers received mucosal prime immunisation with ALVAC vaccine and i.m. boost immunisation

with gp120 subunit vaccine and vaccinees failed to show strong mucosal antibody responses (Wright et al., 2004). Taken together, this limited number of studies in NHP and human confirms the capacity of mucosal immunisation to induce protection against HIV-1/SHIV infection, but also re-emphasises the need for further investigation and optimisation of mucosal vaccination strategies.

1.12 A general overview of innate and adaptive immunity

Before discussing adjuvants it is important for the subject to be placed into an appropriate immunological context. I will therefore introduce a brief section that describes the function of the innate and adaptive immune systems. This information will be helpful for the subsequent Sections on adjuvant discovery and mechanism of action.

For a long time the innate immune system was considered a less important branch of immunology, compared to the adaptive immune system. Innate immunity was thought to be important in lower organisms, but was assumed to be superseded in importance by the complex adaptive immune system in higher organisms and it was considered only a quick early line of defence, allowing more time for adaptive immunity to develop (Coffman et al., 2010). Over recent years however, it has become increasingly clear that innate immunity plays a crucial role in stimulating and guiding the more slowly developing adaptive immune response (Coffman et al., 2010). This new knowledge has had important implications for vaccine development and has added substantially to our understanding of adjuvant mechanisms (Coffman et al 2010).

At the centre of immunology are Antigen Presenting Cells (APCs) that bridge innate and adaptive immunity, the most important of which are DCs (Steinman et al., 1999). The changed view on innate immunity started with the discovery of the Toll-like receptor (TLR) family and other pattern recognition receptors (PRR) (de Veer and Meeusen, 2011). PRR are expressed by a variety of cells and tissues, including DCs, and are

specialised in recognising pathogen-associated-molecular-patterns (PAMPs). In addition to TLRs, other PPR families have been discovered, such as nucleotide-oligomerisation domain (NOD)-like receptors (NLRs), C-type lectin receptors (CLRs), and retinoic acid-inducible gene I (RIG-I)-like RNA helicases (RLHs) (Bauernfeind et al., 2011; Franchi et al., 2009; Palm and Medzhitov, 2009). PRRs can be present on the cell surface, in the cell cytosol, or in endosomal compartments, and recognise a wide variety of microbial structures as well as their synthetic analogues (Figure 1.14). In addition to PAMPs, some of these receptors, such as NLRs, are also capable of recognising endogenous damage-associated molecular patterns (DAMPs), which are endogenous molecules usually stored inside cells and thus invisible for the immune system, but can be released upon stimulation, such as cell damage or necrosis. Examples include monosodium urate crystals (MSU), double-stranded genomic DNA, and heat-shock proteins (Kono and Rock, 2008; Martinon et al., 2006; Shi et al., 2003).

Recently, the so-called inflammasome pathway was found to respond to particulates and DAMPs, including MSU crystals and ATP, which amongst others, can be released from cells upon necrosis (Kono and Rock, 2008). The inflammasome complex is formed following activation of NLR, such as NLR family pyrin domain containing 3 (NLRP3) and NLR family CARD domain containing 4 (NLRC4). When activated, NLRP3 recruits the adapter molecule ASC, which in turn activates procaspase-1 (Pelka and Latz, 2011). Active caspase 1 induces the cleavage of IL-1**β** preforms into its active form (Martinon et al., 2002). In addition to necrosis-induced DAMP release, this activation can take place upon microbial ligand binding. As such, the NLRP3 inflammasome pathway is one example of an immune activating route that can link cell death and tissue damage to immune activation and an inflammatory response (Coffman et al., 2010; Hornung et al., 2008).

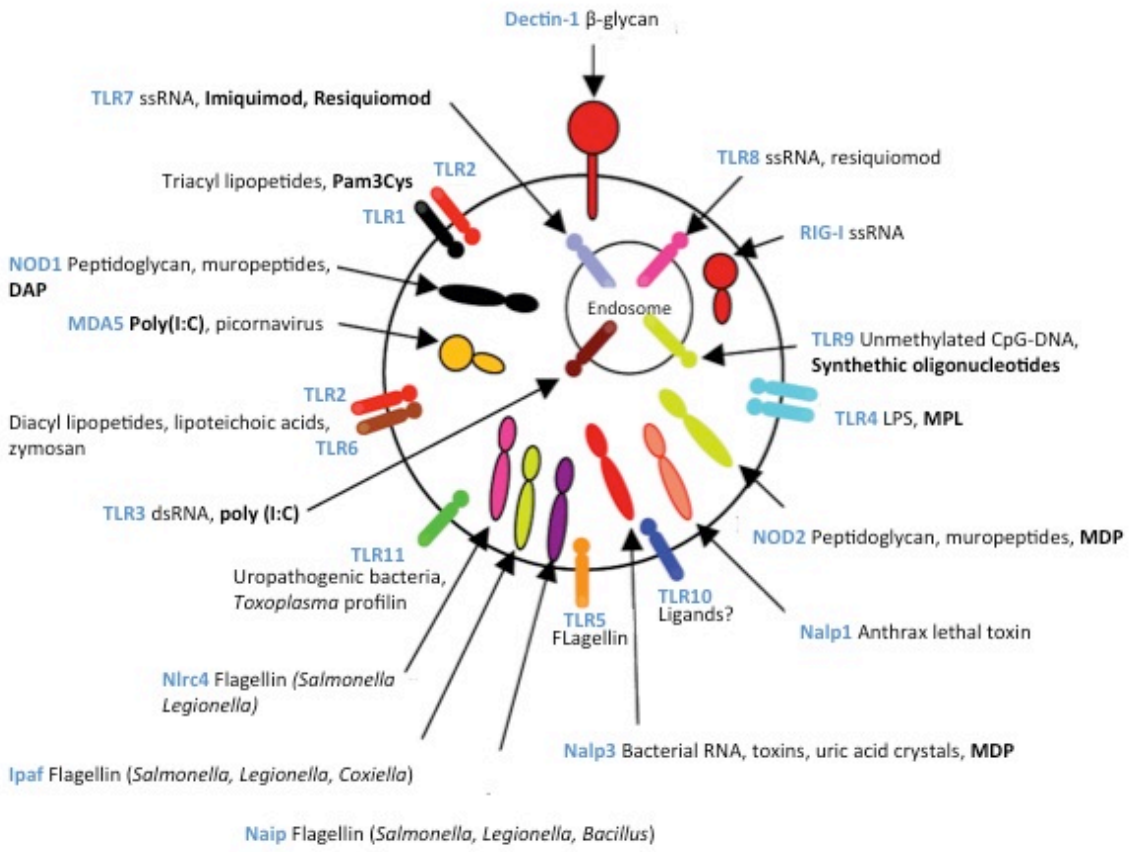


Figure 1.14. Overview of PRRs and a selection of their natural and synthetic ligands. (Adapted from Guy, 2007).

Activation of this recently discovered PRR family can coordinate the expression of various transcription factors in immune cells, resulting in the production of immunomodulatory molecules, such as cytokines, prostaglandins, leukotrienes, and chemokines (de Veer and Meeusen, 2011). These molecules in turn induce activation and recruitment of cells to the infection site. In addition, they are able to fine-tune the activation of specific T_h subsets and the resulting adaptive immune response. Furthermore, innate immune activation often enhances expression of molecules involved in antigen presentation, such as co-stimulatory molecules, and in this way strengthens and accelerates the adaptive immune response (de Veer and Meeusen, 2011).

In the case of DCs, PRR stimulation leads to DC activation resulting in upregulation and release of a variety of molecules that are required for efficient priming of naïve T cells. Figure 1.15 depicts a simplified schematic overview of the key events that occur during DC-mediated T_h cell priming. DCs are present in many tissues in immature form waiting to respond to stimuli including DAMPs and PAMPs. Once activated the DCs undergo a maturation process that prepares them for optimal priming of naïve $CD4^+$ T cells in secondary lymphoid structures (Mellman and Steinman, 2001). During the maturation process an upregulation of peptide-loaded MHC class II molecules as well as co-stimulatory molecules including CD40, CD80 and CD86 on the DC surface takes place (Cella et al., 1997; Cella et al., 1996; Inaba et al., 1992; Larsen et al., 1992; Pierre et al., 1997). Complete DC priming of naïve $CD4^+$ T cells involves 3 types of signal. The first stimulatory signal (signal 1) is provided by interaction of the peptide/MHC II complexes with the T cell receptor (TCR) and is required for determining the specificity of the response (Kapsenberg, 2003). A second signal (signal 2) is provided by engagement of co-stimulatory molecules with their receptors present on T cells, which is essential for supporting the ongoing T cell activation and to start clonal expansion of antigen-specific T cells (Kapsenberg, 2003). In the absence of signal 2 T_h cells become anergic, which can result in tolerance (Kapsenberg, 2003). A third signal (signal 3) is needed to polarise the T cell response and for the T cell to gain effector functions (Kapsenberg, 2003). This third T_h-polarising signal is determined by soluble factors, so-called T_h-polarising molecules, including cytokines, that can be provided in a DC-dependent or DC-independent manner (Kapsenberg, 2003). Important in determining the composition and outcome of signal 3 are the conditions in which the DCs were activated (Kapsenberg, 2003). Interleukin-12 (IL-12) is the main DC-derived cytokine with Th1-polarising capacity *in vitro* and *in vivo* (Cooper et al., 1985; Macatonia et al., 1995; Magram et al., 1996; Maldonado-Lopez et al., 1999; Seder et al., 1993). The most well known DC-derived cytokine with the ability to trigger the development of T_h2 cells *in vitro* and *in vivo* is IL4 (Kopf et al., 1993; Swain et al., 1990). It is generally agreed that development of $CD4^+$ T cells in to T_h17 cells requires three conditions: 1) the presence of transforming growth factor **β** (TGF-**β**) and IL-6 triggers T_h17

differentiation, 2) IL-21 stimulates further commitment to T_h17 polarisation, 3) IL-23 provides a final confirming and stabilising signal for the T_h17 phenotype (Chen and O'Shea, 2008). Regulatory T helper cell (T_{reg}) and T_h9 cell subsets have also been identified, which are thought to be dependent upon the presence of TGF-**β** and a combination of TGF-**β**, IL-4, and IL-21 cytokines during $CD4^+$ T cell activation respectively (Ma et al., 2010; O'Shea and Paul, 2010; Veldhoen et al., 2008; Weaver et al., 2007; Zhu and Paul, 2010). The exact environmental trigger for differentiation into another form of T_h cells, follicular helper T cell (T_{FH}), has not yet been defined, but probably involves the presence of IL-21 (King, 2009).

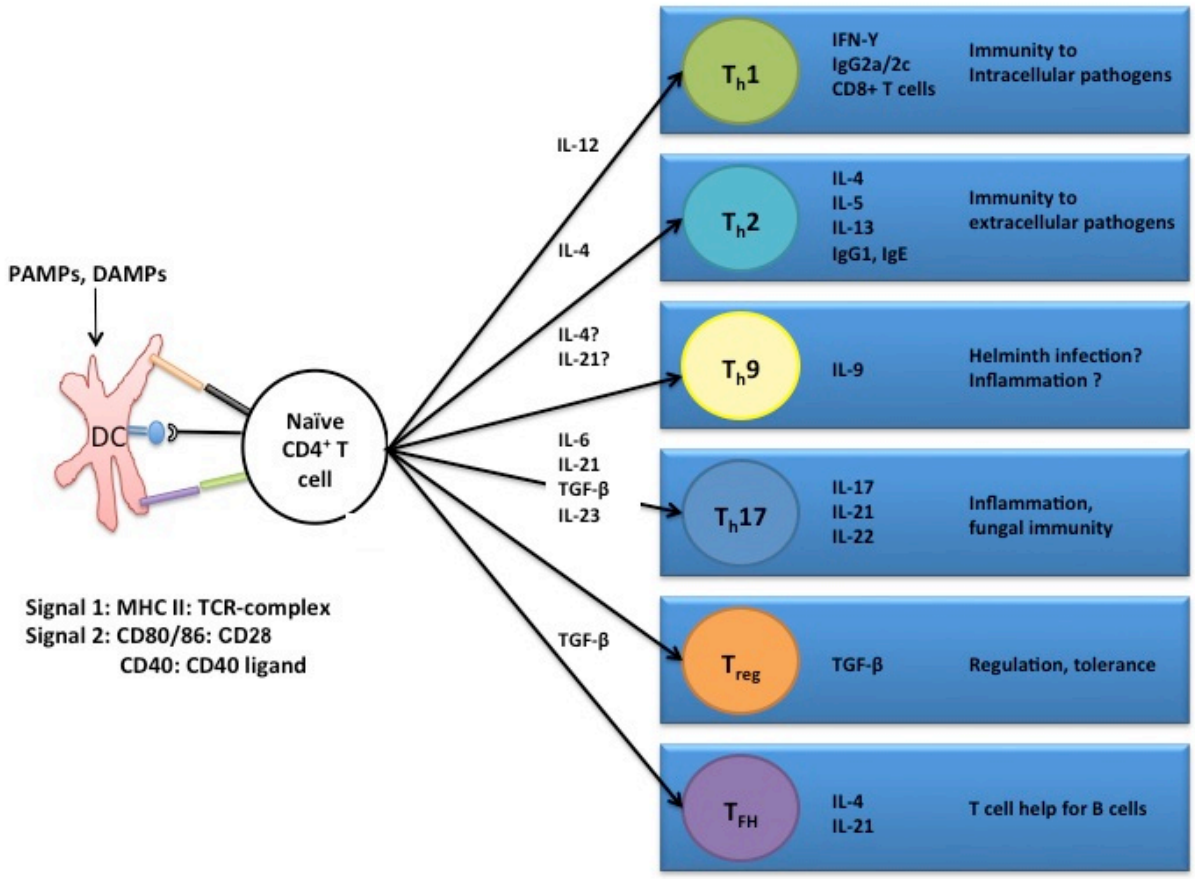


Figure 1.15. Simplified overview of priming and differentiation of CD4⁺ T cells. Activation of DCs by PAMPs and/or DAMPs leads to elevated expression of peptide-loaded MHC II molecules (signal 1) and co-stimulatory molecules CD80, CD86, CD40 (signal 2) which can induce the differentiation of CD4⁺ T cells into T_h1, T_h2, T_h9, T_h17, T_{reg}, or T_{FH} cells, depending on the action of cytokines (signal 3). Each T_h subset is typified by a distinct cytokine profile and effector functions. Adapted from (King et al., 2008) and (Ma et al., 2010).

Each T_h-subset is accompanied by a distinct cytokine profile and functions. Interferon-γ (IFN-γ) is a hallmark of a T_h1 response (Stevens et al., 1988). This cytokine is largely involved in the induction of isotype class switch recombination to IgG2a, or IgG2 in mouse strains that lack IgG2a, B cell production, and the stimulation of antimicrobial activity in macrophages (Finkelman et al., 1988; Martin et al., 1998; Suzuki et al., 1988). Another important role in the T_h1 response is played by $CD8^+$ T cells (Wan and Flavell, 2009). Induction of a T_h1 response and IFN-γ are thought to be important in the defence against viruses, bacteria and tumours, while uncontrolled T_h1 responses might add to the development of autoimmune diseases, such as type-1 diabetes and arthritis (Bungener et al., 2008; Graham et al., 1994; Kobayashi et al., 1997; Wan and Flavell, 2009).

A T_h2 response is characterised by IL-4 and IL-5 production (Wan and Flavell, 2009). IL-4 is thought to be involved in IgE production while IL-5 has been shown to trigger eosinophil recruitment (Coffman et al., 1989; Kopf et al., 1993). IgE-mediated activation of basophils and mast cells as well as eosinophil recruitment are thought to play an important role in protection against infection by extracellular pathogens, including helminths (Abbas et al., 1996). Another T_h2 cytokine IL-13 was reported to be involved in defence against helminths (Finkelman et al., 1999). T_h2 cells have been demonstrated to be implicated in IgG1 isotype antibody production by B cells (Stevens et al., 1988). Finally, T_h2 responses are crucially involved in asthma and allergies (Zhu and Paul, 2008).

A T_h17 response, that has only been discovered more recently, is characterised by the release of IL-17, IL-21, and IL-22 (Chen and O'Shea, 2008; Harrington et al., 2005; Park et al., 2005). T_h17 cells have been reported to be involved in protection against infections caused by a variety of pathogens, including extracellular bacteria and fungi (McGeachy and Cua, 2008), while T_h17 responses have also been implicated in the development of autoimmune diseases including MS, psoriasis, and arthritis (Korn et al., 2009). T_h9 cells produce IL-9 and the exact function of this T_h subset still needs to be elucidated, but may involve immunity against helminth

infection and/or the induction of inflammatory conditions (Ma et al., 2010). T_{FH} cells play a role in the generation of high affinity B cell responses and class-switching and express high levels of IL-4 and IL-21 (King et al., 2009).

1.13. Adjuvants

With the gradual shift in the vaccination field from traditional attenuated or killed whole organism vaccines towards safer but often poorly immunogenic subunit vaccination strategies, the development of adjuvants (from *adjuvare*, to help), which are vaccine components that are formulated together with the immunogen in a vaccine to help stimulate protective immunity (Coffman et al., 2010), has become more important. Furthermore, already relatively weak subunit immunogens generally perform even less well when administered mucosally, thus with the increasing need for mucosal immunisation, even more pressure is exerted on adjuvant development (Neutra and Kozlowski, 2006; Ryan et al., 2001). On top of this, adjuvants can allow certain improvements to existing vaccines such as the reduction of antigen dose or the number of immunisations or the benefit of vaccination in older and/or immunocompromised populations (O'Hagan and De Gregorio, 2009).

1.13.1. Adjuvants: mode of action

Traditionally adjuvants have been used to enhance the magnitude of the adaptive immune response elicited by a vaccine (Coffman et al., 2010), but more recently a second role for adjuvants has become increasingly important: to guide the quality of the immune response towards the most effective form of immunity for the particular pathogen (Coffman et al., 2010). Adjuvants are increasingly used to achieve a type of immunity that

is not successfully induced by the non-adjuvanted antigen. As such, adjuvants have been included in pre-clinically and clinically-tested vaccines to: 1) guide towards functionally appropriate immune responses; 2) increase the induction of memory responses; 3) enhance the speed of the initial response; 4) modify the breadth, specificity, affinity or compartmentalisation of the response; 5) enable mucosal delivery of a vaccine. These effects can be achieved via two distinct adjuvant mechanisms (Coffman et al., 2010; de Veer and Meeusen, 2011): direct stimulation of the innate immune system or through effects on the antigen, including: antigen protection against denaturation or degradation; formation of a depot and slow release of antigen; increased targeting of vaccine antigen to immune cells; antigen clustering; particle formation and effect on particle size.

For mucosal immunisation (I) is particularly important, because of the potentially harsh pH and enzyme conditions at the mucosa. On top of the above, an important feature for development of a mucosal vaccine is the enhancement of antigen uptake by M cells or mucosal epithelial cells (Ryan et al., 2001).

Recently improved elucidation of the innate immune system discussed in the previous Section has lead to the understanding that the majority of traditionally used adjuvants act at least in part by direct stimulation of innate immune pathways, through activation of DAMP- or PAMP-pathways.

Before this finding, traditional adjuvants were thought to work solely through effects on antigen, such as protection against antigen degradation following administration. Adjuvant interaction with antigen can delay antigen degradation or depletion by proteases or DNAses in the body resulting in enhanced and more durable immunogenicity (Ryan et al., 2001).

The vast majority of non-replicating vaccines require a prime-boost regimen, possibly because of antigen depletion or insufficient initial immunogenicity of the vaccine. Many approaches have been tried to develop depot-forming adjuvants that contain antigen, such as degradable microparticles and slow-release implants and in general these depot forming adjuvants are more effective than non-depot forming adjuvants (Kemp et al., 2002).

Another mechanism underlying adjuvanticity can be the effective targeting of antigen to specific cell types, for example DCs or other APCs (Bonifaz et al., 2004; Schijns, 2000). Adjuvants have for example been engineered to include targeting ligands that bind to specific cell type receptors and therefore preferentially bind certain cell types (Bonifaz et al., 2004). Particulate vaccines, including Alum and MF59, have been shown to enhance the local number of immune cells available for interaction through recruitment and to facilitate antigen uptake by APCs (Dupuis et al., 2001; Kool et al., 2008a; Morefield et al., 2005).

The immune system has evolved to detect ordered structures and antigen clustering is suggested to be a way in which adjuvants could improve antigen recognition by the immune system (de Veer and Meeusen, 2011). Most pathogenic surfaces are composed of highly ordered and repetitive structures, such as the regular matrix that is formed by viral outer proteins (de Veer and Meeusen, 2011). Many immune components such as IgM, and complement and C-type lectins recognise multimeric repetitive antigens and enhance uptake by B cells and macrophages (de Veer and Meeusen, 2011). Clustered antigens have been found to efficiently activate B cells resulting in higher B cell proliferation and differentiation and increased antibody responses (Bachmann and Zinkernagel, 1997).

The particle size of a vaccine formulation influences its immunogenicity through effects on migration patterns and cellular uptake, so adjuvants can play a role in affecting immunogenicity through particle formation. DCs generally pick up particles between 40–80 nm (Bachmann and Zinkernagel, 1997). In contrast, even smaller

particles of ≤ 25 nm drain immediately into lymphatic vessels to later be taken up by APCs in the LNs (Fifis et al., 2004; Gamvrellis et al., 2004). Larger particles of ≥ 200 nm get caught at the immunisation site and can only be transported to the LN by monocytes, neutrophils and DCs (Bachmann and Jennings, 2010; de Veer et al., 2010; de Veer et al., 2007). Furthermore within the LN, particle migration is dependent on size, with small antigens immediately going into the lymphatic conduit while larger particles remain longer in the subcapsular sinus and are taken up by macrophages that line the sinus. In addition, particle size influences the type of immune response, as smaller ≤ 40 nm particles are more likely to elicit IFN-**γ** while larger ≥ 200 nm generally elicit more IL-4 release from T_h-cells (Fifis et al., 2004). These aspects are taken into consideration in vaccine development and an increasing number of distinct chemical particles, polymers, liposomes, and structures are being studied for use as delivery vehicles for vaccine development (Guy, 2007; Heegaard et al., 2011).

An ideally formulated adjuvant will need a few requirements, in addition to the effective enhancement of immunogenicity. It will need to be well tolerated, structurally stable prior to immunisation, easily biodegraded and eliminated, and it should be cheap and easy to manufacture. Furthermore, it will need to maintain adjuvant activity via the mucosal route (Coffman et al., 2010; de Veer and Meeusen, 2011).

1.13.2. Licensed and experimental adjuvants

Adjuvant research began around 1920 when the veterinarian Gaston Ramon, at The Pasteur Institute in France observed that horses developed a stronger immune response to diphtheria toxin if they had some inflammation at the site of injection. Since then many adjuvant candidates have been investigated and developed, originally mainly through empirical research but more recently our increased understanding of innate immunity (Section 1.12) has led to a more rational approach in adjuvant development (Reed et al., 2009). Although some of these adjuvants are very potent, only very few have been formulated in licensed vaccines, usually due to high toxicity

profiles; finding the right balance between potency and toxicity remains probably the biggest hurdle in adjuvant development (O'Hagan and De Gregorio, 2009). Examples of very potent adjuvants that are considered too toxic for use in humans are the gold standard experimental adjuvants Freund's Complete Adjuvant (FCA) and mucosal adjuvant Cholera Toxin (CT), and its beta subunit Cholera Toxin B (CTB). The most successful adjuvants licensed for human use have by far been aluminium-salt based formulations that for more than 50 years have been incorporated into a wide range of vaccines for worldwide use (Clements and Griffiths, 2002). Examples of newer human adjuvants are the Adjuvant System (AS)-series from GlaxoSmithKline (GSK), MF59 from Novartis, ISCOMS, and QS21, most of which induce markedly higher reactogenicity and systemic toxicity than Alum, implying a need for the development of less reactogenic adjuvants (Petrovsky et al., 2007). In this Section an overview will be provided of the most well known available licensed and experimental adjuvants, including a selection of the latest reports on reactogenicity and possible mechanisms of action underlying their adjuvanticity.

1.13.2.1. Aluminium salts

Aluminium salts, sometimes referred to as alum, is the collective name for a group of adjuvant molecules, including aluminium hydroxide ($Al(OH)_3$), aluminium phosphate ($AlPO_4$) and aluminium sulphate ($Al_2(SO_4)_3$), that have been used for almost a century, since 1926, to improve Ab responses in animals and humans (Glenny, 1930; McKee et al., 2009). Aluminium salts induce a T_h2 response (Mbow et al., 2011). The range of prophylactic human vaccines it is used in includes diphtheria, tetanus, pertussis, *H Influenzae* type B, hepatitis A and B (Mbow et al., 2011). For a long time there was very little understanding of how alum works and it was hypothesised that depot formation and prolonged antigen exposure was the main mechanism underlying its adjuvant effect (Coffman et al., 2010; Mbow et al., 2011). However, it has now become evident that activation of the innate immune system plays a major role in alum's adjuvant activity. Several *in vitro*

studies in macrophages and DCs have reported alum-induced activation of the NLRP3 inflammasome following LPS priming (Li et al., 2007). It was suggested that cellular uptake of alum crystals results in reactive oxygen production and lysosomal damage resulting in the release of cathepsin B into the cytosol, which is thought to act upstream of the NLRP3 inflammasome (Dostert et al., 2008; Hornung et al., 2008). Additionally, aluminium salt-induced cytotoxicity can indirectly activate the NLRP3 inflammasome via the release of uric acid (UA) and ATP (Kool et al., 2008b). Although it is generally agreed that alum-induced NLRP3 inflammasome activation is responsible for IL-1β release, its contribution to alum's T_h2 adjuvant effect remains controversial (Lambrecht et al., 2009; Marrack et al., 2009; Pelka and Latz, 2011). Some studies reported NLRP3, ASC, and caspase-1 to be essential for alum's adjuvant effect (Eisenbarth et al., 2008), but others could not confirm a role for NLRP3 (Franchi and Nunez, 2008). Stimulation of T_h2 polarising cytokines IL-4, IL-6, and IL-25 could also account for alum's T_h2 adjuvant effect (Lambrecht et al., 2009; Marrack et al., 2009; Serre et al., 2008). Earlier this year, Kuroda *et* al and Kool *et* al attempted to decipher the molecular mechanism induced by alum and both pointed at an NLRP3-independent mechanism (Kool et al., 2011; Kuroda et al., 2011). Kuroda *et* al demonstrated the production of prostaglandin E2 (PGE2) through a syk and p38 MAP kinase–dependent pathway but independently of the inflammasome pathway, following alum administration (Kuroda et al., 2011). PGE2 was produced following lysosomal damage and release of lysosomal enzymes (Kuroda et al., 2011). It was suggested that PGE2 has either T_h1- or T_h2- polarising effects depending on the present cytokines, for instance IL-4 or IL-12. Another study by Marichal *et* al earlier this year revolutionised the understanding of adjuvants by showing that it is probably alum's cytoxicity resulting in the release of host cell DNA that can act as DAMP on Irf3-dependent and independent pathways to activate immunity (Marichal et al., 2011). Despite alum's good safety record and widespread use, the elevated risk of vaccine allergy, anaphylaxis, and macrophagic myofascitis (MMF), a chronic inflammation syndrome, as well as the observed formation of granulomas at the site of injection might pose a problem (Carter and Reed, 2010;

Petrovsky, 2007). Additionally, its incapacity to induce significant T_h1 and $CD8^+$ T cell responses renders it incompatible with many vaccine uses including HIV-1 vaccination designed to elicit $CD8^+$ T cells.

1.13.2.2. MF59

MF59 has been licensed since 1997 for use in influenza vaccines for the elderly (Pulendran and Ahmed, 2011). It is composed of small oil-in-water emulsion droplets of <250 nm degradable oil squalene and two surfactants, polyethylene sorbitan monoleate (Tween 80) and sorbitan triolate (Span 85). Compared to Alum, MF59 induces stronger antibody responses and allows a mixed T_h1- + T_h2- memory response (Ott et al., 1995). The exact mechanism underlying MF59 adjuvanticity is unknown but MF59 is thought to form a depot and directly increase the release of certain chemokines and cytokines (Carter and Reed, 2010). Also, MF59 has been shown to trigger recruitment of DCs, granulocytes, elicit the differentiation of monocytes to DCs (Seubert et al., 2008), and enhance antigen uptake by DCs (Dupuis et al., 1998). Furthermore, intramuscular use of MF59 was shown to induce a wider and different array of genes than alum or a TLR9 agonist (Mosca et al., 2008). MF59 has an acceptable safety record and has been investigated in the context of various vaccine formulations, including mucosal immunisation, as previously mentioned (Section 1.11.8)(Barnett et al., 2010). Nevertheless, MF59 is known to be more reactogenic than Alum and possibly increases the risk of inflammatory arthritis (Frey et al., 2003).

1.13.2.3. The Adjuvant System (AS) series: AS03 and AS04

AS03 is another squalene-based oil-in-water emulsion that is licensed for use in an influenza vaccine in Europe (Carter and Reed, 2010). It is composed of DL-**α**-tocopherol in squalene and polysorbate 80 (Morel et al., 2011). The exact mode of action of AS03 is unknown, but key features include transient production of

cytokines at the local injection site and draining LNs, and increased recruitment of granulocytes and antigen-associated monocytes in the draining LNs (Morel et al., 2011). It is believed that DL-**α**-tocopherol is required for most of these functions. AS03 is well tolerated, but is subject of similar reactogenicity concerns as MF59 (Carter and Reed, 2010; Petrovsky, 2007).

The AS04 adjuvant is a combination of aluminium salt and TLR-4 ligand MPL (3-O-desacyl-4'-monophosphoryl lipid A), which is a detoxified derivative of LPS from the *Salmonella minnesota* R595 isolate. It is the first and currently only TRL ligand licensed for human vaccination (Cervarix for HPV and Fendrix for HBV in USA) (Carter and Reed, 2010) and is a potent inducer of both humoral and cellular immunity. MPL binds to TLR4 on macrophages, which results in the release of pro-inflammatory cytokine TNF-**α**, as well as IL-2, IFN-**γ**, and a T_h1 response. As a downside, AS04 production is costly and lacks an easily reproducible protocol. Furthermore, similar to most newer adjuvants, AS04 is more reactogenic than Alum (Petrovsky, 2007).

The AS-series has been investigated in a range of HIV-1 immunisation studies, including monomeric and trimeric Env subunit and DNA prime/protein boost immunisation in mouse, rabbit, guinea pig, and NHP models (Li et al., 2006; Rollman et al., 2007; Zhang et al., 2007). The adjuvants effectively induced antibody titres including nAb responses and partial protection, with AS02 being the most effective adjuvant of the series, but as of yet no full protection was observed in any of these models (Carter and Reed, 2010).

1.13.2.4. Liposomes

Liposomes are composed of phospholipid spheres of lipid layers that can encapsulate antigen. They are thought to achieve adjuvant activity through fusion with macrophage membranes and delivery of antigen into the macrophages, which allows protein antigens to enter the cytoplasm and to be processed via the MHC class I

pathway so that $CD8^+$ CTL responses can be triggered (Owais and Gupta, 2000; Zheng et al., 1999). The adjuvant efficacy of liposomes depends amongst others on the number of lipid layers, electric charge and composition (Heath et al., 1976; Tyrrell et al., 1976). In Europe liposomes have been licensed for use in hepatitis A and influenza vaccines (Petrovsky, 2007), but their use is limited by manufacturing difficulties, high cost, instability, injection site pain and the fact that potent adjuvant activity often requires co-formulation with other adjuvants such as MPL (Petrovsky, 2007).

1.13.2.5. Freund's complete adjuvant (FCA)/Freund's incomplete adjuvant (FIA)

Freund's adjuvant is a water-in-oil emulsion of paraffin oil and surfactant. FCA contains heat-killed *Mycobacterium tuberculosis* or *M. butrycium* and is used only for experimental and veterinary purposes (Coffman et al., 2010). FIA is usually used for boosting after FCA prime immunisation and does not contain the mycobacterium component. FIA is therefore although less potent, also less reactogenic and for example used in an influenza vaccine in the UK (Vogel and Powell, 1995). Also, FIA is currently under investigation in the US for HIV-1 vaccination (Vogel and Powell, 1995). FCA induces a strong T_h1 and T_h17 cell response that is MYD88 dependent in the context of protein antigens (Mayer-Barber et al., 2010; Su et al., 2005). The inflammasome pathway and IL-1β release are crucially involved in FCA-induced T cell responses and are thought to be responsible for the MYD88-dependancy of T cell immunity, instead of the many TLR ligands present on mycobacterium, such as TLR2, 4, and 9, which are though to be dispensable for FCA adjuvant activity (Mayer-Barber et al., 2010). In contrast, the FCA-induced humoral response is inflammasome-independent and studies have been controversial about the requirement of MYD88- and TRIF-dependent signalling pathways (Coffman et al., 2010; Eisenbarth et al., 2008; Gavin et al., 2006). The mode of action of FIA and water-in-oil emulsions in general is poorly understood. It is thought that NOD2 is required (Moreira et al., 2008), but since these emulsions are likely to induce cellular damage, it is also possible that endogenous

signals released during cell death also play a role (Coffman et al., 2010). The combination of FCA/FIA is a gold standard experimental adjuvant for use in animal models.

1.13.2.6. Cholera Toxin Beta subunit (CTB)

Another gold standard experimental adjuvant, but one with mucosal adjuvant activity, is CT, and its beta subunit CTB. CTB is the cell-binding subunit of CT and belongs to a group of strongly potent toxins derived from *Vibrio Cholera* and *Escherichia Coli* that are the most effective mucosal adjuvants currently available (Lycke and Holmgren, 1986). CTB enters cells via binding to cell surface monosialogangliosides (GM1) that are present on various cells, including epithelial cells, lymphocytes, and APCs (Sack et al., 1980). Its adjuvant activity is characterised by a mixed T_h1/T_h2 response, as well as T_h17 activation and involves the induction of serum IgG1 and IgG2b, serum and mucosal IgA, as well as $CD4^+$ T cell and minor $Cd8^+$ T cell responses (Imaoka et al., 1998; Lee et al., 2009; Marinaro et al., 1995; Su et al., 2004; Wilson et al., 1991; Xu-Amano et al., 1993). *In vitro* CTB has been shown to enhance DC maturation and expression of maturation markers CD80 and CD86, induce suppression of IL-12 production and enhancement of IL-10 production (Braun et al., 1999; Cong et al., 2001; Gagliardi et al., 2000). However, despite the production of less toxic variants, the use of these adjuvants in human immunisation is still hampered by side effects and safety concerns, including diarrhoea (Elson, 2005) and CNS effects (Mutsch et al., 2004; van Ginkel et al., 2000).

1.13.2.7. Saponin-based adjuvants: ISCOMs, ISCOMATRIX, Quil-A, and QS-21

Saponins are natural glycosides of steroid or triterpene purified from a South American tree, *Quillaja saponaria* and saponin-based adjuvants include Immune Stimulating Complexes (ISCOMS), ISCOMATRIX, and Quil-A and its derivatives such as QS-21. The ISCOM adjuvant was first described by Bror Morein in 1982 (Morein et al., 1984). It is a nanoparticle composed of saponins, cholesterol, phospholipid, and antigen. The

incorporation of the antigen into the complex is not necessary for adjuvant activity and therefore the most widely used formulation is ISCOMATRIX (ISCOM without antigen) and soluble antigen (Coffman et al., 2010). ISCOMS are strong inducers of T_h cell responses but do not induce a bias towards T_h1 or T_h2 (Coffman et al., 2010). ISCOMS have not been shown to stimulate any identified PRR, but were reported to enhance antigen uptake and extend retention by DCs in draining LNs, trigger DC activation, and induce strong humoral and cellular responses (Maraskovsky et al., 2009). In contrast to most adjuvants, ISCOMS can induce strong MHC class I presentation of soluble protein antigens resulting in both $CD8^+$ and $CD4^+$ T cell responses (Davis et al., 2004). This ability is thought to be due to endosomal destabilisation, which enables cytoplasmic access of antigens (Schnurr et al., 2009). ISCOM adjuvants can be used for mucosal delivery (Abusugra and Morein, 1999; Hu et al., 1998). As a downside of this adjuvant group, incorporation of antigen into ISCOM is likely to result in antigen denaturation, which is highly undesirable for HIV-1 Env antigens. Furthermore, saponins interact irreversibly with cholesterol in cell membranes, which can result in adverse effects at the administration site. Despite the development of new generation ISCOM adjuvants with less adverse effects, ISCOM adjuvants still suffer from a range of problems, including cost, instability, production difficulties, reactogenicity and safety concerns (Claassen and Osterhaus, 1992; Ronnberg et al., 1995).

Quil-A is a heterogenous mixture of at least twenty chemically related tri-terpenoid saponins that is used in veterinary vaccines. Quil A adjuvant activity is dependent on an aldehyde group present on the saponins that forms a Schiff base with amino groups on the co-stimulatory T cell receptors (Kensil, 1996). Possibly, Quil A can replace co-stimulatory signal CD80 (B7.1) and thus trigger T_h1 type immune responses (Kensil, 1996). QS-21 is a highly purified saponin that is currently investigated in a range of human immunisation studies (Coffman et al., 2010). In a number of clinical trials, QS-21 was found to induce serious side effects, including severe injection pain, hemolysis, and possibly even death, rendering it unsuitable for use in human prophylactic

vaccination (Chapman et al., 2000; Ghochikyan et al., 2006; Kashala et al., 2002; Livingston et al., 1994; Mbawuike et al., 2007).

1.13.2.8. Other experimental adjuvants

In addition to the above adjuvants, other and newly developed adjuvants are constantly being investigated and applied for experimental use. A major group of immunestimulants are the TLR-based adjuvants, including Flagellin (TLR5), CpG oligodeoxynucleotides (TLR9) and Poly-IC (TLR3), which achieve adjuvant activity through activation of TLR innate immune pathways. Generally, the use of these adjuvants in human is hampered by safety concerns (Petrovsky et al., 2007). Another interesting group of new adjuvants are the ADVAX adjuvants that are based on natural plant-derived inulin in nanocrystalline particles form (Cristillo et al., 2011). They have been shown to induce mixed T_h1 + T_h2 responses and certain ADVAX adjuvants, such as ADVAX-M work as mucosal adjuvant (Cristillo et al., 2011). ADVAX adjuvants completely lack reactogenicity, which is unique among the currently identified adjuvants and makes this group particularly promising, yet further investigation into the potency of these adjuvants is required.

Adjuvant name:	**Main constituents:**	**Immune bias*:**	**(selected examples of) actions found in experiments**	**Note:**	**Reactogenicity**:**	**References:**
Alum	Aluminium salts	T_h2	• Induced cell death resulting in release of host cell DNA that acts as DAMP Other potential factors: • NLRP3-inflammasome activation? (UA release, ATP release, and lysosomal damage, ROS) • Lysosomal damage and release PGE2 • Immune cell recruitment • Cytokine and chemokine release • Particulate vaccine	Not effective in HIV-1 immunisation	++	(Azizi et al., 2010; Dostert et al., 2008; Hornung et al., 2008; Kool et al., 2008a; Kool et al., 2008b; Kool et al., 2011; Kuroda et al., 2011; Lambrecht et al., 2009; Li et al., 2007; Marichal et al., 2011; Mbow et al., 2011; Petrovsky, 2007; Ryan et al., 2001; Serre et al., 2008)
MF59 and ASO3	Squalene-in-water emulsions	T_h1 + T_h2	Undefined, probably: • Depot formation • Immune cell recruitment (eg.DCs,granulocytes, macrophages) • Apoptosis induction • Cytokine and chemokine release • Particulate vaccine • Tissue inflammation	**Mucosal** adjuvant activity Used with ΔV2 **HIV-1** $gp140_{SF162}$ trimer in NHP model, showed protection but not against heterologous virus challenge	++++ (Not licensed in the US because considered too unsafe for use in human)	(Barnett et al., 2010; Carter and Reed, 2010; Dupuis et al., 2001; Dupuis et al., 1998; Frey et al., 2003; Morel et al., 2011; Ott et al., 1995; Petrovsky, 2007; Seubert et al., 2008)
AS04 **(MPL and formulations)**	MPL-A and aluminium salt	T_h1	• TLR4 ligand • …and see above for Alum..		++++	(Carter and Reed, 2010; Moore et al., 1999; Ryan et al., 2001; Salkowski et al., 1997)
liposomes	Combination of lipids and cholesterol	T_h1	• Delivery vehicle: Macrophage membrane fusion and delivery of antigens into macrophages	Ability to **cross-present** protein antigens Potent activity requires co-formulation with other adjuvant, eg. MPL	++	(Ichikawa et al., 2002) (Anders et al., 2004; Baldridge et al., 2000; Prinz et al., 2006) (Hartmann et al., 2000; Heath et al., 1976; Owais and Gupta, 2000; Petrovsky, 2007; Tyrrell et al., 1976; Zheng et al., 1999)
CFA/IFA	Mineral or paraffin oil and surfactant peptidoglycan	T_h1 + T_h17	Undefined, possibly: Inflammasome NLR, TLR ?	Gold standard experimental adjuvant	++++	(Coffman et al., 2010; Gavin et al., 2006; Mayer-Barber et al., 2010; Moreira et al., 2008; Su et al., 2005; Vogel and Powell, 1995)

CT, LT, and Pertussis Toxin	Cholera toxin from *Vibrio Cholera*, heat labile toxin from *E.Coli* and Pertussis toxin from *Bordetella Pertussis*	$T_h1 + T_h2$	<u>Undefined, partially:</u> • ADP-robosyltransferace activity and intracellular cAMP by subunit A, binding of PRR GM1 ganglioside by subunit B • DC maturation *in vitro*	Gold standard **mucosal** adjuvant Too toxic for use in man	++++	(Braun et al., 1999; Cong et al., 2001; Elson, 2005; Gagliardi et al., 2000; Imaoka et al., 1998; Lee et al., 2009; Lycke and Holmgren, 1986; Mutsch et al., 2004; Sack et al., 1980; van Ginkel et al., 2000)
ISCOMs and **ISCOMATRIX**	saponins	$T_h1 + T_h2$	<u>Undefined, possibly:</u> • Ag delivery: interaction with cholesterol en pore formation in cell membrane • Recruitment neutrophils, mast cells, macrophages, DCs and lymphocytes	Can **cross-present** protein antigens Works as **mucosal** adjuvant in mice Induced nAb responses to **HIV-1** gp120 in rabbits	++++	(Abusugra and Morein, 1999; Browning et al., 1992; Claassen and Osterhaus, 1992; Coffman et al., 2010; Davis et al., 2004; Hu et al., 1998; Maraskovsky et al., 2009; Morein et al., 1984; Ronnberg et al., 1995; Schnurr et al., 2009)
Flagellin	Flagellin from *S.typhimurium*	$T_h1 + T_h2$	• TLR5 ligand	**Mucosal** adjuvant activity in mice	+++	(Bates et al., 2008; Lee et al., 2006; Petrovsky, 2007)
CpG oligodeoxynucleotides	CpG motifs	T_h1	• TLR9 ligand		+++	(Ichikawa et al., 2002; Petrovsky, 2007; Prinz et al., 2006; Sparwasser et al., 1999)
Poly-IC	dsRNA derivative	T_h1	• TLR3 ligand and MDA5 ligand		+++	(Petrovsky, 2007)
ADVAX	Inulin	$T_h1 + T_h2$	Undefined	ADVAX-M (consisting of **α**-galactosyl ceramide) can work **mucosally**	Lack of reactogenicity	(Cristillo et al., 2011; Petrovsky, 2007)

Table 1.6. Overview of licensed and widespread experimentally used adjuvants. * Experimental evidence of immune bias. ** Based on the literature, particularly (Petrovsky et al., 2007). Blue: licensed adjuvants. Adapted from: (Coffman et al., 2010; Petrovsky, 2007; Ryan et al., 2001).

1.14. Scope and context of this thesis

In the absence of an effective HIV-1 vaccine the AIDS pandemic will not be halted. Subunit HIV-1 vaccination or heterologous prime–boost combination regimens will need an adjuvant that: 1) enhances the immunogenicity of HIV-1 immunogens, such as HIV-1 Env; 2) allows the induction of a T_h1/T_h2 response compatible with both antibody and $CD8^+$ T cell responses; 3) allows mucosal delivery and the induction of mucosal HIV-1 specific immune responses; 4) is safe, preferably biodegradable, and less reactogenic than the most recently developed adjuvant molecules.

My research will be focused on investigating the potential adjuvant capacity of the widely used transfection agent polyethyeleneimine (PEI). PEI is an organic polymer with the highest described cationic charge density: every third atom is a nitrogen atom that can be protonated, giving PEI a strong buffering capacity at virtually all pH values (Figure 1.16) (Boussif et al., 1995). The high cationic charge density and buffering capacity are thought to partially underlie its unique transfection efficiency, as outlined in Figure 1.17 PEI transfection is mediated through the formation of PEI-DNA polyplexes resulting from the interaction of PEI with electronegative oxygen groups on the DNA backbone (Sun et al., 2011). Cell entry and delivery of the nucleic acids into the cells is thought to occur through interaction of the positively charged PEI-DNA polyplexes with negatively charged sulphated proteoglycans on the target cell surface, which induces endocytosis (Khalil et al., 2006; Mislick and Baldeschwieler, 1996). Additionally, PEI can disrupt membranes and long branches of PEI are thought to pass cell membranes directly (Behr, 1997; Yue et al., 2011). Inside the cell, polyplexes are initially present in endosomes, but PEI induces eventual endosome rupture resulting in release of DNA and PEI into the cell cytosol (Boussif et al., 1995; Behr, 1997; Akinc et al., 2005; Yue et al., 2011a; Yue et al., 2011) and targeting into the nucleus (Suh et al., 2003).

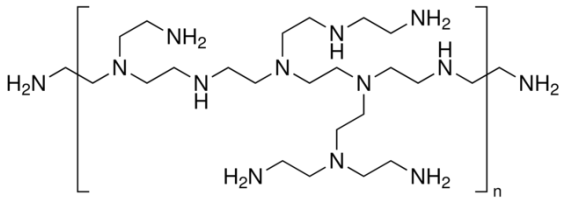


Figure 1.16. Molecular structure of branched PEI (bPEI). Every third atom is a protonable nitrogen atom, which makes PEI the organic macromolecule with the highest cationic charge density and gives it a strong pH buffering capacity. Taken from: Sigma Aldrich website.

Because of its transfection activity, PEI has also been studied in the context of DNA vaccination. Recently, PEI was for example incorporated in a HIV-1 therapeutic DNA vaccine, called Dermavir® (Lisziewicz et al., 2005a; Lisziewicz et al., 2005b). DermaVir® is a combination of a linear mannobiosylated PEI (lPEIm) and a DNA plasmid that encodes 15 HIV protein antigens in ~100 nm particle form in a glucose solution (Lori, 2011). It is administered with a new medical device called DermaPrep that causes transient erythema and mild inflammation, which is thought to provide the necessary 'danger' signal to achieve activation and targeting of epidermal LCs, which is thought to induce major immune stimulation (Lori, 2011). Generally in this Dermavir® formulation and similar DNA therapy studies, PEI is regarded solely as a delivery agent. Although it has been incorporated in a wide variety of gene delivery formulations and despite widely being studied in this context, including a small number of studies on its immune activation and potential side effects (Regnstrom et al., 2003; Chen et al., 2010; Cubillos-Ruiz et al., 2009), its *in vivo* adjuvant activity in the context of glycoprotein antigens has not been properly investigated[3].

[3] During the course of my DPhil studies a few articles have been published on potential PEI-mediated TLR-4 or TLR-5 stimulation and complement activation, as well as on PEI-mediated cross-presentation of protein antigens (Chen et al., 2010; Chen et al., 2011; Cubillos-Ruiz et al., 2009; Merkel et al., 2011), but the in *vivo* PEI adjuvant activity in the context of glycoprotein immunisation has still not been adequately explored. The mentioned studies will be discussed throughout and in the concluding chapter of this thesis.

In our lab, the ability of PEI and other transfection agents to protect and deliver TLR-ligand PolyI:C as a vaccine adjuvant was investigated in an immunisation assay. In this assay, PEI alone was found to have unique adjuvant activity compared to the other transfection agents.

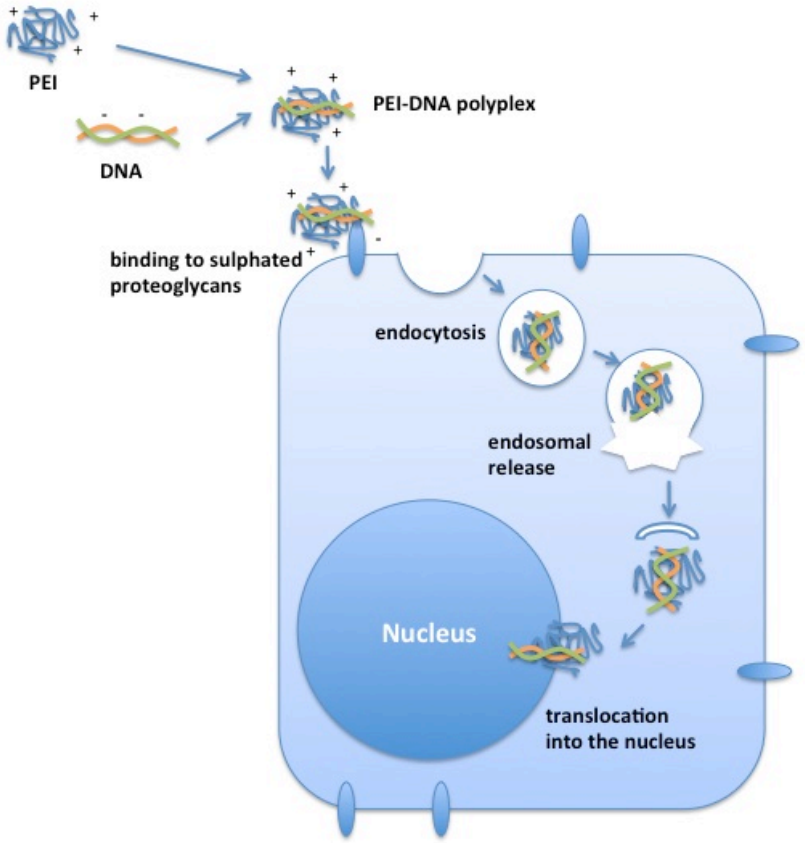


Figure 1.17. Suggested mechanism of PEI-induced DNA delivery. PEI interacts with nucleic acids mainly through binding to electronegative oxygen groups on the DNA backbone, resulting in PEI-DNA polyplex formation (Sun et al., 2011). Polyplexes interact with negatively charged sulphated proteoglycans on the target cell surface, which induces endocytosis (Khalil et al., 2006; Mislick and Baldeschwieler, 1996). Additionally, PEI can disrupt membranes and long branches of PEI are thought to pass cell membranes also directly (Behr, 1997; Yue et al., 2011). Inside the cell polyplexes are initially present in endosomes, but PEI induces eventual endosome rupture resulting in release of DNA and PEI into the cell cytosol (Yue et al., 2011) and targeting into the nucleus (Suh et al., 2003).

Based on this finding, I decided to study PEI adjuvant activity and to determine whether PEI has adjuvant activity in the context of *in vivo* glycoprotein subunit immunisation, motivated by the following questions and observations: 1) The strong capacity of PEI to deliver nucleic acids into cells, through complex formation with nucleic acids, interaction with target cell surfaces, and endosome-rupture, raises the question of whether PEI could similarly interact with glycoprotein immunogens and direct them (in)to immune cells, such as APCs; 2) The observed PEI-mediated lysosomal rupture is an activity that in the context of other adjuvants, such as Alum, has been shown to activate NLRP3-inflammasome immune pathways and result in the induction of humoral immune responses (Hornung et al., 2008), suggesting that PEI may have similar capacities to enhance immunogenicity through activation of such pathways; 3) The successful use of PEI in DNA vaccine formulations has, amongst other results, allowed the induction of mucosal immune responses, following mucosal administration (Huang et al., 2007; Torrieri-Dramard et al., 2011), suggesting that PEI maintains the capacity to deliver nucleic acids via the mucosal route and may allow the induction of mucosal glycoprotein-specific immune responses; 4) In addition to potential stimulation of innate immune pathways through PEI-mediated lysosome-endosome rupture activity, this activity of PEI suggests that PEI may be able to engage both MHC class I and class II pathways in antigen presentation, something that would be desirable to allow the activation of both humoral and cellular immune responses to glycoprotein antigen, which is desired for HIV-1 immunisation; 5) The widespread use of PEI for *in vitro* and *in vivo* transfection purposes, including the formulation of PEI in Dermavir® and cancer treatments that have proven safe and effective in Phase I and II trials (Lisziewicz et al., 2005a; Lisziewicz et al., 2005b; Ohana, 2004), as well as the limited release of pro-inflammatory cytokines following PEI administration in studies on DNA delivery (Bonnet et al., 2008) suggest that PEI can safely be used for applications in humans). Finally, PEI meets many of the practical requirements to become a successful vaccine adjuvant (Section 1.13.1.): it is cheap, chemically well-defined, easy to manufacture, and stable prior to administration (Moghimi et al., 2005).

Because PEI has been investigated in the context of DNA vaccination and therapies that mainly aim to induce T_h1 and CTL immune responses (Bivas-Benita et al., 2010; Huang et al., 2007; Lisziewicz et al., 2005a; Lisziewicz et al., 2005b; Lori, 2011; Ohana, 2004), even though PEI is mostly regarded solely as a delivery agent in these studies, I decided to investigate PEI adjuvant activity in a different context: HIV-1 glycoprotein subunit immunisation and particularly its capacity to elicit humoral responses in a mixed T_h1 + T_h2 immune environment.

Specific aims of the thesis

- ***Test whether PEI can act as an adjuvant with HIV-1 Env glycoprotein antigen in systemic rabbit immunisation, characterise specific antibody responses induced by PEI, and study effects of PEI on gp140 antigenicity in vitro (chapter 3).***
- ***Discovery and characterisation of PEI as a mucosal adjuvant for gp140, and the effect of PEI size, structure, and biodegradability on adjuvanticity (chapter 4).***
- ***Analysis of cellular mechanisms of PEI-induced immune responses (chapter 5).***

CHAPTER 2

Materials and Methods

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2.1. General notes

All buffer constituent percentages (%) quoted refer to weight per volume (w/v) unless otherwise indicated. Cells were incubated in 5% CO_2 at 37°C. All molecular weights (MW) quoted for PEI represent weight-average molecular weights based on light scattering and all Abs were purified, except where otherwise indicated.

2.2. Suppliers

General suppliers:

- AbD Serotec, UK
- Abcam, UK
- Abott, UK
- Anachem, UK
- Becton Dickinson (BD), UK
- BIAcore, Sweden
- Bio-Rad laboratories, UK
- Brenntag-Biosector, Denmark
- eBioscience, USA
- GE Healthcare, UK
- GeneTex, USA
- Greiner Bio-one, UK
- Hyglos, Germany
- Invitrogen, UK
- Invivogen, France
- Jackson Immunoresearch Laboratories (Jackson), USA
- Millipore, UK
- National Institute for Biological Standard and Control (NIBSC), UK
- National Institute of Health (NIH), USA
- PAA, UK
- Perbio Science, UK
- Perkin-Elmer, USA
- Polymer Chemistry Innovations, USA
- Polymun Scientific, Austria
- Polyplus Transfection, France
- R & D Systems, UK
- Rockland Immunochemicals, USA
- Sigma-Aldrich, UK
- Terumo, UK
- Thermo Scientific, USA
- Vector Laboratories, USA
- VWR International, USA

Individual suppliers*:*

- D.R. Burton, The Scripps Research Institute, CA, USA
- D. Corti, Institute for Research in Biomedicine, Switzerland
- C. Dohmen, Department of Pharmacy, Munich, Germany
- S. Jeffs, Imperial College London, UK
- I. Jones, University of Reading, UK
- J.E. Robinson, Tulane Medical Centre, USA
- B.Verrier, Institute of Biology and Chemistry of Proteins, France

2.3. General materials and reagents with suppliers

- [^{3}H] Thymidine — Perkin-Elmer
- 0.22 µm Millex GP filter — Millipore
- 2-mercaptoethanol (2-ME) — Sigma-Aldrich
- 3,3,5,5-tetramethylbenzidine (TMB) ELISA substrate — Thermo Scientific
- 24 well tissue culture plate (Costar) — Sigma-Aldrich
- 96 well flat/V-bottom plates — Greinier Bio-one
- 70 µm/100 µm cell strainers — BD
- ACK lysing buffer — Invitrogen
- Amicon ultra-4 centrifugal filter unit (10, 25 kDa) — Millipore
- Bovine serum albumin (BSA, MW 66 kDa) — Sigma-Aldrich
- CM5 biosensor chip — BIAcore
- Concanavalin A (Con A) — Sigma-Aldrich
- Coverslips (13 µm) — Thermo Scientific
- DL-Dithiothreitol (DTT) — Sigma-Aldrich
- Dulbecco's Phosphate Buffered Saline (dPBS) (endotoxin-free) — Gibco, Invitrogen
- 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) — BIAcore
- Ethylenediaminetetraacetic acid (EDTA) — Gibco, Invitrogen

• Endotoxin-free H_2O	Sigma-Aldrich
• Ethanol	VWR International
• Ethanolamine-HCL (pH 8.5)	BIAcore
• Falcon cell strainer	BD
• Fetal Calf Serum (FCS)	Invitrogen
• Filter paper (Whatman)	GE Healthcare
• Fine pH electrode	Jencons
• Formaldehyde	Sigma-Aldrich
• Gilson positive displacement pipette (Microman)	Anachem
• Glycine (pH 2.5)	BIAcore
• HEL (MW 14 kDa)	Rockland
• HEPES	PAA
• HEPES buffered saline (HBS-EP)	BIAcore
• Haemocytometer	Sigma-Aldrich
• L–Glutamine	Gibco, Invitrogen
• Microscope slides (0.8-1mm)	Thermo Scientific
• Microtainer SST ™ tubes for blood collection	BD
• Needles/ syringes	Terumo
• N-hydroxysulfosuccinimide (NHS)	BIAcore
• Non-fat dried milk (Marvel)	Sigma-Aldrich
• Penicillin/Streptomycin (P/S)	Gibco, Invitrogen
• Petri dish	BD
• Recombinant mouse GM-CSF	R & D
• RPMI 1640 with L-glutamine	PAA
• Salmon sperm DNA (ref 15632011)	Invitrogen
• Saponin	Sigma-Aldrich
• Sodium dodecyl sulphate (SDS)	Sigma-Aldrich
• Sulphuric acid (H_2SO_4)	Sigma-Aldrich
• Trypan Blue	Sigma-Aldrich
• Tween-20	Sigma-Aldrich
• VECTASHIELD mounting medium with Dapi	Vector Laboratories

2.4. HIV-1 Env-specific and other antibodies with suppliers

• Goat anti-mouse IgG (Fc-specific) (STAR 120P)(HRP)		AbD Serotec
• Mouse anti-rabbit IgG (Fc-specific) (HRP)		Sigma-Aldrich
• Biotinylated goat anti-mouse IgA		AbD Serotec
• Streptavidin (HRP)		BD
• Rat anti-mouse IgG1 (HRP)		BD
• Rat anti-mouse IgG2a (HRP)		BD
• Donkey anti-human IgG (HRP) minimal cross-reaction to various species, including bovine, chicken, goat, mouse, rabbit, and rat serum proteins		Jackson
• Rat anti-mouse CD16/CD32 (Fc Block)		BD
• Human A32	(C1-C4)	J.E.Robinson[4]
• Human B12	(CD4bs)	NIBSC [5]
• Human 17B	(CD4i)	NIBSC [6]
• Human 19B	(V3)	D.R. Burton [7]
• Human 4E10	(gp41 MPER)	NIH [8]
• Human 4.8D	(CD4i)	J.E. Robinson[9]

[4] Moore JP, McCutchan FE, Poon SW, Mascola J, Liu J, Cao Y and Ho DD (1994) Exploration of antigenic variation in gp120 from clades A through F of human immunodeficiency virus type 1 by using monoclonal antibodies. *J Virol* **68**:8350-8364.

[5] Burton DR, Barbas CF, 3rd, Persson MA, Koenig S, Chanock RM and Lerner RA (1991) A large array of human monoclonal antibodies to type 1 human immunodeficiency virus from combinatorial libraries of asymptomatic seropositive individuals. *Proc Natl Acad Sci U S A* **88**:10134-10137.

[6] Thali M, Moore JP, Furman C, Charles M, Ho DD, Robinson J and Sodroski J (1993) Characterization of conserved human immunodeficiency virus type 1 gp120 neutralization epitopes exposed upon gp120-CD4 binding. *J Virol* **67**:3978-3988.

[7] Scott CF, Jr., Silver S, Profy AT, Putney SD, Langlois A, Weinhold K and Robinson JE (1990) Human monoclonal antibody that recognizes the V3 region of human immunodeficiency virus gp120 and neutralizes the human T-lymphotropic virus type IIIMN strain. *Proc Natl Acad Sci U S A* **87**:8597-8601.

[8] Stiegler G, Kunert R, Purtscher M, Wolbank S, Voglauer R, Steindl F and Katinger H (2001) A potent cross-clade neutralizing human monoclonal antibody against a novel epitope on gp41 of human immunodeficiency virus type 1. *AIDS Res Hum Retroviruses* **17**:1757-1765.

[9] Thali M, Moore JP, Furman C, Charles M, Ho DD, Robinson J and Sodroski J (1993) Characterization of conserved human immunodeficiency virus type 1 gp120 neutralization epitopes exposed upon gp120-CD4 binding. *J Virol* **67**:3978-3988.

- Human 412D (CD4i) NIBSC [10]
- Human 8B (C1-C5) J.E.Robinson
- Human CD4IgG2 Polygenics, IAVI
- Human HGP68 D. Corti[11]
- Human HJ16 (CD4bs) D. Corti[12]
- Human HR10 (V3) D. Corti[13]
- Human X5 (CD4i) NIBSC[14]
- Human 447-52D (tissue supernatant) (V3) NIBSC [15]
- Rabbit Polyclonal Ab (pAb) antiserum to $gp120_{IIIb}$ NIBSC

Fluorescently-labelled antibodies for flow cytometry (all anti-mouse)

Hamster CD3-APC	AbD Serotec
Rat CD3-PE	BD
Rat CD11b-PerCP	AbD Serotec
Hamster CD11c-$Alexa_{647}$	BD
Hamster CD11c-APC	AbD Serotec

[10] Choe H, Li W, Wright PL, Vasilieva N, Venturi M, Huang CC, Grundner C, Dorfman T, Zwick MB, Wang L, Rosenberg ES, Kwong PD, Burton DR, Robinson JE, Sodroski JG and Farzan M (2003) Tyrosine sulfation of human antibodies contributes to recognition of the CCR5 binding region of HIV-1 gp120. *Cell* **114**:161-170.

[11] Corti D, Langedijk JP, Hinz A, Seaman MS, Vanzetta F, Fernandez-Rodriguez BM, Silacci C, Pinna D, Jarrossay D, Balla-Jhagjhoorsingh S, Willems B, Zekveld MJ, Dreja H, O'Sullivan E, Pade C, Orkin C, Jeffs SA, Montefiori DC, Davis D, Weissenhorn W, McKnight A, Heeney JL, Sallusto F, Sattentau QJ, Weiss RA and Lanzavecchia A (2010) Analysis of memory B cell responses and isolation of novel monoclonal antibodies with neutralizing breadth from HIV-1-infected individuals. *PLoS One* **5**:e8805.

[12] Ibid.

[13] Ibid.

[14] Moulard M, Phogat SK, Shu Y, Labrijn AF, Xiao X, Binley JM, Zhang MY, Sidorov IA, Broder CC, Robinson J, Parren PW, Burton DR and Dimitrov DS (2002) Broadly cross-reactive HIV-1-neutralizing human monoclonal Fab selected for binding to gp120-CD4-CCR5 complexes. *Proc Natl Acad Sci U S A* **99**:6913-6918.

[15] Gorny MK, Williams C, Volsky B, Revesz K, Cohen S, Polonis VR, Honnen WJ, Kayman SC, Krachmarov C, Pinter A and Zolla-Pazner S (2002) Human monoclonal antibodies specific for conformation-sensitive epitopes of V3 neutralize human immunodeficiency virus type 1 primary isolates from various clades. *J Virol* **76**:9035-9045.

Hamster CD11c-FITC	AbD Serotec
Rat CD19-PE	AbD Serotec
Rat CD40-PE	BD
Rat CD80-FITC	BD
Rat CD86-PE	BD
Rat F4/80-PE	BD
Rat Ly6C-APC	AbD Serotec
Rat LY6C-FITC	AbD Serotec
Rat MHCII-FITC	eBioscience

Isotype controls:

Rat IgG2a-FITC, **κ** isotype control (for MHCII-FITC)	AbD Serotec
Rat IgG2a-PE, **κ** isotype control (for CD19-PE)	AbD Serotec
Hamster IgG1- APC, **κ** isotype control (for CD3-APC)	AbD Serotec
Rat IgM- FITC, **κ** isotype control (for Ly6C-APC)	AbD Serotec
Rat IgG2b – PE, isotype control (for F4/80-PE)	AbD Serotec

Fluorescently-labelled antibodies for LSCM

Biotinylated anti-mouse CD107A (Lamp-1)	ebioscience
Rabbit anti-mouse EEA1	Abcam
Biotinylated goat anti-mouse Cathepsin D	R & D Systems
Biotinylated anti-mouse F4/80	BD
Goat anti-rabbit (Cy5)	Jackson
Streptavidin (TRITC)	AbD Serotec

2.5. Software & Online databases

- BIAevaluation (V 3.2 RC1)
- Bio-plex manager (V 4.0)
- CellQuest (V 3.3)
- ExPasY and ProtParam (online)
- Fold and Function Assignment (FFAS) database (online)
- Flowjo (V 9.2)
- FV10-ASW 2.0 Viewer (V 2.0b)
- Genebank database (online)
- GraphPad Prism (V 5.0)
- ImageJ (and JaCoP plugin) (V 1.44o)
- Macvector software (V 7.2.3, C2004 Accelrys
- Nanodrop ND 1000 (V3 1.2)
- PDB Protein Data Bank database (online)
- MacPymol (and APBS plugin)

2.6. Standard antigens

2.6.1. HIV-1 gp140$_{97CN54}$

The standard antigen used for immunisation and *in vitro* assays throughout this project is a naturally uncleaved HIV-1 Env gp140 derived from HIV-1$_{97CN54}$, a recombinant CCR5-using isolate of Chinese clade B/C (Cranage et al., 2010; Rodenburg et al., 2001; Su et al., 2000) and kindly provided by S. Jeffs. HIV-1 gp140$_{97CN54}$ comprises gp120 and the external domain of gp41 and was expressed in a Chinese Hamster Ovary cell line (CHO-K1) and purified using an antibody (5F3)-affinity column. Following purification gp140$_{97CN54}$ was exposed to size exclusion chromatography (SEC) to further isolate trimeric gp140$_{97CN54.}$ For *in vivo* experiments gp140$_{97CN54}$ was manufactured to endotoxin-free (GMP) specification by Polymun. If required, gp140$_{97CN54}$ was concentrated using Amicon 10kDa filters (Millipore) according to manufacturer's protocol. The resulting concentration was determined using Nanodrop (ext. coefficient 125400, MW 150 kDa).

In addition to $gp140_{97CN54}$, the following other Env glycoproteins were used [source, expression system, purified]: $gp140_{92UG37}$ [S.Jeffs, CHO, purified] (Jeffs et al., 2004); $gp140_{IIIb}$ [S.Jeffs, CHO, purified] (Jeffs et al., 2004); $gp140_{BX08}$ [B.Verrier, CHO, purified] Moog et al., 1997); $gp120_{W61D}$ [NIBSC, EVA648, Dr G.Voss, CHO, purified] (Groenink et al., 1992); $gp120_{BaL}$ [NIBSC 6007, Human Embryonic Kidney 293 (HEK-293) cells, purified](Gartner et al., 1986); $gp120_{SF162}$ [Immune Technology Corp., HEK-293 cells, purified](Cheng-Mayer et al., 1998); $gp120_{IIIb}$ [NIBSC 0607, Baculovirus expression system, purified](Jeffs et al., 2004);$gp120_{97CN54}$ [I.Jones , 293 Freestyle (293F) expressed, purified] (Jeffs et al., 2004).

2.6.2. Hen egg white ovalbumin (OVA)

For antigen-uptake studies in the peritoneal model a fluorescently labelled form of the model antigen hen egg white OVA, OVA-$Alexa_{647}$ was purchased from Molecular Probes, Invitrogen. For binding studies endograde (low-endotoxin) OVA was purchased from Hyglos.

2.7. Adjuvant preparation

2.7.1. Adjuvant forms

All PEI forms were dissolved in PBS and neutralised to pH ~7.2 with 5 M NaOH, prepared in endotoxin-free H_2O. The bPEI 25 kDa form for standard use was purchased from Sigma-Aldrich. **LPEI forms:** lPEI salt 5 (4 kDa), lPEI salt 50 (40kDa), lPEI salt 200 (160 kDa), and lPEI salt 500 (400 kDa) were purchased from Polymer Chemistry Innovations, Inc.. InvivoJet-PEI (22 kDa) was purchased from Polyplus. **BPEI forms:** PEI solution 03880 (Mr 600 –1000), PEI solution 482595 (MW 1.3 kDa), PEI solution 408700 (MW 2 kDa), and PEI solution P3143 (MW 750 kDa) were purchased from Sigma-Aldrich. **Biodegradable PEI forms:** InvivoJet-PEI SS (22 kDa) was kindly provided by Polyplus; G2-HD-OEI and OEI-BAA-OEI were kindly provided by C. Dohmen. **Fluorescently labelled PEI forms:** Jet-PEI-FluoF (fluorescein, exc. 490 nm, em. 520 nm) and Jet-PEI-FluoR (tetramethylrhodamine, exc. 490 nm, em. 520 nm) were purchased from Polyplus. PEI was tested at the clinical biomanufacturing facility (University of Oxford, UK) to confirm that the presence of endotoxin was <0.5 Endotoxin Units (EU)/mL. The amount of PEI used for immunisation was chosen based on titration studies. Cholera Toxin Beta subunit (CTB) (Sigma-Aldrich) was dialysed with Slide-A-Lyser cassettes (Pierce Scientific) against PBS to remove sodium azide and concentrated as required using Amicon 10kDa filters (Millipore). The resulting concentration was determined using Nanodrop (Ext. coefficient: 11522.5–average of situation with 0% or 100% reduction of cysteines- and MW 12 kDa). Alhydrogel® Aluminium Hydroxide Gel Adjuvant was purchased from Brenntag and dissolved in PBS. For rabbit immunisation Imject Alum $Al(OH)_3$ $Mg(OH)_2$ was purchased from Pierce. Freund's Complete Adjuvant (FCA) and Freund's Incomplete Adjuvant (FIA) were purchased from Sigma-Aldrich. For Bone Marrow Derived

Dendritic Cell (BMDC) analysis lipopolysaccharide (LPS) from E. Coli adjuvant was purchased from Invivogen.

2.8. Fluorescent labelling of PEI and gp140$_{97CN54}$

In the current section the Alexa$_{488}$-labelling of PEI and gp140$_{97CN54}$ antigen is discussed and the rationale behind the choice of commercially available OVA$_{647}$ over the manually labelled forms for use in the *in vivo* association experiments is outlined (Section 5.2.2.2). The described Alexa$_{488}$-conjugated forms of PEI and gp140$_{97CN54}$ were used for the *in vitro* peritoneal macrophage assay (Section 5.2.2.4).

The Alexa-Fluor$_{488}$ (exc. 494 nm, em. 519 nm) microscale-protein labelling kit was chosen because of the superiority of the Alexa$_{488}$ signal and the suitability of the carboxylic acid, tetrafluorophenyl (TFP) ester conjugate for the coupling of amine groups in PEI and gp140$_{97CN54}$.

2.8.1. Alexa$_{488}$-conjugation of gp140$_{97CN54}$ antigen

The gp140$_{97CN54}$ stock was buffer exchanged from Tris buffer into PBS to remove the present primary amines from the buffer, and the conjugation procedure was conducted based on the manufacturer's protocol. A molar ratio (MR) (dye:protein) of 55 was chosen for an optimal degree of labelling (DOL). The resulting Alexa$_{488}$-gp140$_{97CN54}$ was then again buffer exchanged into PBS to remove free unconjugated Alexa$_{488}$-Fluor ester from the solution. To optimise the amount of gp140$_{97CN54}$ for further experiments and to investigate whether the resulting Alexa$_{488}$-gp140$_{97CN54}$ was suitable for use in the injection and flow cytometric assays of the following sections, two aspects needed to be tested: 1) the brightness of the Alexa$_{488}$-gp140$_{97CN54}$ and its detectability in our FACSCalibur cytometer; 2) a

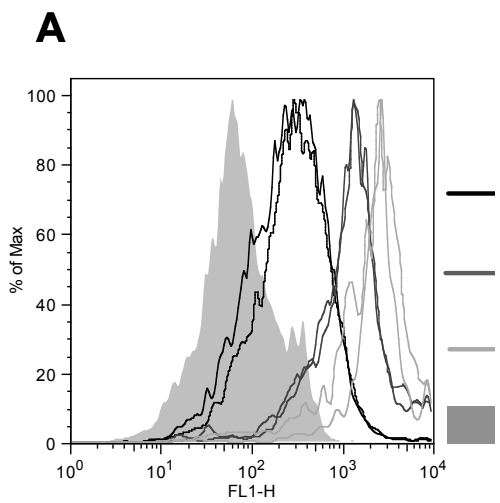
pilot i.p. injection needed to be conducted to ensure that the $Alexa_{488}$-$gp140_{97CN54}$ at the chosen amount did not impair leukocyte recruitment into the peritoneal cavity.

To investigate these aspects mice were i.p. injected with various doses of $Alexa_{488}$-conjugated $gp140_{97CN54}$ (10 µg, 50 µg, 116 µg) or unconjugated $gp140_{97CN54}$ (10 µg) mixed with PEI (70 µg). At 16 h post-injection, mice were sacrificed and peritoneal lavages collected. Peritoneal cells were stained with leukocyte surface marker F4/80 and analysed using flow cytometry. F4/80 high cell population (including macrophages and monocytes) was selected and $Alexa_{488}$-fluoresence intensity was analysed in Fl-1 histograms (Figure 2.1A). The frequency and mean fluorescence intensity (MFI) for each group is depicted in Figure 2.1B.

All $Alexa_{488}$-$gp140_{97CN54}$ doses induced a detectable change in $Alexa_{488}$ fluorescence intensity compared to the control group of unconjugated $gp140_{97CN54}$ and PEI. As expected, the size of the shift in the FL-1 histogram increased with increasing concentration of $Alexa_{488}$-$gp140_{97CN54}$, but this was accompanied by an effect of $F4/80^{HI}$ population frequency. The frequency of the $F4/80^{HI}$ cell population was most similar to the unlabelled $gp140_{97CN54}$ control group in the group that received the lowest dose of $Alexa_{488}$-$gp140_{97CN54}$ (10 µg, F4/80 high count ~4971/5116 versus 5828/4428 for the control group). At this lowest $Alexa_{488}$-$gp140_{97CN54}$ dose, $Alexa_{488}$ MFI was still detectably higher (242, 285) than the control group (73.9, 67.2). Because of this optimal ratio of signal to adjuvant impairment, the 10 µg $Alexa_{488}$-$gp140_{97CN54}$ dose was chosen for further studies.

Despite the above observations the signal of 10 µg was repeatedly undetectable in later flow cytometry assays. Choosing a higher dose of $Alexa_{488}$-$gp140_{97CN54}$ was undesirable because of its observed effect on the leukocyte profile that implied impairment of adjuvant activity. Therefore, a commercially available form of $Alexa_{647}$-OVA, a model antigen was chosen for the i.p. studies in

Section 4.2.2.2, while the Alexa$_{488}$-gp140$_{97CN54}$ stock was used for *in vitro* analysis of peritoneal macrophage gp140$_{97CN54}$ uptake (Section 5.2.2.4).



B

	F4/80 ^{hi} count	F4/80 ^{hi} freq of parent	geom mean MFI Fl-1
10 µg gp140-alexa$_{488}$ & PEI	4971	24.6	242
	5116	25.2	285
50 µg gp140-alexa$_{488}$ & PEI	2062	10.2	1129
	1613	7.96	1163
116 µg gp140-alexa$_{488}$ & PEI	945	4.66	2323
	1286	6.35	1861
10 µg gp140 & PEI	5828	28.8	73.9
	4428	21.8	67.2

Figure 2.1. Optimisation of Alexa$_{488}$-conjugation of gp140$_{97CN54}$through analysis in the peritoneal model and flow cytometry. Mice (*n*=2) were i.p. injected with various amounts of Alexa$_{488}$-gp140$_{97CN54}$ (10 µg, 50 µg, 116 µg) or unconjugated gp140$_{97CN54}$ (10 µg) mixed with PEI (70 µg). At 16 h post injection mice were sacrificed and peritoneal lavages collected. Peritoneal cells were stained with fluorescent leukocyte surface marker F4/80 and analysed using FACSCalibur cytometer**. A) Alexa$_{488}$ fluorescence intensity of peritoneal F4/80^{HI} population. B) Frequency and Alexa$_{488}$ MFI for peritoneal F4/80^{HI} population.**

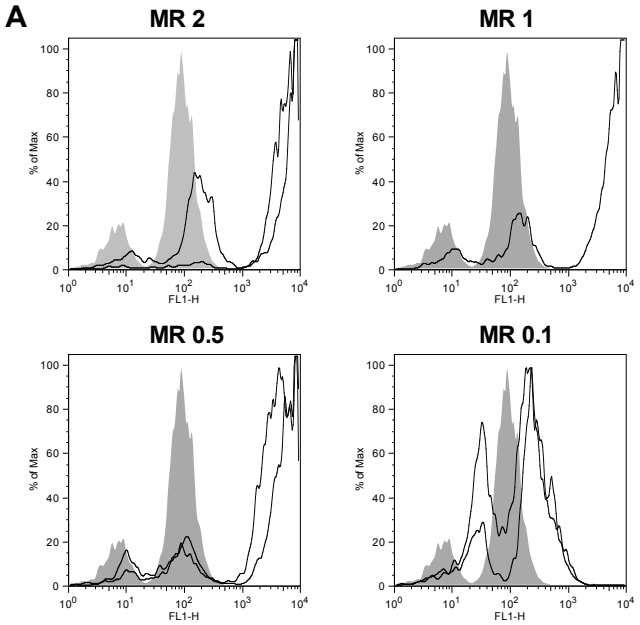
2.8.2. Alexa$_{488}$-conjugation of PEI

Alexa$_{488}$-conjugation of PEI was also conducted based on the manufacturer's protocol. Because of the presence of exceptionally high numbers of amine groups in PEI and the unpredictability of the effect of this on the DOL, PEI conjugation to Alexa$_{488}$ was carried out at several different MRs of 10, 5, 2, 1, 0.5, and 0.1. Again, to optimise the DOL for further experiments 1) the brightness of the Alexa$_{488}$-PEI in our FACSCalibur cytometer was determined to ensure that the fluorescence signal was not to bright to allow compensation between fluorescence channels and 2) a pilot i.p. injection was

conducted to ensure that the Alexa$_{488}$-PEI at the chosen amount did not impair leukocyte recruitment into the peritoneum. Mice were i.p. injected with 70 µg Alexa$_{488}$-PEI at the above MR or as a control one group of mice received an equivalent amount of unconjugated Alexa$_{488}$ Fluor with PEI. At 16 h post-injection, mice were sacrificed and peritoneal lavages collected. Peritoneal cells were stained with leukocyte surface marker F4/80 and analysed using flow cytometry as before. F4/80 high cell population (including macrophages and monocytes) was selected and FITC Alexa$_{488}$-fluorescence intensity was analysed in Fl-1 histograms (Figure 2.2A). The frequency and mean fluorescence intensity for each group is depicted in Figure 2.2B. The i.p. injection was not completely successful for one mice in the MR 1 group and this sample was therefore excluded from the analysis.

The Alexa$_{488}$-PEI forms prepared at MR 10 and MR 5 were too bright for detection with the FACSCalibur cytometer in a four-colour assay (off-scale signal, see Figure 2.2A). For all MRs a shift in the Alexa$_{488}$ fluorescence histogram was detectable compared control group. The size of the shift and the MFI increased with increasing MR, as expected. The lowest MR group still showed a higher MFI (111-154) than the control group (57.3). Similar to the Alexa$_{488}$-gp140$_{97CN54}$ pilot study, the increase in Alexa$_{488}$-PEI fluorescence intensity was accompanied by a drop in F4/80 high frequency and cell counts, suggesting an impaired adjuvant activity with the highly labelled PEI forms. Therefore, the Alexa$_{488}$-PEI form that was conjugated at the lowest MR was chosen for further use. Subsequent assays in the i.p. model using this MR 0.1 Alexa$_{488}$-PEI form resulted in the Alexa$_{488}$-PEI being too bright on our FACSCalibur cytometer for detection within quantifiable range and compensation in a four-colour assay. Following attempts to conjugate a less bright form of Alexa$_{488}$-PEI and subsequent analysis showed that the DOL was variable despite the adjustment of conjugation conditions. Based on this, it was decided to use a commercially available form of lPEI, Jet–PEI–FluoF® that has a known DOL and therefore would add to the consistency of subsequent

flow cytometry experiments. Alexa$_{488}$-PEI was used for the *in vitro* peritoneally-derived macrophage assay (Section 5.2.2.4).



B

	F4/80 ^{hi} count	F4/80 ^{hi} freq of parent	geom mean MFI Fl-1
alexa$_{488}$ –PEI (MR 2)	2724	16.1	4134
	2608	14.7	1187
alexa$_{488}$ - PEI (MR 1)	*2097*	*12.4*	*53.3*
	2922	15.9	1525
alexa$_{488}$ -PEI (MR 0.5)	2155	11.9	1371
	3464	19.5	1834
alexa$_{488}$ -PEI (MR 0.1)	3752	20.6	154
	3476	19.5	111
Alexa$_{488}$ and PEI (unconjugated)	3058	19.1	57.3

Figure 2.2. Alexa$_{488}$-conjugation PEI, analysis using peritoneal model and flow cytometry. Mice (n=1-2) were i.p. injected with 70 µg Alexa$_{488}$-PEI of molar ratios (MR) of 0.1, 0.5, 1, or 2, or unconjugated Alexa $_{488}$ Fluor mixed with 70 µg PEI (control). At 16 h post injection mice were sacrificed and peritoneal lavages collected. Peritoneal cells were stained with leukocyte surface marker F4/80 and analysed using a FACSCalibur flow cytometer **A) Alexa$_{488}$ fluorescence intensity of peritoneal F4/80^{HI} population. B) Frequency and mean Alexa$_{488}$ fluorescence intensity for F4/80^{HI} population.**

2.9. Animal procedures

2.9.1. Animals

6-12 week old female BALB/c mice were obtained from the specified pathogen-free breeding colony at the Pathology Support Building at the Sir William Dunn School of Pathology, University of Oxford, UK. These mice were housed in microisolator cages with filtered air. Female New Zealand White (NZW) rabbits were housed at the Department of Veterinary Medicine, University of Cambridge. All experiments were performed under appropriate licenses in accordance with the UK Animals (Scientific Procedures) Act 1986. Animals were monitored for adverse reactions.

2.9.2 *In vivo* administration protocols

Samples of immunogens, adjuvants and buffers were tested for endotoxin at the Clinical BioManufacturing Facility (University of Oxford, UK). Any immunogens that contained ≥ 1 EU/mL endotoxin were passed through a Detoxi-Gel™ endotoxin affinity column (Perbio Science UK Ltd) according to manufacturer's instructions to minimise endotoxin content. Formulations for immunisation were prepared under sterile conditions. The total administered volume per mouse was made up to 10-11 µL for intranasal (i.n.) immunisations, 100 µL for subcutaneous (s.c) immunisations, and 500 µL for intraperitoneal (i.p.) administration, by addition of sterile endotoxin-free PBS. For i.n. immunisation, mice were lightly anaesthetised using Isoflurane (Abott) and the formulation was instilled into both nostrils using a micropipette. S.c. immunisations and i.p. administration were carried out using 0.5 or 1 mL micro-fine insulin syringes. Prior to immunisation, adjuvant and $gp140_{97CN54}$ antigen were mixed according to manufacturer's instruction. PBS-dissolved and pH-neutralised PEI was added dropwise to antigen and left to

incubate for 15 min before administration. In the case of Alhydrogel or Imject®Alum, adjuvant was added dropwise to $gp140_{97CN54}$ antigen followed by continuous mixing for 30 min at RT to allow the adjuvant to efficiently adsorb the antigen. Rabbit immunisations were carried out with a total volume of 94–200 µL per injection. FCA or FIA were emulsified using a 19G needle and 1:1 (v:v) mixed with $gp140_{97CN54}$ antigen. Antigen was similarly adsorbed 1:1 (v:v) to Imject®Alum. The dose of antigen, the dose of adjuvant, the immunisation route and the immunisation schedule are indicated in the figure legends and text of each individual chapter.

2.10. Blood, vaginal lavage, peritoneal lavage, and tissue sampling

2.10.1. Blood collection

Blood samples were collected at baseline, at one or two timepoints prior to boost immunisation and at suitable timepoints post boost immunisation to allow measurement of peak antibody endpoint titre and long-term antibody titres (up to 6 months). 50 µL samples of blood were collected in Microtainer SST ™ (BD) by venous puncture of the basal tail vein. Samples were allowed to clot for 30 min at 37°C before centrifugation at 6200 rotations per minute (RPM) at 4°C for 10 min. Serum was collected and stored in 96-well V-bottom plates at-20°C until analysis.

2.10.2. Vaginal lavage collection

Similar to blood samples, vaginal lavages were collected at baseline, at one or two timepoints prior to boost immunisation and at suitable timepoints post boost immunisation to allow measurement of peak antibody endpoint titre and long –term antibody titres. Mice were lightly anaesthetised using isoflurane (Abott) and vaginal mucosal lavage samples were obtained by pipetting 2 x 50 µL of sterile

PBS in and out of the vagina several times using sterile blunted micropipette tips. Vaginal lavage samples were cleared by centrifugation at 14000 RPM at 4°C for 10 min and stored in 96-well V-bottom plates at-20°C until analysis.

2.10.3. Peritoneal lavage collection

Peritoneal lavage was collected at 4, 12, 16 or 24 h after peritoneal administration of adjuvant and/or antigen for analysis of leukocyte populations and leukocyte adjuvant/antigen association. Peritoneal lavages were obtained as previously described (Cash et al., 2009). Briefly, mice were sacrificed with a rising CO_2 concentration and a small (~0.5 cm) incision was made at the base of the diaphragm that allowed the skin to be pulled back so that the abdomen was exposed. The exposed abdomen was sprayed with 70% ethanol and 7 mL of icecold PBS-EDTA (2 mM)-FCS (1-2%) were injected into the peritoneal cavity using a 10 mL syringe and a 25 G needle. After gently shaking the abdomen by pulling the mouse at each leg 5mL of the peritoneal lavage fluid was carefully collected using the same syringe and needle as before. Lavage samples were stored on ice until centrifugation at 1700 RPM for 5 min at 4°C to collect the cell pellet. Total cell numbers were counted using trypan blue and a haemocytometer or cell Liquid Cell Counting beads (BD Cell Viability kit). Cells were prepared for flow cytometry analysis or LSCM. Alternatively, for analysis of cytokine/chemokine profiling, a volume of 2mL of peritoneal fluid was collected instead of the above 5mL. Peritoneal lavages were stored on ice before centrifugation at 1700 RPM for 5 min at 4°C to collect supernatant. Supernatant samples were stored at-20°C until analysed using the Bio-Plex cytokine assay (Bio-Rad) according to the manufacturer's instructions.

2.10.4. Spleen and LN collection

To harvest splenocytes and cervical lymph node (CLN) cells, mice were sacrificed with a rising CO_2 concentration and the spleen and CLN were removed under sterile conditions and placed into ice-cold complete RPMI medium (RPMI 1640 with 4mM L-glutamine, 10% (v/v) FCS, 100 µg/mL P/S, 50 µM 2-ME) and kept on ice. Splenocytes were obtained by gently applying pressure to the spleen capsule using the back of a 1mL syringe plunger. The resulting cell suspension was centrifuged at 1700 RPM for 5 min at 4°C. The cell pellet was resuspended in ACK lysing buffer (Invitrogen) and incubated for 5 min at 37°C to achieve red blood cell lysis before the suspension was filtered through a 70 µm Falcon cell strainer (BD). The cell suspension was washed by centrifugation at 1700 RPM for 4 min at 4°C and resuspended in ice-cold complete RPMI medium. Splenocytes in each sample were counted using trypan blue and a haemocytometer. CLN cells were obtained by cutting the CLN with 25 G needle (Terumo 1.1 x 40 mm). The resulting cell suspension was sieved through a 70 µm Falcon cell strainer (BD), centrifuged at 1700 RPM for 4 min at 4°C, resuspended in complete RPMI and CLN cells were counted as above.

2.10.4.1. Thymocyte restimulation and proliferation

To measure $gp140_{97CN54}$-specific responses, splenocytes or CLN cells were cultured in the presence or absence of 10 µg/mL antigen and cultured for 48h (splenocytes) or 72h (CLN). Cells were pulsed with 1 µCi of [^{3}H] thymidine per well (Perkin-Elmer) for the last 8-12 h of incubation. The cellular DNA was harvested on glass-fibre filters and then assayed by liquid scintillation counting. Data are expressed as counts per minutes (cpm). For cytokine profiling, supernatants were obtained after 48h of antigen-restimulation and stored at-80°C until analysed using a Bio-Plex cytokine assay (Bio-Rad) according to the manufacturers instructions.

2.11. Enzyme-linked immunosorbent assays (ELISAs)

Between individual steps, wells were washed with 300 µL/well ELISA wash buffer (0.05% Tween-20 in PBS).

2.11.1. Detection of gp140-specific/gp120-specific serum antibodies

Flat-bottom 96-well Immulon II ELISA plates were coated overnight at 4°C with 50 µL/well of 0.5 µg/mL $gp140_{97CN54}$, gp140 of other HIV-1 isolates, or gp120 of other HIV-1 isolates, or HEL (negative control) in PBS (pH 7.4) buffer. Coated plates were blocked with 200 µL/well ELISA blocking buffer (2% non-fat dried milk, 0.05% Tween-20 in PBS) for 2 h. Serum samples were serially diluted in ELISA sample buffer (1% BSA, 0.05% Tween-20 in PBS) and transferred to antigen-coated plates (50 µL/well) for 1 h incubation. HIV-1 gp140/gp120-specific antibodies were detected by incubating 50 µL/well of one of the following appropriately diluted HRP-conjugated secondary antibodies for 1 h: goat anti-mouse IgG (AbD Serotec), mouse anti-rabbit IgG (Sigma-Aldrich, Fcγ-conjugated). Plates were developed using 50 µL/well TMB ELISA substrate (Thermo Scientific). The reaction was stopped with 50 µL/well of 0.5 M H_2SO_4. The OD_{450} was measured using a Molecular Devices M5 plate reader according to manufacturer's instructions. Antigen-specific antibody responses were expressed as endpoint titres that were calculated non-linear regression (five parameter) curve fitting using GraphPad Prism software. The endpoint titre was defined as the highest serum dilution giving an absorbance greater than the cut-off threshold (background & 0.02 ≥ background & 2 SD). Pre-immunisation sera consistently gave a signal below detection (below 2 SD above average background signal).

2.11.2. Detection of vaginal lavage gp140-specific IgA and IgG responses

Flat-bottom 96-well Immulon II ELISA plates were coated overnight at 4°C with 50 µL/well of 2 µg/mL $gp140_{97CN54.}$ Coated plates were blocked with 200 µL/well ELISA blocking buffer for 2 h. Post- and pre-immunisation vaginal lavage samples were serially diluted in ELISA sample buffer and transferred to antigen-coated plates (50 µL/well) for 1 h incubation. HIV-1 gp140-specific IgG and IgA antibodies were detected by incubating 50 µL/well of 1: 10 000 diluted HRP-conjugated goat anti-mouse IgG and a biotin-conjugated goat anti-mouse IgA antibody (both AbD Serotec, 1: 10 000 and 1: 8000 dilution in ELISA sample buffer, 50 µL/well) respectively for 1 h. Plates incubated with the HRP-conjugated goat anti-mouse IgG were developed and read at OD_{450}. Plates incubated with biotin-conjugated goat anti-mouse IgA antibody were incubated with HRP-conjugated streptavidin (BD, 1:10 000 dilution in ELISA sample buffer) for 1 h followed by development and OD_{450} reading as before. HIV-1 gp140-specific antibody responses were expressed as endpoint titres that were calculated by non-linear regression (five parameter) curve fitting using GraphPad Prism software.

2.11.3. Studying the accessibility of $gp140_{97CN54}$ epitopes

Flat-bottom 96-well Immulon II ELISA plates were coated overnight at 4°C with 50 µL/well of 0.2 µg/mL $gp140_{97CN54.}$ Coated plates were blocked with 200 µL/well ELISA blocking buffer for 2 h. Half of the wells were incubated with ELISA sample buffer only. The other half of the wells were incubated with a saturating amount of PEI (0.354 mg/mL based on MW $gp140_{97CN54}$ 140 kDa including glycosylation, MW PEI 25 kDa) in ELISA sample buffer for 1 h. Human B12 mAb was serially diluted in ELISA sample buffer and transferred to antigen-coated plates (50 µL/well) for 1 h incubation. HRP-conjugated donkey anti-human secondary antibody (Jackson) 1: 10 000 diluted in

ELISA sample buffer) was incubated for 1 h. As a negative control, $gp140_{97CN54}$-coated plates were incubated with a saturating amount of PEI, followed by incubation with the above detection antibody. Plates were developed and read at OD_{450} as before (Section 2.11.1). The protocol was tested with and without washing in between incubation steps and results were the same. Results were converted into % of max binding (OD_{450} at plateau of nonlinear regression fitted curve).

2.11.4. Assessing the conformation stability or potential denaturation of $gp140_{97CN54}$ antigen

2.11.4.1. Preparation of heat-denatured $gp140_{97CN54}$

Original stock (0.5 mg/mL) was diluted 1:100 to 5 µg/mL in 1% SDS, 50mM DTT and heated at 95 °C for 5 min.

2.11.4.2. ELISA

Flat-bottom 96-well Immulon II ELISA plates were coated with native or heat-denatured $gp140_{97CN54}$ (0.5 µg/mL in PBS or $NaHCO_3$ respectively, 50 µL/well) overnight at 4°C. Coated plates were blocked with 200 µL/well ELISA blocking buffer for 2 h. Rabbit serum samples (50 µL/well) were serially diluted, in ELISA samples buffer and incubated for 1 h. The starting dilution was low so that a plateau in nonlinear regression fitting would be reached. As a control for native $gp140_{97CN54}$ binding, human B12 antibody, that recognises a conformation-dependent epitope, was serially diluted in ELISA sample buffer and incubated (50 µL/well) for 1h. As a control for heat-denatured $gp140_{97CN54}$ binding rabbit polyclonal antiserum to IIIB gp120 was serially diluted in ELISA sample buffer and incubated (50 µL/well) for 1h. HRP-conjugated mouse anti-rabbit IgG (Fc-specific, Sigma-Aldrich, 1: 10 000 diluted in ELISA sample buffer) or HRP-conjugated donkey anti-human secondary antibody (Jackson, 1: 10 000 dilution in ELISA sample buffer) in case of the B12 control,

wells were incubated for 1 h. Plates were developed and read at OD_{450} as before (Section 2.11.1). The EC_{50} for both native $gp140_{97CN5}$ and heat-denatured $gp140_{97CN54}$ binding were determined using non-linear regression (five parameter) curve fitting (GraphPad Prism software). The ratio of EC_{50} for native divided by the EC_{50} for heat-denatured $gp140_{97CN54}$ was calculated and depicted.

2.11.5. Cross-competition ELISA with human mAbs

Flat-bottom 96-well Immulon II ELISA plates were coated with 0.2 µg/mL $gp140_{97CN54}$ in PBS (50 µL/well) overnight at 4°C. Coated plates were blocked with 200 µL/well ELISA blocking buffer for 2 h. Rabbit serum samples (50 µL/well) were serially diluted in ELISA sample buffer and incubated for 1 h. The starting dilution was kept low so that a maximum binding plateau in the nonlinear regression curve fitting model would be reached. Prebleed samples were used as a negative control. Human mAbs were added at EC_{50} (determined prior to this assay in titration assays) and incubated for 2 h. Pilot studies had been used to confirm that results in the herein described protocol did not differ substantially from results obtained with protocols in which mAb and dera were incubated simultaneously. HRP-conjugated donkey anti-human secondary antibody (Jackson, 1: 10 000 dilution in ELISA sample buffer) was incubated for 1 h. Plates were developed and read at OD_{450} as before (Section 2.11.1). IC_{50} values were calculated based on the nonlinear regression curve fitting analysis (GraphPad Prism software) and normalised to total $gp140_{97CN54}$-specific IgG endpoint titre.

2.12. Generation and stimulation of bone marrow-derived DCs (BMDCs)

Female BALB/c mice were sacrificed using rising CO_2 concentration. Femur and tibia bones were surgically removed, separated from the surrounding muscle tissue, and sequentially incubated in 70% ethanol (1 min), sterile endotoxin-free PBS (1 min), sterile endotoxine-free PBS (1 min), and warm 37 °C handling medium (RPMI 1640 with L- glutamine, 10 % FCS, 1 % P/S). Both ends of the bones were removed using scissors. The marrow was flushed out by placing a 5 mL syringe filled with handling medium and a 25 G needle at the top end of the bone and by injecting the handling medium through the bone central canal. Bone marrow derived cells (BMDC) were filtered through a 70 µm cell strainer into a 50 mL tube and washed by centrifugation (1200 rpm for 10 min at 4°C). Cell pellets were resuspended in handling medium, counted using trypan blue and a haemocytometer, and then resuspended in culture medium (RPMI 1640, 10% FCS, 2mM L-glutamine, 1% P/S, 0.00035 % 2-ME, 20 ng/mL recombinant mouse GM–CSF) at a final concentration of 1 x 10^6 cells/mL. BMDC were placed in 24-well flat bottom plates (1 mL/well) and incubated in 5% CO_2 at 37°C. On day 3, culture medium was replaced by fresh pre-warmed culture medium (37°C). On day 6, culture medium was similarly refreshed and immature BMDC were stimulated with PBS, PEI (0.1 µg/mL, 1 µg/mL, 10 µg/mL) or LPS from E.Coli (1 µg/mL) or culture medium only (control). Cells were harvested after 16 h (on day 7) in fresh handling medium by gently pipetting up and down until BMDC were dislodged.

2.13. Flow cytometry

2.13.1. Flow cytometry analysis for phenotypic markers on stimulated BMDCs

After overnight stimulation BMDCs were harvested using ice-cold handling medium (RPMI 1640 with L- glutamine, 10 % FCS, 1 % P/S), centrifuged (1700 RPM for 5 min) and resuspended in handling medium. BMDCs were counted using trypan blue and a haemocytometer. Approximately 2 x 10^5 cells/well were then placed into a 96-well V bottom plate and washed once with FACS wash buffer 1 (1-2% FCS in PBS, 4 °C, 200 µL/well) and centrifuged (1700 RPM for 5 min at 4°C). Plates were aspirated and cells were incubated with rat anti- mouse CD16/CD32 (Fc block) antibody (1: 200 dilution in FACS washing buffer 1, 25 µL) for 5 min on ice. After the Fc block, cells were incubated with the following appropriately diluted Ab combinations or respective isotype controls (total 25 µL in FACS washing buffer 1) for 30 min on ice (4°C, in the dark): **1**) FITC-conjugated rat anti-mouse MHCII & PE-conjugated rat anti-mouse CD40 & PerCP-conjugated rat anti-mouse CD11b & APC-conjugated hamster anti-mouse CD11c; **2**) FITC-conjugated rat anti-mouse CD80 & PE-conjugated rat anti-mouse CD86 & APC-conjugated hamster anti-mouse CD11c; **3**) isotype controls: FITC-conjugated rat IgG2a (equals FITC-conjugated rat anti-mouse CD80 and FITC-conjugated rat anti-mouse MHCII) & PE-conjugated rat IgG2a (equals PE-conjugated rat anti-mouse CD86 and PE-conjugated rat anti-mouse CD40) & APC-conjugated hamster IgGa (equals APC-conjugated hamster anti-mouse CD11c). Following Ab incubation, cells were washed twice with FACS washing buffer 1 (200 µL/well, centrifugation at 1700 RPM for 5 min at 4°C, aspiration) and subsequently fixed in 4% paraformaldehyde in PBS (200 µL/well) for 30 min in the dark at 4°C. Cells were washed twice with FACS washing buffer 1 as above, resuspended in FACS washing buffer

1 and stored at 4°C until flow cytometry using the FACSCalibur flow cytometer (BD) in combination with CellQuest and FlowJo software the next day.

2.13.2. Evaluation of peritoneal leukocyte populations by flow cytometry

Peritoneal cells were harvested from female BALB/c mice 4, 12 and 24 h post injection with PEI (70 µg/mouse), Alhydrogel (1mg/mouse) or PBS (500 µL/mouse, see Section 2.9.2) and prepared and counted as described in Section 2.10.3. Approximately 2 x 10^5 cells/well were placed into a 96-well V bottom plate and washed once with FACS wash buffer 2 (1-2% FCS, 2 mM EDTA in PBS, 4 °C, 200 µL/well) and centrifuged (1700 RPM for 5 min at 4°C). Plates were aspirated and cells were incubated with rat anti- mouse CD16/CD32 (Fc block) antibody (1: 200 dilution in FACS washing buffer 2, 25 µL) for 5 min on ice. After the Fc block, cells were incubated with the following appropriately diluted Ab combinations or respective isotype controls (total 25 µL in FACS washing buffer 2) for 30 min on ice (4°C, in the dark): **1**) FITC-conjugated rat anti-mouse DX5 (CD49B) & PE-conjugated rat anti-mouse CD19 & APC-conjugated rat anti-mouse CD3 & rat anti-mouse CD16/CD32 (Fc block); **2**) FITC-conjugated rat anti-mouse MR & PE-conjugated rat anti-mouse F4/80 & $Alexa_{647}$-conjugated rat anti-mouse CD11c & rat anti-mouse CD16/CD32 (Fc block); **3**) FITC-conjugated rat anti-mouse Ly6C & PE-conjugated rat anti-mouse Ly6G & APC-conjugated rat anti-mouse F4/80 &rat anti-mouse CD16/CD32. Following Ab incubation cells were washed twice with FACS washing buffer 2 (200 µL/well, centrifugation at 1700 RPM for 5 min at 4°C, aspiration). Propidium Iodide (PI) was added (1:200 diluted in FACS washing buffer 2, 200 µL/well) and cells were analysed using a FACSCalibur flow cytometer (BD) in combination with CellQuest and FlowJo software.

2.13.3. Evaluation of peritoneal leukocyte association with OVA_{647} by flow cytometry

Peritoneal cells were harvested from female BALB/c mice 4 and 24 h post injection with PEI (70 µg) plus OVA_{647} (10 µg), Alhydrogel (1mg) plus OVA_{647} (10 µg), PBS and OVA-$Alexa_{647}$ (10 µg), or as control: PEI only (70 µg), Alhydrogel only (1 mg) or PBS only (500 µL/mouse) and prepared and counted as described in Section 2.10.3. Approximately 2 x 10^5 cells/well were placed into a 96-well V bottom plate and washed once with FACS wash buffer 2 (1-2% FCS, 2 mM EDTA in PBS, 4 °C, 200 µL/well) and centrifuged (1700 RPM for 5 min at 4°C). Plates were aspirated and cells were incubated with rat anti- mouse CD16/CD32 (Fc block) antibody (1: 200 dilution in FACS washing buffer 2, 25 µL) for 5 min on ice. After the Fc block, cells were incubated with the following appropriately diluted Ab combinations (total 25 µL in FACS washing buffer 2) for 30 min on ice (4°C, in the dark): **1**) FITC-conjugated hamster anti-mouse CD11c & PE-conjugated rat anti-mouse F4/80 & PerCP-conjugated rat anti-mouse CD11b & rat ant-mouse CD16/CD32 (Fc block); **2**) FITC-conjugated rat anti-mouse Ly6C & PE-conjugated rat anti-mouse F4/80 & PerCP-conjugated rat anti-mouse CD11b & rat anti-mouse CD16/CD32 (Fc block); **3**) PE-conjugated rat anti-mouse CD19 & PerCP-conjugated rat anti-mouse CD11b & rat anti-mouse CD16/CD32 (Fc block); **4**) PE-conjugated rat anti-mouse CD3 & PerCP-conjugated rat anti-mouse C11B & rat anti-mouse CD16/CD32 (Fc block); **5**) FITC-conjugated rat anti-mouse MHCII & PE-conjugated hamster anti-mouse CD11c & PerCP-conjugated rat anti-mouse CD11b & rat anti-mouse CD16/CD32 (Fc block); **6**) isotype controls: FITC-conjugated rat IgM & PE-conjugated rat IgG2B & rat anti-mouse CD16/CD32 (Fc block). Following Ab incubation, cells were washed twice with FACS washing buffer 2 (200 µL/well, centrifugation at 1700 RPM for 5 min at 4°C, aspiration) and subsequently fixed in 4% paraformaldehyde in PBS (100 µL/well) for 30 min in the dark at RT. Cells were washed twice with FACS washing buffer 2 as above, resuspended in FACS washing buffer 2 and stored at

4°C until flow cytometry using a FACSCalibur flow cytometer (BD) in combination with CellQuest and FlowJo software.

2.13.4. Evaluation of peritoneal leukocyte association with Jet-PEI FluoF® by flow cytometry

Peritoneal cells were harvested from female BALB/c mice 24 h post injection with unlabelled Jet-PEI (70 µg), FluoF® FITC-labelled Jet-PEI (70 µg) or PBS (500 µL/mouse) and prepared and counted as described in Section 2.10.3. Approximately 2 x 10^5 cells/well were placed into a 96-well V bottom plate and washed once with FACS wash buffer (1-2% FCS in PBS, 4 °C, 200 µL/well) and centrifuged (1700 RPM for 5 min at 4°C). Plates were aspirated and cells were incubated with rat anti- mouse CD16/CD32 (Fc block) antibody (1: 200 dilution in FACS washing buffer, 25 µL) for 5 min on ice. After the Fc block, cells were incubated with the following appropriately diluted Ab combinations (total 25 µL in FACS washing buffer) for 30 min on ice (4°C, in the dark): **1**) PE-conjugated rat anti-mouse F4/80 & PerCP-conjugated rat anti-mouse CD11b & APC-conjugated hamster anti-mouse CD11c & rat anti-mouse CD16/CD32 (Fc block); **2**) PE-conjugated rat anti-mouse CD19 & PerCP-conjugated rat anti-mouse CD11b & APC-conjugated rat anti-mouse CD3 & rat anti-mouse CD16/CD32 (Fc block); **3**) PE-conjugated rat anti-mouse F4/80 & PerCP-conjugated rat anti-mouse CD11b & APC-conjugated rat anti-mouse Ly6C & rat anti-mouse CD16/CD32 (Fc block); **4**) isotype controls I: FITC-conjugated rat IgM **κ** isotype & PE-conjugated rat anti-mouse F4/80 & APC-conjugated hamster IgG1 **κ** isotype & rat anti-mouse CD16/CD32 (Fc block); **5**) isotype controls II: PE-conjugated rat IgG2B & APC-conjugated rat anti-mouse Ly6C & rat anti-mouse CD16/CD32 (Fc block). Following Ab incubation, cells were washed twice with FACS washing buffer 2 (200 µL/well, centrifugation at 1700 RPM for 5 min at 4°C, aspiration) and

subsequently fixed in 4% paraformaldehyde in PBS (100 µL/well) for 30 min in the dark at RT. Cells were washed twice with FACS washing buffer 2 as above, resuspended in FACS washing buffer 2 and stored at 4°C prior to flow cytometry using a FACSCalibur flow cytometer (BD).

2.14. *Ex vivo* restimulation of splenocytes and LN T cells

Single cell suspension from spleen and CLN were prepared as described in Section 2.10.4. Splenocytes and CLN cells were placed in 96-well round bottom plates (0.5 x 10^6 cells/well and 2.5 x 10^5 cells/well respectively) in complete RPMI medium (RPMI 1640 with 4mM L-glutamine, 10% (v/v) FCS, 100 µg/mL P/S, 50 µM 2-ME) alone or supplemented with 20 µg/mL $gp140_{97CN54}$ or 5 µg/mL Concanavalin A (Con A) as a control (volume per well: 100 µL for splenocytes, 200 µL for CLN cells). Cells were incubated in 5% CO_2 at 37°C for 5 days. After 48 h cells were centrifuged at 1700 RPM for 5 min and 60 µL supernatant was collected and replaced with 60 µL fresh complete RPMI. The collected supernatants were stored in V bottom plates at-80°C until cytokine profiling (Section 2.15.1.2). After 48 h (splenocytes) or 72 h (CLN cells) cells were analysed using [^{3}H]-thymidine incorporation assay (Section 2.14.1).

2.14.1. [^{3}H]-thymidine incorporation assay

Freshly-isolated splenocytes and CLN cells were restimulated as described in Section 2.14 and incubated in 5% CO_2 at 37°C for 48 h (splenocytes) or 72 h (CLN cells). Cells were pulsed with 1 µCi/well [^{3}H]-thymidine (Perkin-Elmer, 1: 20 dilution, 20 µL/well) for the last 8–12 h of incubation. The cellular DNA was harvested on glass-fibre filters and then assayed by liquid scintillation counting. Data are expressed as cpm.

2.15. Multiplex bead-based Luminex™ assays

2.15.1. Cytokine and chemokine quantification

2.15.1.1. Peritoneal lavage samples

Peritoneal lavage samples 4, 12 and 24 h following PEI or Alhydrogel or PBS control administration were screened for IL-1**α**, IL-1**β**, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-17, interferon-**γ** (IFN-**γ**), tumour necrosis factor-**α** (TNF-**α**), granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony stimulating factor (GM-CSF), Eotaxin, KC (CXCL1), monocyte chemotactic protein-1 (MCP-1, CCL2), macrophage inflammatory protein-1**α** (MIP-1**α**, CCL3), macrophage inflammatory protein-1**β** (MIP-1**β**, CCL4) and Regulated upon Activation, Normal T cell Expressed, and Secreted (RANTES, CCL5). Analyses were conducted according to manufacturer's instructions (Bio-Rad). Briefly, the cytokine standards were reconstituted in 500 µL of the appropriate matrix, in this case FACS washing buffer 2, left at 4°C for 30 min, and serially diluted in the same matrix. Each well of a 96-well filter plate was washed once with bio-plex wash buffer (100 µL/well) using a vacuum manifold pump. Anti-cytokine labelled beads were added (0.5 x in bio-plex assay buffer, 50 µL/well) and plates were washed twice with bio-plex wash buffer (100 µL/well), as before. Samples and standards (50 µL/well) or anti-cytokine beads in matrix alone as background control were added. The assay plate was shaken at 1100 rpm for 30 s and 300 rpm for 30 min at RT in the dark. The wells were washed three times using bio-plex wash buffer as before, and 0.5 x detection antibody (in antibody diluent, 25 µL/well) was incubated in the dark while vortexing at low speed as before. Wells were washed three times using bio-plex wash buffer, as before, and 0.5 x streptavidin–PE antibody (in bio-plex assay buffer, 25 µL/well) was incubated for 10 min at RT in the dark while vortexing at low speed. The wells were washed three times as before and the beads were resuspended in bio-plex assay buffer (125 µL/well). Prior to

analysis using the Luminex 2000 machine (Luminex Corporation, UK), the plate was vortexed at 1100 rpm for 30 s. The results were extrapolated from the cytokine/chemokine standard curve, using the Bio-plex manager software.

2.15.1.2. Supernatant of gp140$_{97CN54}$-restimulated splenocytes and CLN cells

Supernatants from gp140$_{97CN54}$-restimulated splenocytes and cervical CLN cells were screened for IL-2, IL-4, IL-5, IL-13, IL-17, IFN-**γ**, and TNF-**α**. Analysis was conducted according to manufacturer's instructions (Bio-Rad) (Section 2.15.1.1.). The matrix used was complete RPMI.

2.15.2. Quantification of total IgG and IgA content in vaginal lavage samples

The total IgA and IgG levels in vaginal lavage samples were determined using multiplex bead-based Luminex^{TM} assay according to manufacturer's protocol (Millipore). Briefly, the mouse multi-immunoglobulin standard (IgG1, IgG2a, IgG2b, IgG3, IgA, IgE, and IgM) was diluted in mouse isotype serum diluent (MISD), vortexed at high speed for 15 s and incubated at 4°C for 15 min. Each well of a 96-well filter plate was washed once with cytokine washing buffer (0.05% Tween-20 in PBS, 50 µL/well) using a vacuum manifold pump. Vaginal lavage samples (1:124 diluted) and standards in cytokine assay buffer (1% BSA, 0.05% Tween-20 in PBS, 50 µL/well) or cytokine assay buffer only as background control were added. Anti-immunoglobulin beads were added (0.5 x, 25 µL/well). The plate was sealed, vortexed at 1100 rpm for 30 s and incubated in the dark for 30 min at 350 rpm at RT. Samples were incubated with diluted PE-conjugated anti-mouse **λ** light chain antibody (1: 100 in cytokine assay buffer) for 30 min at 350 rpm in the dark, after brief vortexing at 1100 rpm. Wells were washed twice with cytokine washing buffer using a vacuum manifold pump, briefly vortexed at high speed, and analysed using the Luminex 2000 instrument (Luminex

Corporation, UK). Results were extrapolated from the IgG and IgA standard curves, using the Bio-plex manager software.

2.16. Microscopy

2.16.1. Transmission Electron Microscopy (TEM)

Standard bPEI form (12 µg, 25 kDa Sigma-Aldrich) was dissolved in 5% glucose solution. PEI alone was evaluated and additionally, $gp140_{97CN54}$ (4 µg, Polymun) or sheered salmon sperm DNA (at various weights, Invitrogen) was added to PEI solution for evaluation of complex formation. Samples were loaded onto glow-discharged formvar-coated 400-mesh copper grids and negatively stained with 2% aqueous uranyl acetate. Excess stain was removed by blotting with filter paper and the grids were air-dried before examination in a FEI Tecnai 12 electron microscope.

2.16.2. Laser Scanning Confocal Microscopy (LSCM)

Peritoneal lavages of female naïve BALB/c mice were obtained as described in Section 2.10.3. Following cell counting, cells were resuspended to 1 x 10^6 cells/mL in complete RPMI (RPMI 1640 with 4mM L-glutamine, 10% (v/v) FCS, 100 µg/mL P/S, 50 µM 2-ME) and approximately 0.5 x 10^6 cells/well were added to a 24-well flat bottom plate (500 µL/well) containing sterile coverslips. Cells were incubated in 5% CO_2 at 37°C for 1-3 h to allow adherence to coverslips (incubation was stopped when adherence was observed under the microscope). Each well was gently washed three times with cell wash buffer (5% FCS in RPMI 1640 with 4mM L-glutamine) to remove non-adherent cells. Fresh complete RPMI was then added to the cells with one of the following: PEI_{488} (0.1 µg/mL, 1 µg/mL, 10 µg/mL), PEI & $Alexa_{488}$ label (quenched using Tris solution and

unconjugated, concentration equal to amount of $Alexa_{488}$ in 10 µg/mL PEI_{488}), $Alexa_{488}$ label alone (concentration equal to amount of $Alexa_{488}$ in 10 µg/mL PEI.$Alexa_{488}$), complete RPMI alone, $gp140_{97CN54}$–$Alexa_{488}$ (2 µg/mL) plus PEI (0.1 µg/mL, 1 µg/mL, 10 µg/mL), $gp140_{97CN54}$–$Alexa_{488}$ alone (2 µg/mL). Cells were incubated in 5% CO_2 at 37°C for 2 h. Each well was gently washed three times with cell wash buffer (5% FCS in RPMI 1640 with L-glutamine) and fixed using 4 % paraformaldehyde in PBS for 0.5 h at RT (1 mL/well). Each well was gently washed three times with cell wash buffer and 0.5% BSA in PBS was added to the fixed cells (1 mL/well) that were then stored at 4°C prior to fluorescent antibody staining. To prepare for antibody staining, cells were permeabilised using permeabilisation solution (0.1 % Saponin/0.5 % BSA/5 % FCS in PBS) for 60 min at RT. The rest of the staining procedure was carried out by removing the coverslips from the 24-well plate and placing them on a 55-µL drop of the particular staining or washing solution on parafilm. Coverslips with adhered cells were stained for 1 h in the dark at RT with one of the following appropriately diluted primary antibodies (55 µL/coverslip in permeabilisation solution): biotinylated goat anti-mouse Cathepsin D (R & D Systems), biotinylated rat anti-mouse F4/80 (AbD Serotec), or biotinylated rat anti-mouse CD107 (Lamp-1) (eBioscience). Coverslips with cells were then washed three times with permeabilisation solution followed by incubation with TRITC-conjugated streptavidin (BD) (55 µL/coverslip in permeabilisation solution). Coverslips were washed three times as before using permeabilisation solution and were mounted in Vectashield mounting medium with Dapi (25 µL/coverslip) on microscope slides. Microscope slides were stored upright at 4°C until analysis using an Olympus confocal microscope fv1000 and IX81 (N/A 1.35). Colocalisation analysis was conducted using ImageJ software and the JaCoP plugin. The Costes' threshold was applied.

2.17. Surface Plasmon Resonance (SPR)

SPR experiments were conducted using a BIAcore 3000 biosensor (GE Healthcare) in HEPES running buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% surfactant polysorbate20 (p20, v/v), pH 7.4) at 25°C.

2.17.1. Assessing PEI binding to $gp140_{97CN54}$ and OVA antigen

For $gp140_{97CN54}$–PEI interaction analysis, PEI (10 mM sodium acetate pH 5.0) and $gp140_{97CN54}$ (10 mM sodium acetate pH 4.5) were immobilised in approximately equivalent molar amounts on separate BIAcore CM5 sensor chip flow cells using amine couping chemistry to a level of 4 000 Response Units (RU; pg/mm^2) and 17 000 RU respectively (target RU were calculated based on PEI MW 25 kDa, $gp140_{97CN54}$ monomer MW 140 kDa). As a positive control for PEI binding, BSA (MW 66 kDa) was immobilised (10 mM sodium acetate pH 5.0, 11 000 RU) on a separate BIAcore flow cell using standard amine coupling. An NHS-EDC activated and ethanolamine-HCL deactivated empty flow cell was used as negative control. For association phases one of the following solutions was injected for 5 min at a flow rate of 5 µL per minute: PEI in HEPES running buffer (50 µg/mL), $gp140_{97CN54}$ in HEPES running buffer (50 µg/mL), OVA in HEPES running buffer (50 µg/mL), HEL in HEPES running buffer (50 µg/mL), or BSA in HEPES running buffer (50 µg/mL). Dissociation phases were monitored for up to 200 seconds by injection of HEPES running buffer only. Flow cell surface recovery was carried out using glycine (pH 2.0). Interaction analysis was carried out using BiaEvaluation Software.

2.17.2. Assessing the interaction between PEI and human mAbs

For PEI-mAb interaction analysis PEI (10 mM sodium acetate pH 5.0) was immobilised on BIAcore CM5 sensor chip flow cells using amine couping chemistry to a level of 4 000 RU. An NHS-EDC activated and ethanolamine-HCL deactivated empty flow cell was used as a background control. For association phases one of the following human mAb in solution (50 µg/mL in HEPES running buffer) was flowed over: B12, HJ16, X5, 412D, 4.8D, 17B, 8B, A32, 4E10, HGP68, 19B, HR10, CD4IgG2, sCD4. Dissociation phases were monitored for up to 200 s by injection of HEPES running buffer only. Flow cell surface recovery was carried out using glycine (pH 2.5 or pH 2.5). Interaction analysis was carried out using BiaEvaluation Software.

2.18. Sequence alignments

Primary sequence alignments for gp120 and gp140 forms were carried out using Macvector software.

2.19. Homology modelling and calculation of electrostatic surface potential

The sequence of HIV-1 $gp120_{97CN54}$ (genebank: AF286226.1) was entered into the FFAS server at http://ffas.ljcrf.edu/ffas-cgi/cgi/ffas.pl searching the PDB as of August 2011 for structures of homologous sequences. The search retrieved and aligned the structures of gp120: 3LQA, 3NGB and 3U7Y (Diskin et al., 2010; Diskin et al., 2011; Zhou et al., 2010). The homology modelling program SCWRL was used to produce 3D homology models of $gp120_{97CN54}$ based on those three structures. These three sets of coordinates were used in the Poisson-Boltzmann solving program APBS within the PyMOL software to compute the electrostatic potential. The potential was represented on the molecular surface, contoured at +3 (blue) and-3 kT/e (red).

2.20. Statistical analysis

Statistical analyses were performed using GraphPad Prism software. For comparison of two data sets a non-parametric test (Mann – Whitney test, two-tailed) was used. For comparison of more than two data sets a non-parametric one-way analysis of variance (Kruskal-Wallis test) followed by a Dunn's multiple comparison post-test was used. Correlation between antibody surface potential and binding to PEI was analysed using a two-tailed Spearmann correlation test. The statistical test used in each individual experiment and the respective *p* values are given in the main text and figure legends.

CHAPTER 3 [#]

Characterisation of PEI-induced gp140$_{97CN54}$-specific antibody responses

&

Analysis of the effects of PEI-gp140$_{97CN54}$ binding on gp140$_{97CN54}$ structure and epitope accessibility

[#] Portions of this work have been submitted for publication

CHAPTER CONTENT LIST

CHAPTER 3 - CHARACTERISATION OF PEI-INDUCED GP140$_{97CN54}$-SPECIFIC ANTIBODY RESPONSES AND THE EFFECTS OF PEI–GP140$_{97CN54}$ BINDING ON GP140$_{97CN54}$ STRUCTURE AND EPITOPE ACCESSIBILITY 157

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POLYETHYLENEIMINE AS AN ADJUVANT FOR ENV-BASED HIV-1 IMMUNISATION

- RESULTS CHAPTERS-

Equipped with his five senses, man explores the universe around him and calls the adventure Science.-Edwin Powell Hubble, The Nature of Science, 1954

The most exciting phrase to hear in science, the one that heralds the most discoveries, is not "Eureka!" (I found it!) but "That's funny..." . -Isaac Asimov, 1920-1992.

Never, never, never give up. -Winston Churchill, 1874-1965.

3.1. Chapter introduction

The branched 25 kDa form of PEI (bPEI 25) has emerged as a gold standard transfection agent, and is the form that most of my research has focused on. Therefore, unless indicated otherwise, PEI refers to bPEI 25.

To analyse PEI adjuvant activity in the context of an antigen with relevance to HIV-1 immunisation, I chose a recombinant form of the extra-viral soluble portion of Env, called gp140. The gp140 of choice was derived from HIV-1 isolate 97CN54, which is a CCR5-tropic clade B/C recombinant form (Cranage et al., 2010; Rodenburg et al., 2001; Su et al., 2000). This $gp140_{97CN54}$ immunogen was selected for several reasons: a) as the primary target of nAb responses to HIV-1, Env is the most attractive candidate antigen for HIV-1 subunit immunisation; b) HIV-1 Env, like most soluble proteins, is by itself only weakly immunogenic, so this antigen will need strong adjuvantation; c) it is expressed in a trimeric form, which is considered to be beneficial for induction of nAb responses; d) it is derived from a HIV-1 isolate that is, except from a part of the leader sequence, completely of clade C origin, which is the clade responsible for ~50% of worldwide infections; e) it is available as a quality controlled, endotoxin-free Good Manufacturing Practice (GMP) batch.

HIV-1 $gp140_{97CN54}$ comprises gp120 and the external domain of gp41 (Figure 3.1A). HIV-1_{97CN54} is a recombinant form of clade C and clade B'. Clade B' viruses are similar to clade B viruses but have a distinct V3 loop sequence (Kalish et al., 1994). This clade was the major contributor to the pandemic in China, but later the recombinant C/B' form became the most prevalent circulating isolate in China (Graf et al., 1998). Genetic analysis demonstrated the presence of 10 predicted breakpoints of recombination between the clade B' and clade C

sequences in HIV-1_{97CN54} (Figure 3.1B). The env gene is except from a minor part of the leader sequence, completely of clade C origin. Recombinant $gp140_{97CN54}$ is produced in a CHO-K1 expression system, purified using an antibody (5F3)-affinity column, and subsequently exposed to SEC to further isolate trimeric $gp140_{97CN54}$ (Figure 3.1C).

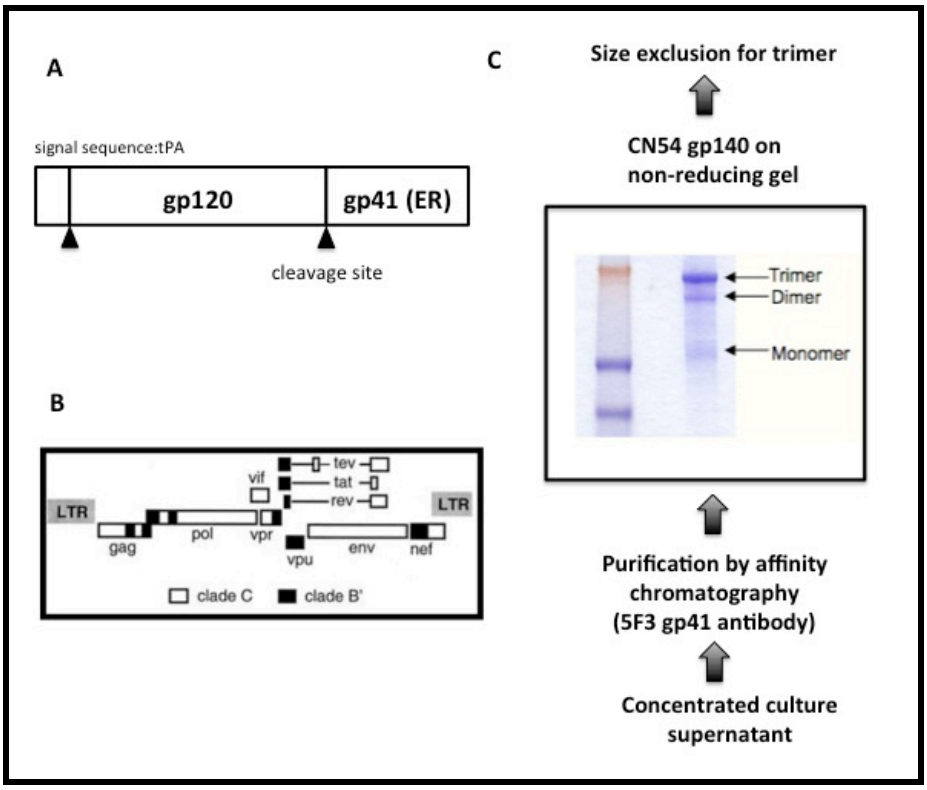


Figure 3.1. HIV-1 $gp140_{97CN54}$ gene construct and purification process. A. The HIV-1 $gp140_{97CN54}$ gene construct starts with a human tissue plasminogen activator (tPA) signal sequence and consists of the gp120 coding sequence and the gp41 external region (ER) coding sequence. **B. Schematic diagram showing the mosaic genome organisation of HIV-1_{97CN54}.**Taken from: (Su et al., 2000). **C. Purification process.** The recombinant $gp140_{97CN54}$ is expressed in CHO-K1 cells and subsequently purified using 5F3-mAb affinity chromatography, a non-reducing gel, and SEC.

A major proportion of the currently identified bnmAbs derived against HIV-1 have long complementarity-determining region heavy chain 3 (CDR-H3) hypervariable loops that some rodents, including mice, cannot produce (Cardoso et al., 2005; Collis et al., 2003; Ofek et al.,

2004; Saphire et al., 2001; Wu et al., 1993). Rabbits, in contrast, have been found to produce CDR-H3 loops up to 22 amino acids long (Popkov, 2003). Therefore, and to raise higher amounts of serum for in-depth analysis, it was decided to investigate whether PEI enhanced the induction of $gp140_{97CN54}$–specific antibody responses in a rabbit immunisation model.

Since effective HIV-1 Env subunit immunisation would require induction of broad (neutralising) antibody responses that can target the widely diverse range of circulating HIV-1 isolates and that are directed against functionally important, for instance neutralisation- or ADCC- associated, epitopes, it was decided to investigate the PEI-induced antibody responses in this context (Section 1.10). In an extensive ELISA study it was investigated whether PEI-induced antibodies can recognise Env proteins of various, non-homologous, HIV-1 strains. The results were compared to the gold standard adjuvant FCA/FIA and the widely used and licensed adjuvant Alum. By means of a cross-competition ELISA with human mAbs of known specificity an attempt was made to identify epitope regions that were targeted by PEI-induced serum antibodies. Again results were compared to FCA/FIA and Alum adjuvants.

As adjuvants can, in addition to direct immune stimulation, achieve immune enhancement though effects on the co-administered vaccine antigen, including protection against degradation, depot formation, or enhanced antigen targeting to immune cells, direct interaction between adjuvant and antigen might be beneficial (Section 1.12)(Calabro et al., 2011; de Veer and Meeusen, 2011). However, it should be ensured that, if present, direct interaction between adjuvant and antigen does not impair antigenicity through, for example, denaturing effects or steric hindrance of epitopes, which could result in a loss of accessible or conformation-dependent epitopes. Here, potential direct interaction between PEI and $gp140_{97CN54}$ or the

model hen egg white ovalbumin (OVA) antigen* was examined in SPR assays. PEI was previously shown to bind to negatively charged molecules, including serum albumins, DNA, and sialic acid-containing sulphated proteoglycans (Mislick et al., 1996; Pan, 2009; Sun et al., 2011). I therefore hypothesised that PEI similarly interacts with protein antigens, possibly through electrostatic interactions with negatively charged surface patches, such as the sialic acid residues on $gp140_{97CN54}$ (Kong et al., 2010). By means of ELISA assays and human mAbs with known epitope specificity as probes an attempt was made to study the effect of PEI on the accessibility of the conformation-dependent mAb B12 epitope. Also, the ability of PEI-induced serum antibodies to bind native $gp140_{97CN54}$ versus heat-denatured $gp140_{97CN54}$ was examined as an indication of the presence or absence of PEI-mediated $gp140_{97CN54}$ denaturing effects during immunisation. Again, the results were compared to the adjuvants FCA/FIA and Alum.

In summary, current literature and understanding in the field suggest that an effective adjuvant for HIV-1 Env-based immunisation will need to: 1) enhance HIV-1 Env immunogenicity and induce serum Env-based specific antibody titres that a) recognise the majority of divergent circulating HIV-1 Envs and b) are involved in immune responses that could help providing HIV-1 protection, such as neutralisation or ADCC, and target the relevant epitopes; 2) conserve the structural conformation of Env immunogen and the accessibility of relevant epitopes.

Since in some of the planned assays human mAbs would be present with PEI, potential interactions between PEI and human mAbs that might confound assay interpretation were analysed using SPR assays (Didenko et al., 2005). To expand on the previously mentioned

* Model OVA antigen will be used in the next thesis chapter due to technical reasons that will be specified in the text.

studies that suggested electrostatic interaction between PEI and negatively charged molecules, a possible role of electrostatic interactions in PEI-mAb binding was investigated by calculation of the pI of the human Fab fragments and by analysing possible correlations between this pI and the binding results.

Principal aims of this chapter:

1) **Investigate whether PEI has adjuvant activity in the context of HIV-1 gp140$_{97CN54}$ antigen**
2) **Analysis of PEI-induced Env-specific antibodies:**
   - Investigate whether PEI-induced Env-specific antibodies can recognise heterologous divergent HIV-1 Env glycoproteins;
   - Investigate which epitope regions on HIV-1 gp140$_{97CN54}$ were targeted by PEI-induced antibodies.
3) **Investigate the interaction between PEI and gp140$_{97CN54}$ and OVA antigens**
4) **Analysis of PEI effects on antigen structure:**
   - Investigate the effect of *in vitro* PEI-gp140$_{97CN54}$ complex formation on the accessibility of gp140$_{97CN54}$ epitopes using human mAbs of known epitope specificity as probes;
   - Analyse the recognition of native and denatured gp140$_{97CN54}$ by PEI-induced antibodies compared to FCA/FIA-induced and Imject®Alum-induced antibodies as an indication of gp140$_{97CN54}$ antigen structure conservation during immunisation.
5) **Investigate the interaction between PEI and mAbs**
   - Study the potential direct interaction between PEI and human mAbs;
   - Analyse a potential correlation between PEI-mAb binding and isoelectric point (pI) of the mAb Fab fragment.

Chapter hypotheses:

- **PEI enhances HIV-1 gp140$_{97CN54}$ immunogenicity in a rabbit immunisation model**

Justification: PEI showed adjuvanticity with TLR ligand Poly I:C in mice model, resulting in increased total IgG and IgG1 endpoint titres following PEI administration alone or with Poly I:C and increased IgG2a endpoint titres following the combination of PEI and Poly I:C.

- **PEI directly interacts with HIV-1 gp140$_{97CN54}$, possibly via electrostatic interactions**

Justification: PEI has been shown to bind to sialic acid-containing sulphated proteoglycans (Hu *et* al., 2001; Mislick *et* al., 1996) as well as negatively charged, low pI, molecules, including human serum albumin (HSA), BSA, and nucleic acids. Although experimental evidence is falling short in the case of BSA and HSA, the general belief is that this binding occurs through electrostatic interactions, which has been confirmed for binding between PEI and nucleic acids/DNA. Although HIV-1 gp140$_{97CN54}$ has a pI of 8.78, its glycans contain sialic acid residues that might facilitate binding to PEI.

- **PEI binds to mAbs that have low pI Fab fragments**

Justification: PEI was shown to bind to mAbs in one previously published study (Didenko et al., 2005). See previous hypothesis for the assumed involvement of electrostatic interactions in PEI-DNA and PEI-BSA binding. This would suggest that having a lower pI, which implies a positive charge at neutral pH, facilitates binding to PEI.

3.2. Results

3.2.1. PEI adjuvant activity with gp140$_{97CN54}$

Two rabbit immunisation studies were conducted to investigate whether PEI has adjuvant activity with HIV-1 gp140$_{97CN54}$ after systemic immunisation. In both studies, and in all following immunisation models in this thesis, boost immunisations were conducted within 3 to 4 weeks following preceding immunisations. Pilot studies in our laboratory have shown repeatedly that this timeframe allows optimal kinetics of the antibody response, including class switching and affinity maturation, possibly because a temporary depot of antigen is maintained inside the germinal centres for 3 -4 weeks. The same studies have shown that variations of the immunisation schedule within this timeframe do not tend to substantially affect the results obtained. The serum samples obtained from the final study were used for the subsequent assays described here, since the serum from the earlier immunisation study only became available at a later stage.

Our collaborators Paul Tonks and Mark Sheppard in Prof. Jonathan Heeney's group at Cambridge University, UK, carried out the first rabbit immunisation. Rabbits were primed by the subcutaneous (s.c.) route with 25 µg gp140$_{97CN54}$ alone, antigen adsorbed to Imject®Alum (1:1, 1.88 mg Aluminium hydroxide ($Al(OH)_3$), 1.88 mg Magnesium hydroxide ($Mg(OH)_2$), or antigen mixed with 2 mg PEI. After 4, 8, and 14 weeks rabbits were boost immunised with equivalent immune preparations. Total serum gp140$_{97CN54}$-specific IgG responses were measured pre-prime, 2 weeks post each immunisation, as well as at week 1 and week 20 and are depicted in Figure 3.2.

HIV-1 $gp140_{97CN54}$ antigen alone was only weakly immunogenic and only yielded IgG responses after boosting. The median $gp140_{97CN54}$-specific IgG titres in the unadjuvanted group were circa 10^2 after the boost. PEI elicited median $gp140_{97CN54}$-specific IgG titres of circa $10^{2.2}$ as early as 1 week after prime immunisation that were statistically indistinguishable from the Imject®Alum-adjuvanted group and continued to increase until and after boosting. Already at 2 weeks after the prime, the difference in IgG titre between unadjuvanted and PEI- or Imject®Alum-adjuvanted groups reached statistical significance (P<0.01) (Figure 3.1.B) and this trend was maintained after boosting. HIV-1 $gp140_{97CN54}$-specific IgG antibody levels in adjuvanted rabbits peaked at between 10 to 15 weeks (4–9 weeks after boost) and slowly started falling after week 15. However, at week 20 the $gp140_{97CN54}$-specific IgG titres were still significantly (P<0.01) higher in the adjuvanted groups compared to the unadjuvanted group.

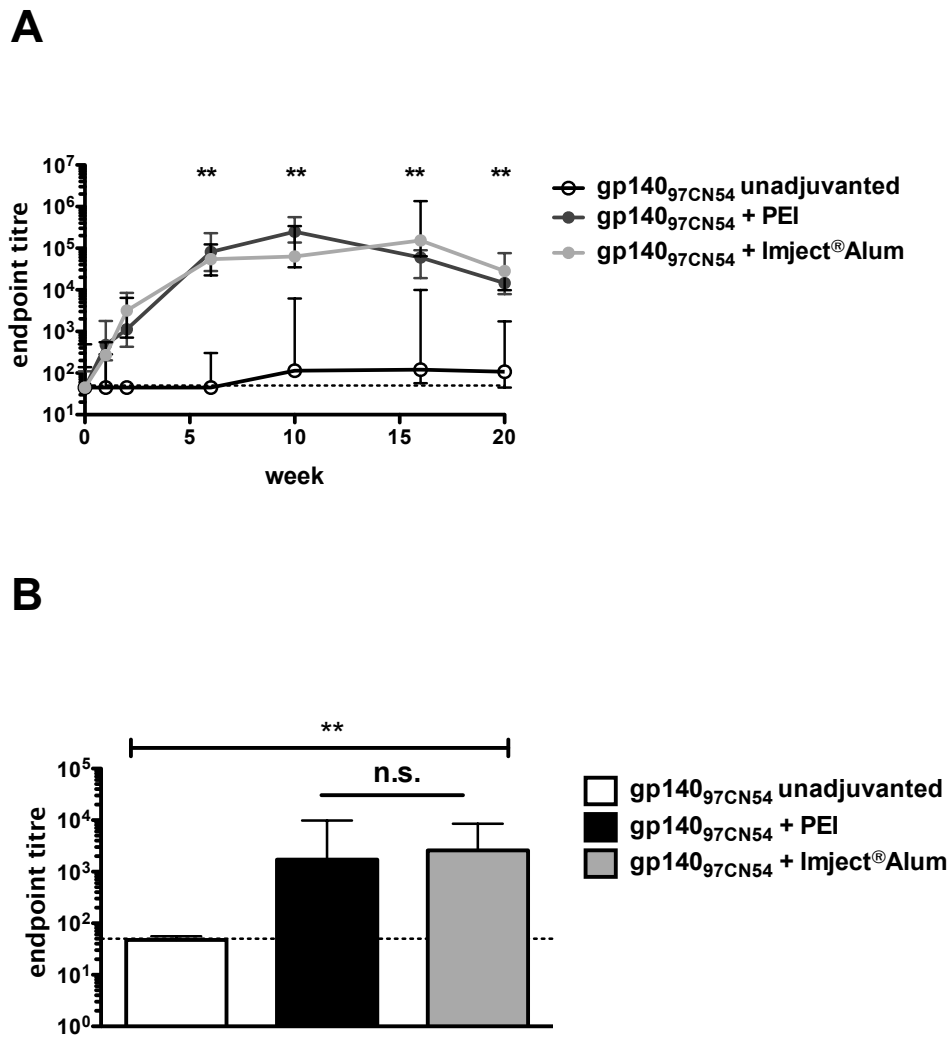


Figure 3.2. Adjuvant activity of PEI in a rabbit gp140$_{97CN54}$ immunisation model. Female NZW rabbits (*n*=5) were immunised s.c. with four doses of 25 µg gp140$_{97CN54}$ combined with 2 mg PEI, Imject®Alum (1:1, 1.88 mg $Al(OH)_3$, 1.88 mg $Mg(OH)_2$) or or in PBS alone at week 0, 4, 8, and 14. Bleeds were collected at week 0,1,2,6,10,16 and 20. A) HIV-1 gp140$_{97CN54}$-specific serum IgG endpoint titre time course. B) HIV-1 gp140$_{97CN54}$-specific serum IgG response at week 2 post prime. Medians ± error (A) or geometric means ± 95% CI (B) of biological replicates (individual rabbits) within the experiment are depicted. Kruskal–Wallis, Dunn's post-test, $^{**}P<0.01$.

In a second immunisation study rabbits were s.c. immunised by Neil Sheppard, a previous member of our lab, with 50 µg $gp140_{97CN54}$ antigen mixed with 202.52 µg PEI, antigen adsorbed to Imject®Alum (1:1, 5 mg $Al(OH)_3$, 5 mg $Mg(OH)_2$), or antigen emulsified in FCA (1:1, 117.5 µg Mycobacterium tuberculosis, 100 µL paraffin oil, 147.5 µL mannide monooleate). The FCA and Imject®Alum weights used in this study were distinct from the weights used in the previously discussed study so as to obtain equivalent volume (antigen): volume (FCA/Imject®Alum adjuvant) ratios. At week 3, rabbits received boost immunisations with 20 µg $gp140_{97CN54}$ antigen in the same amounts of adjuvant as the prime. For the boost immunisation FCA was replaced by FIA. At week 6, 9, and 18 rabbits received boost immunisation with antigen alone. Serum samples were collected at week 0, 3, 6, 9, 12, and 20. Serum $gp140_{97CN54}$-specific IgG endpoint titres measured by ELISA are depicted in Figure 3.3.

In accordance with the previous results, PEI and Imject®Alum induced approximately equivalent $gp140_{97CN54}$-specific serum IgG antibodies after priming that further increased after the first three boost immunisations, and peak titres were reached around week 12. PEI- and Imject®Alum- induced titres were slightly lower than those observed in the first rabbit immunisation study, probably due to the lower number of adjuvanted boost immunisations in the current study. FCA/FIA adjuvantation induced higher serum $gp140_{97CN54}$-specific IgG endpoint titres although this difference was not statistically significant.

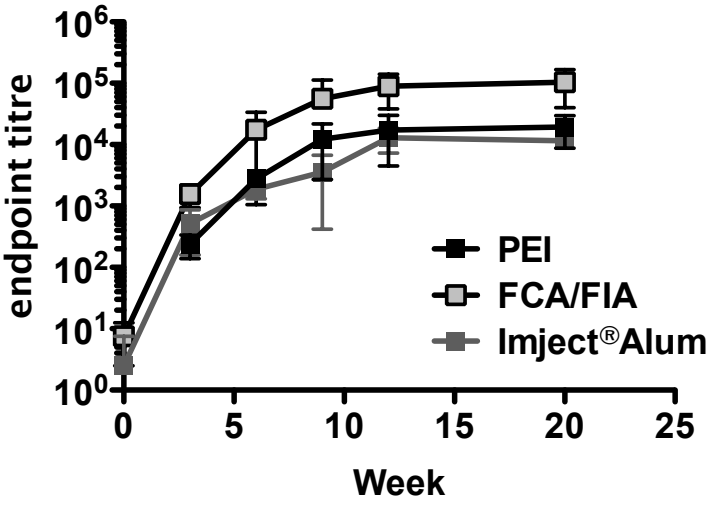


Figure 3.3. Serum gp140$_{97CN54}$-specific IgG responses in a s.c. rabbit immunisation model with PEI, FCA/FIA or Imject®Alum adjuvants. NZW rabbits (*n*=4) were primed with 50 µg HIV-1 gp140$_{97CN54}$ antigen mixed with 202.52 µg PEI, antigen mixed with Imject®Alum (1:1, 5 mg $Al(OH)_3$, 5 mg $Mg(OH)_2$), or antigen emulsified in FCA adjuvant (1:1, 117.5 µg Mycobacterium tuberculosis, 100 µL paraffin oil, 147.5 µL mannide monooleate). At week 3 rabbits received boost immunisation with 20 µg gp140$_{97CN54}$ in equivalent formulations (FCA was replaced by FIA). At week 6, 9, and 18 rabbits received boost immunisations with 20 µg gp140$_{97CN54}$ antigen without adjuvant. Serum samples were collected at week 0, 3, 6, 9, 12, and 20. Depicted are means ± SD of biological replicates (individual rabbits) within the experiment. Kruskal-Wallis and Dunn's post-test, n.s.

3.2.2. Characterisation of PEI-induced gp140$_{97CN54}$-specific antibodies

3.2.2.1. PEI-induced antibodies are directed towards conserved epitopes that allow recognition of heterologous HIV-1 Envs

To investigate whether PEI-induced gp140$_{97CN54}$-specific serum IgG antibodies can bind Env-derived glycoproteins from heterologous clades and distinct circulating HIV-1 isolates a range of direct binding ELISA assays were carried out with week 20 serum samples of the first rabbit immunisation experiment and a range of HIV-1 Envs comprising gp140s and gp120s derived from isolates that belong to distinct clades and were produced in diverse expression systems including mammalian and insect cells (see Table 3.1), which have been shown to affect glycosylation patterns and the presence of sialic acid residues (Kong et al., 2010). The Env-specific IgG endpoint titres are shown in Figure 3.4.

HIV-1 Env glycoprotein:	Clade:	Expression system:	Reference:
$gp140_{97CN54}$	C	CHO	(Jeffs et al., 2004; Su et al., 2000)
$gp140_{92UG37}$	A	CHO	(Bjorndal et al., 1997; Jeffs et al., 2004)
$gp140_{BX08}$	B	CHO	(Moog et al., 1997)
$gp140_{IIIB}$	B	CHO	(Jeffs et al., 2004; Popovic et al., 1984; Ratner et al., 1987)
$gp120_{97CN54}$	C	293F	(Su et al., 2000)
$gp120_{BaL}$	B	HEK-293	(Aricescu et al., 2006; Gartner et al., 1986)
$gp120_{IIIB}$	B	Baculovirus (insect)	(Popovic et al., 1984; Ratner et al., 1987)
$gp120_{W61D}$	B	CHO	(Groenink et al., 1992)
$gp120_{sf162}$	B	HEK-293	(Cheng-Mayer and Levy, 1988)

Table 3.1. Env gp140s and gp120s from divergent HIV-1 isolates used in the current study: isolate glycoprotein, clade, expression system and references.

Total Env-specific IgG endpoint titres ranged from ~10^3 for IIIB gp120 to ~10^6 for BaL gp120. As expected a trend was observed towards higher homologous (clade C) Env-specific IgG endpoint titres compared to heterologous (clade A or B) Env-specific IgG endpoint titres (Figure 3.4A). The observed differences did not reach statistical significance in the case of PEI or Imject®Alum-adjuvantation. By contrast, variation observed between IgG endpoint titres in the FCA/FIA-adjuvanted group was statistically significant (P<0.05), suggesting that the relative proportion of FCA/FIA adjuvant–induced antibodies to conserved HIV-1 Env epitopes may be

smaller compared to PEI- and Imject®Alum adjuvant. For all adjuvants a trend was observed towards lowest clade B Env-specific endpoint titres, with the exception of clade B BaL gp120, therefore in general the ranking was: clade C ≥ A > B. Since it was impossible to test the coating efficacy per Env type, as there is no available mAb that binds all Envs with the exact same affinity and since coating concentrations were too low for direct detection by for example a BCA-test, the possibility that results are partially accounted for by differences in coating efficacy cannot be excluded. Nevertheless, even when care is taken with quantification of the results, the quality of the results indicate that PEI adjuvantation during HIV-1 $gp140_{97CN54}$ immunisation induces IgG serum antibodies that can recognize a wide range of heterologous Envs. No particular differences were detected between gp120- and gp140-directed IgG endpoint titres, suggesting that the induced IgG antibody responses were predominantly directed towards gp120 epitopes, although differences between monomer and trimer behaviour in ELISA coating in the current experiment can again not be ruled out.

The exceptionally high BaL gp120-specific IgG endpoint titres compared to other clade B glycoproteins in all adjuvant groups could not be explained by sequence variation or overall sequence homology with $gp140_{97CN54}$ antigen. Sequence homology was studied using NCBI protein sequences and Macvector software (V 7.2.3, C2004 Accelrys). The results are shown in Table 3.2. BaL gp120 showed 49% identities and 6% similarities to $gp140_{97CN54}$ antigen that is similar to results observed for other clade B glycoproteins, such as SF162 gp120 (49% identities, 6% similarities). Closer examination of sequence homology around particular epitope regions, such as 5F3 and 447-52D epitopes, did also not reveal any differences between BaL gp120 and other clade B glycoproteins. A trend towards lowest recognition of IIIB gp120 was observed. This might be related to the fact that this was the only Env expressed in an insect

system, which is known to elicit different post-translational modifications resulting in different glycan structures (Kwong et al., 2010).

For all glycoproteins approximately 10-fold higher specific IgG endpoint titres were induced by FCA/FIA adjuvantation compared to PEI and Imject®Alum-adjuvantation. These differences were not statistically significant, except for the lower $gp140_{97CN54}$- and $gp140_{UG37}$-specific IgG endpoint titres in the Imject®Alum group (circa $10^{4.2}$ and 10^{4} respectively) compared to the FCA/FIA group (circa $10^{5.5}$ and $10^{5.5}$ respectively) ($P< 0.05$, $P< 0.01$ respectively).

HIV-1 Env glycoprotein	**vs. $gp140_{97CN54}$ Identities-similarities**	**vs. $gp120_{97CN54}$ Identities-similarities**	**5F3 epitope AAGSTMGAASMTLTVQARQ**	**447-52D epitope GPGQ**
BX08 gp140	73% - 8%	-	**AAGSTMGAASLTLTVQARQ**	**GPGR**
IIIB gp140	72% - 10%	-	**AAGSTMGARSMTLTVQARQ**	**GPGR**
BaL gp120	49% - 6%	71% - 9%	**AAGSTMGAASMTLTVQARL**	**GPGR**
IIIB gp120	48% - 7%	70% - 11%	**AAGSTMGARSMTLTVQARQ**	**GPGR**
SF162 gp120	49% - 6%	71% - 9%	**AAGSTMGAASLTLTVQARQ**	**GPGR**

Table 3.2. Env subunit sequence analysis. Sequence comparison of different HIV-1 isolate Envs versus $gp140_{97CN54}$. Overall sequence analysis, and analysis around studied mAb epitopes 5F3 and 447-52D. (The mAb 2F5 epitope is not present on CN54 HIV-1 isolates, and the $gp140_{97CN54}$ sequence terminates before the mAb 4E10 epitope).

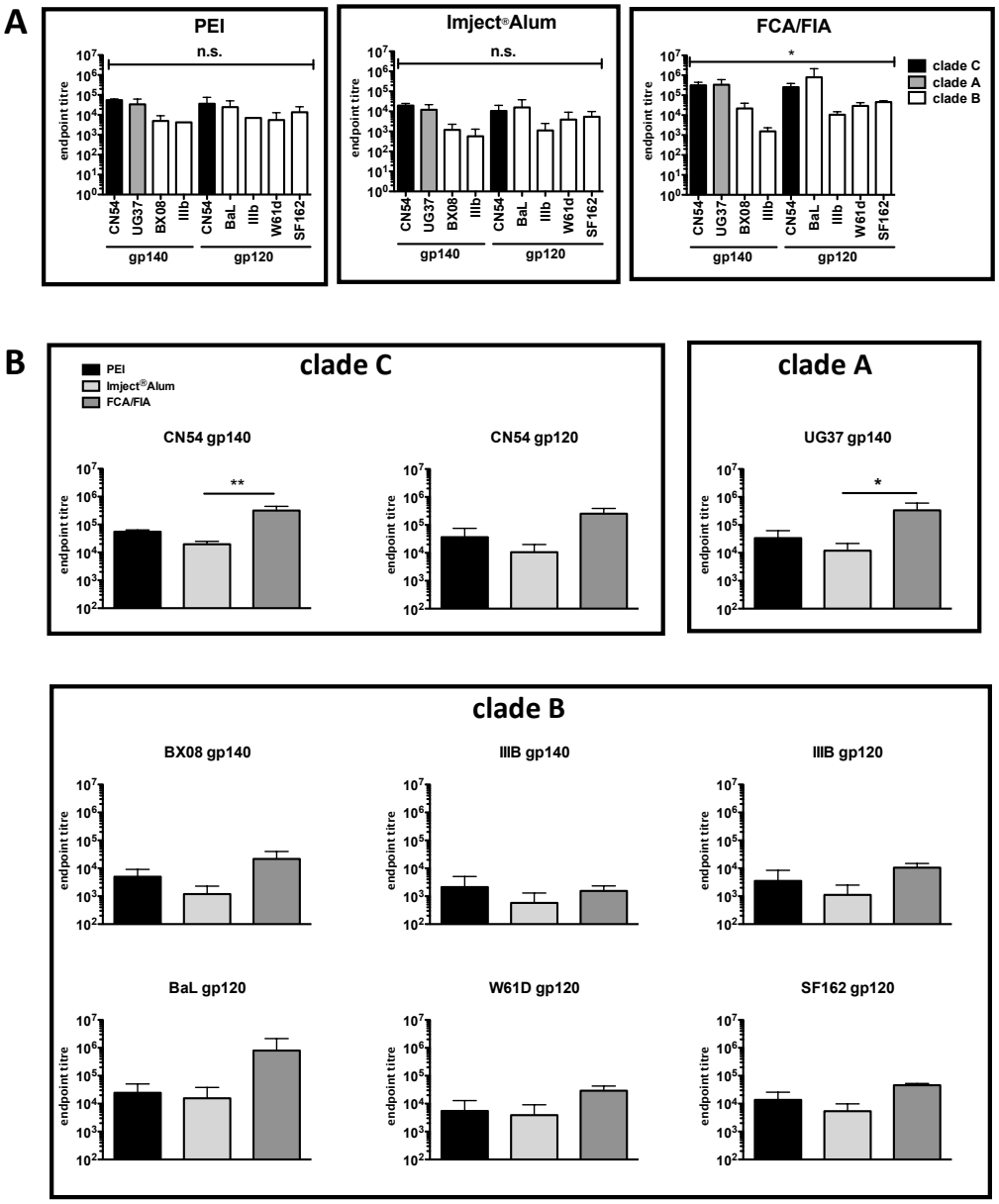


Figure 3.4. Serum homologous and heterologous HIV-1 Env-specific IgG endpoint titres induced after gp140$_{97CN54}$ immunisation of rabbits with PEI, FCA/FIA or Imject®Alum adjuvant. Rabbits (n=4) were primed with 50 µg HIV-1 gp140$_{97CN54}$ antigen mixed with 202.52 50 µg PEI, antigen mixed with 5 mg Imject®Alum, or antigen mixed 1:1 with FCA/FIA. At week 3, 6, 9 and 18 rabbits received boost immunisations with 20 µg gp140$_{97CN54}$ antigen plus adjuvant formulations. Serum samples were collected at week 20. Serum Env-specific IgG endpoint titres were determined using a direct binding ELISA. A) IgG endpoint titres arranged per adjuvant. B) IgG adjuvant titres arranged per Env clade and isolate. Means ± SD of 3 independent biological experimental repeats (independent ELISAs) are depicted. Kruskal-Wallis, Dunn's post-test. *P < 0.05, **P < 0.01.

3.2.2.2. Approximate identification of HIV-1 gp140$_{97CN54}$ epitope regions targeted by PEI-indiced IgG antibodies

In an attempt to identify the antigenic surfaces on gp140$_{97CN54}$ that were targeted by PEI-induced antibodies compared to Imject®Alum- and FCA/FIA-induced antibodies after rabbit immunisation, rabbit sera from the previously described immunisation study were analysed in cross-competition ELISA with human mAbs B12 (Env gp120 CD4bs), 19B (Env gp120 V3 loop), and 447-52D (Env gp120 V3 loop). B12 mAb was chosen because it was the only broad nAb identified at the time of this experiment (Burton et al., 1991; Burton et al., 1994; Burton and Weiss, 2010; Zhou et al., 2007). 19B and 447-52D mAbs were chosen because their epitopes are well defined and located in another region of gp120: the V3 loop (Gorny et al., 1992; Scott et al., 1990). Antibodies directed towards this part of gp120 were originally thought to neutralise only a small subset of HIV-1 isolates as the V3 loop region is characterised by robust sequence variability and is thought to not be well exposed on most native trimeric Envs due to occlusion by glycans (Goudsmit et al., 1988; McCaffrey et al., 2004; Moore et al., 1995; Palker et al., 1988; Pinter et al., 2004), but recent studies and newly identified V3 loop antibodies have shown that targeting of this Env region can result in extremely broad neutralisation (Bonsignori et al., 2011; Hioe et al., 2010; Stanfield et al., 2004; Stanfield et al., 2006; Walker et al., 2009). EC_{50} concentrations were first determined for each human mAb. Serum IC_{50} titres were normalised to total gp140$_{97CN54}$-specific IgG titres (Figure 3.3) and are shown in Figure 3.5.

Serum IC_{50} titres ranged in general from ~10^3 to ~10^6. The mAb B12-inhibiting proportion of PEI-induced serum gp140$_{97CN54}$-specific IgG was larger than that of FCA/FIA-induced sera, but smaller than the inhibiting proportions of Imject® Alum-induced antibodies. However, these

differences were not statistically significant. In contrast, relative inhibition of the V3-loop mAbs 19B and 447-52D was statistically significantly higher in the PEI group than in the Imject® Alum group. Normalised IC_{50} titres for 19B mAb inhibition and 447-52D mAb were almost equivalent for all adjuvant groups, which might be explained by the fact that the respective epitopes are located in the same region of gp120.

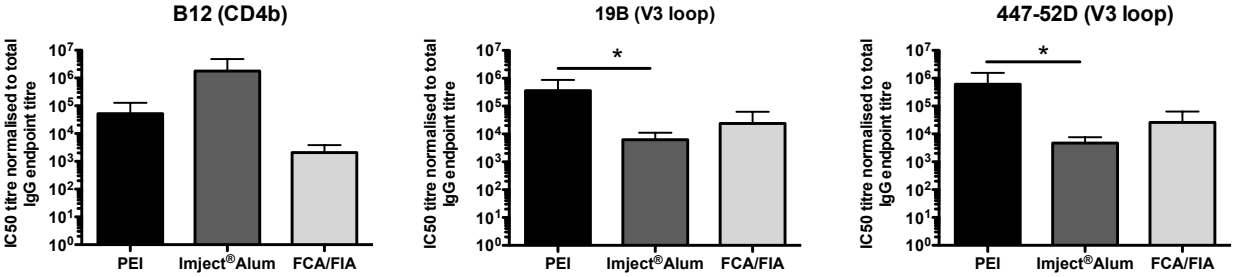
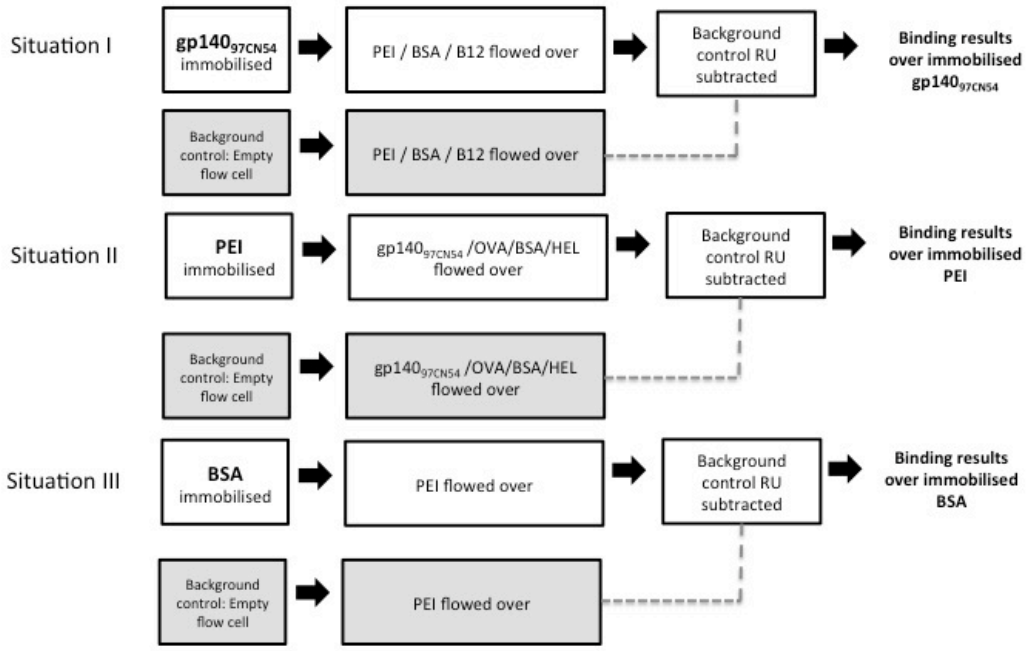


Figure 3.5. Rabbit immune serum inhibition of mAb binding to HIV-1 $gp140_{97CN54}$ antigen: comparison between PEI and standard Imject®Alum and FCA/FIA adjuvants. NZW rabbits (n=4) were prime immunised with 50 µg HIV-1 $gp140_{97CN54}$ antigen mixed with 202.52 50 µg PEI, antigen mixed 1:1 with Imject®Alum, or antigen emulsified 1:1 in FCA adjuvant. At week 3, rabbits received boost immunisations with 20 µg $gp140_{97CN54}$ antigen plus adjuvant formulations (FCA was replaced by FIA). At week 6, 9, and 18 rabbits received boost immunisation with unadjuvanted 20 µg $gp140_{97CN54}$ antigen. Serum was collected at week 20 and used for cross-competition ELISA with human mAbs B12 (Env gp120 CD4bs), 19B (Env gp120 V3 loop), and 447-52D (V3 loop) at EC_{50} concentrations. IC_{50} titres were calculated based on the observed dose-response curves and normalised to total $gp140_{97CN54}$-specific IgG endpoint titres (see Figure 3.3, FCA/FIA-induced IgG endpoint titres were set to 1). Means ± range of two technical replicates (ELISAs) are depicted. Kruskal-Wallis, Dunn's post-test. *$P < 0.05$.

3.2.3. Adjuvant-antigen interaction: PEI binds to gp140$_{97CN54}$ and OVA antigens

One reason why an adjuvant might differentially alter the specificity of antibodies elicited by an antigen is that the adjuvant associates with the antigen and modifies its antigenicity. This might occur by the adjuvant directly masking certain regions of the antigen, or alternatively by inducing conformational changes in the antigen to reveal or mask regions distant from contact sites.

To investigate potential PEI-antigen interactions, SPR binding assays were performed under various conditions using gp140$_{97CN54}$ as an experimental vaccine antigen and OVA as a model antigen. In the first condition tested, gp140$_{97CN54}$ was coupled to the sensor chip and solutions containing PEI, BSA (negative control), or B12 mAb (positive control)(Burton et al., 1991) were flowed over. As a negative control for PEI binding to immobilised gp140$_{97CN54}$ a second empty sensor chip was activated and deactivated. The reading at this sensor chip was subtracted from the test result. Since previous studies have shown that PEI binds BSA (Pan et al, 2009), BSA was used as a positive control and an approximate molar equivalent of BSA (compared to gp140$_{97CN54}$) was coupled to a third sensor chip. In the second condition, PEI was coupled to the sensor chip and solutions containing gp140$_{97CN54}$ or OVA were flowed over. To investigate whether potential binding of PEI to gp140$_{97CN54}$ might be a general characteristic of proteins, another protein, Hen Egg Lysozyme (HEL) was also flowed over. As a negative control, a second empty sensor chip was activated and deactivated and the reading of this sensor chip was subtracted from the results. The strategy used is presented in Flow chart 3.1, and observed binding curves are shown in Figure 3.6.



Flow chart 3.1 Studying the potential interaction between PEI and gp140$_{97CN54}$ in SPR. Since OVA will be used in later assays, OVA was also included in the study. Positive control for gp140$_{97CN54}$ binding: B12 mAb (B12). Negative control for gp140$_{97CN54}$ binding: BSA. Positive control for PEI binding: BSA. Negative control for PEI binding: HEL.

Immobilised gp140$_{97CN54}$ interacted strongly with PEI in solution with fast on- and slow off-rates that are indicative of relatively high avidity binding. As expected, no interaction was observed between immobilised gp140$_{97CN54}$ and soluble BSA while the positive control B12 mAb bound to the immobilised gp140$_{97CN54}$ antigen (~3800 RU)(Figure 3.6B). By contrast with the strong interaction observed between immobilised gp140$_{97CN54}$ and soluble PEI, in the reverse orientation soluble gp140$_{97CN54}$ interacted only weakly with immobilised PEI, with a relatively slow on- and off-rate. Binding was also observed between positive control BSA and immobilised PEI, as well as soluble OVA and immobilised PEI (Figure 3.6C). No interaction was detected between soluble HEL and immobilised PEI, indicating that observed binding was

not global, and was most likely to be a result of electrostatic interactions between the molecules (Figure 3.6A).

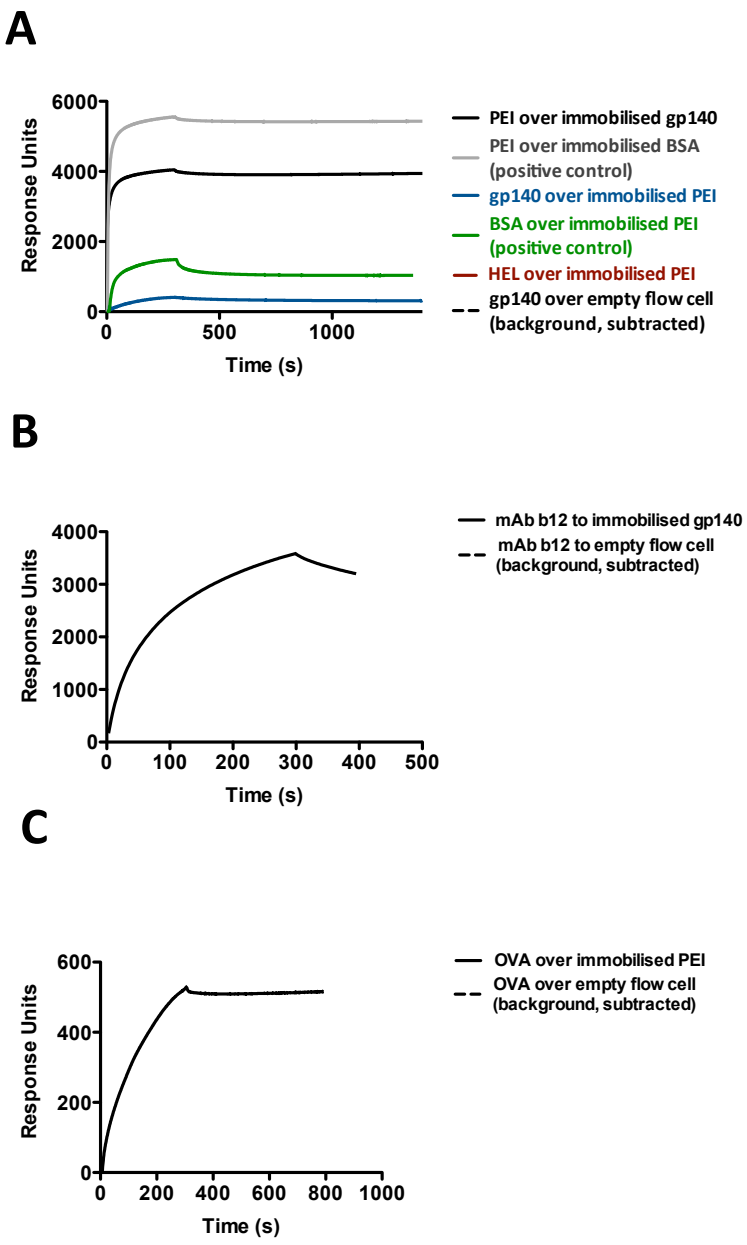
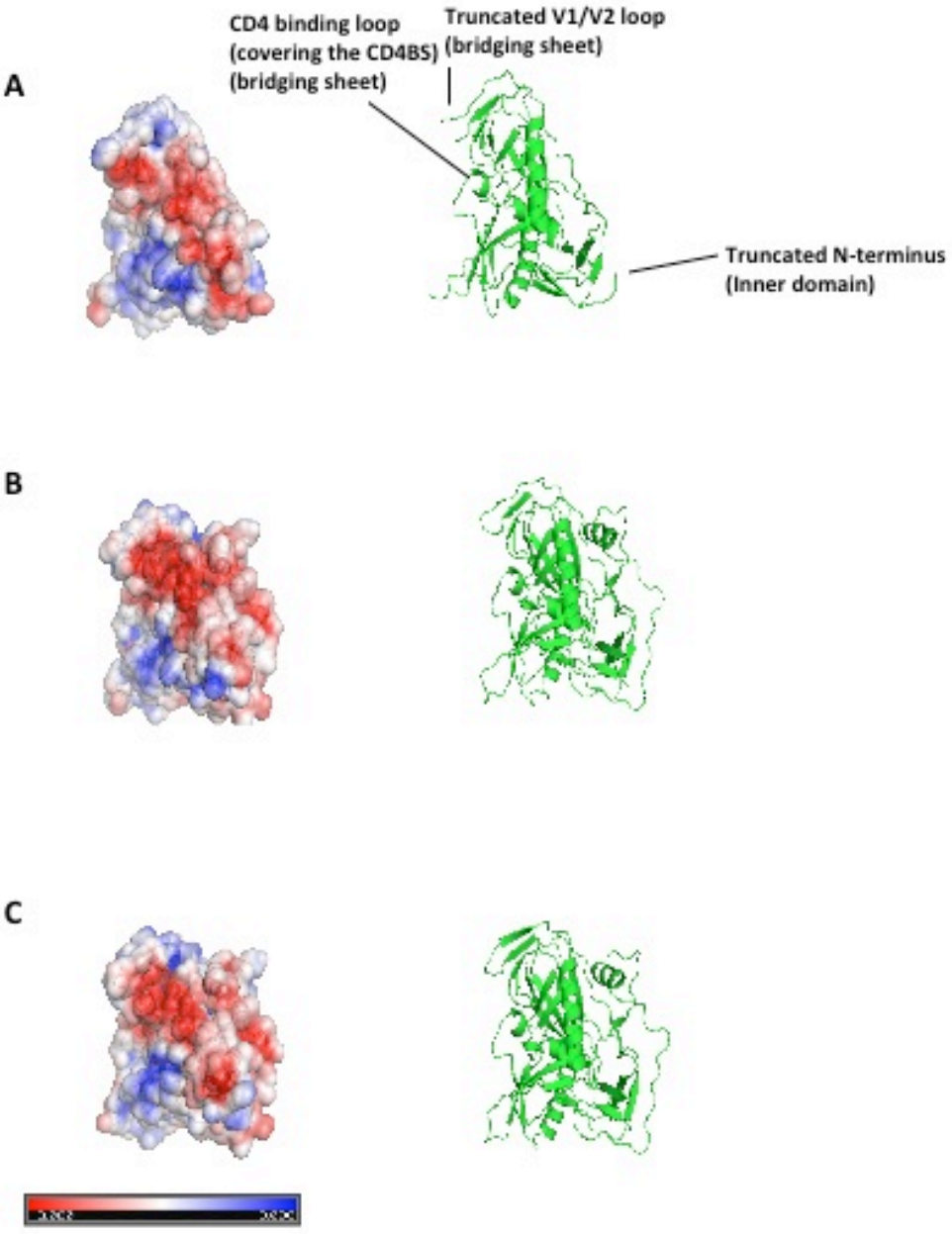


Figure 3.6. SPR binding interactions between PEI and gp140$_{97CN54}$ or OVA. PEI (pI ~10), BSA (pI 5.38), or gp140$_{97CN54}$ (pI 8.78) was surface immobilised and 25 µg/mL PEI, gp140$_{97CN54}$, mAb B12, BSA, or HEL (pI 9.36) were flowed over. A) Direct biophysical interaction between PEI and BSA or PEI and gp140$_{97CN54}$. B) Positive control mAb B12 interaction with immobilised gp140$_{97CN54}$ C) Direct biophysical interaction between OVA (pI 4.7) and surface immobilised PEI. Data are representative of >3 independent experiments. BIAcore 3000.

Since electrostatic potential is an important determinant of intermolecular interactions, it was decided to model the electrostatic potential of $gp140_{97CN54}$. To get an impression of $gp140_{97CN54}$ electrostatic potential and the possible presence of negatively charged surface patches, which could be involved in interaction with positively charged PEI molecules, three homology models were made and electrostatic potential was calculated. The sequence of HIV-1 $gp120_{97CN54}$ (genebank: AF286226.1) was fed to the FFAS server at http://ffas.ljcrf.edu/ffas-cgi/cgi/ffas.pl searching the PDB as of August 2011 for structures of homologous sequences. The search retrieved and aligned the structures of gp120: 3LQA, 3NGB and 3U7Y (Diskin et al., 2010; Diskin et al., 2011; Zhou et al., 2010). The homology modelling program SCWRL was used to produce 3D homology models of $gp120_{97CN54}$ based on those three structures. These three sets of coordinates were used in the Poisson-Boltzmann solving program APBS within the PyMOL software to compute the electrostatic potential. The potential was represented on the molecular surface, contoured at +3 (blue) and -3 kT/e (red) and depicted in Figure 3.7.

In all three homology models a clear electronegative surface patch was visible, probably located around the CD4bs and into the bridging sheet and inner domain. The homology model based on clade C $gp120_{cap102}$ is likely to be most representative for $gp120_{97CN54}$, but since homology modelling is sometimes subject to inaccuracy due to potential differences between the sequence fed to the model and the original sequence used for modelling, it was decided to compose three homology models. The fact that the negative electrostatic surface patch was found on all three of the models suggests that this patch is unlikely to be a result of inaccuracy, indicating that in addition to the negatively charged glycan sial acid residues present on $gp140_{97CN54}$, an electronegative surface patch on $gp120_{97CN54}$ offers another potential interaction site for contact between PEI and $gp140_{97CN54}$.


A
CD4 binding loop
(covering the CD4BS)
(bridging sheet)
Truncated V1/V2 loop
(bridging sheet)
Truncated N-terminus
(Inner domain)
B
C

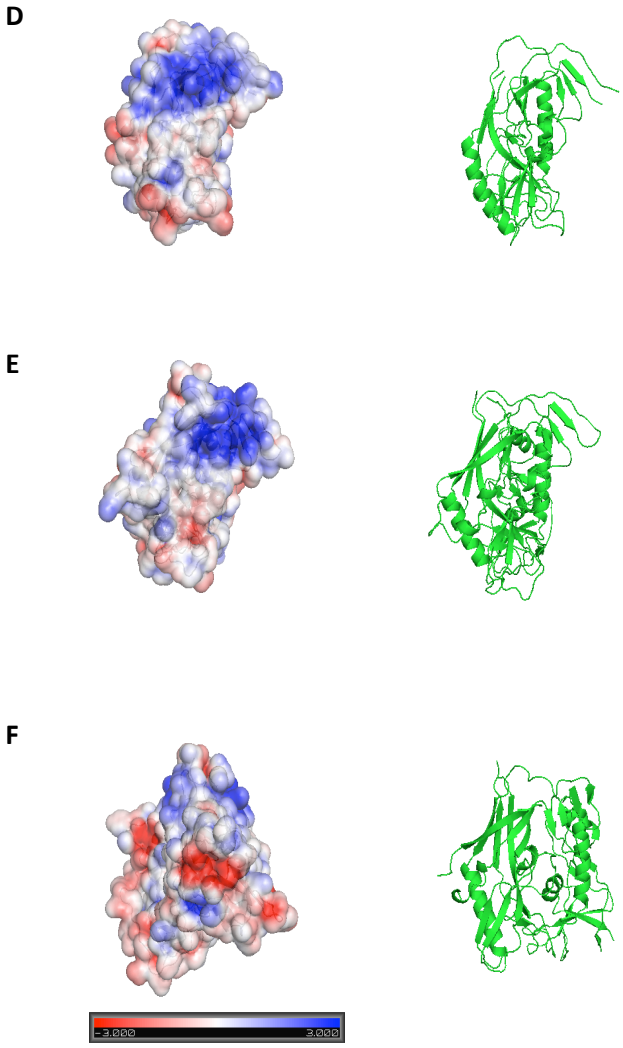


Figure 3.7. Electrostatic surface potential of three gp120$_{97CN54}$ homology models. Red= -3 kT/e, Blue= + 3 kT/e. The sequence of HIV-1 gp120$_{97CN54}$ (genebank:AF286226.1) was fed to the FFAS server at http://ffas.ljcrf.edu/ffas-cgi/cgi/ffas.pl searching the PDB as of August 2011 for structures of homologous sequences. The search retrieved and aligned the structures of gp120: 3LQA, 3NGB and 3U7Y. The homology modelling program SCWRL was used to produce 3D homology models of gp120$_{97CN54}$ based on those three structures. These three sets of coordinates were used in the Poisson-Boltzmann solving program APBS within the PyMOL software to compute the electrostatic potential. The potential was represented on the molecular surface, contoured at +3 (blue) and -3 kT/e (red)**. A+D. Homology model based on 3LQA (clade C gp120$_{CAP102}$)**(Diskin et al., 2010). Depicted sequence: LENVT-KVVEI. **B+E. Homology model based on 3NGB (clade A/E gp120$_{93TH057}$)**(Zhou et al., 2010)**.** Depicted sequence: VWKEA-VVEIK. **C+F. Homology model based on 3U7Y (other clade A/E gp120$_{93TH057}$ model)**(Diskin et al., 2011). Depicted sequence: VWKEA-VVEIK. The two views (A-C versus D-F) differ by a rotation of 180 degrees around the horizontal axis. Truncated N-terminus in the inner domain, and the truncated V1/V2 loop and CD4 binding loop in the bridging sheet region are indicated. The truncated C-terminus, V3, V4, and V5 loops are occluded from these views.

3.2.4. Direct interaction between PEI and human mAbs that correlates with theoretical pI of human Fab fragment

The most obvious assay to use to probe the effect of PEI-gp140 interaction on the antigenicity of gp140 is the binding of mAbs with defined epitopes to the antigen in the presence and absence of PEI. However, a complicating factor in this analysis is the potential for PEI to associate directly with mAbs via electrostatic interactions. Therefore, the potential for interaction between PEI and a selection of human mAbs was analysed using SPR. Human mAbs that did not show direct binding to PEI were selected for use in subsequent antigenicity assays.

PEI was coupled to a sensor chip and solutions containing BSA, HEL (positive and negative controls respectively, calculated theoretical pI 5.38 and 9.36, see previous section), and the following human mAbs were flowed over at pH 7.4: B12, HJ16, X5, 412D, 48D, 17B, 8B, A32, 4E10, HGP68, 19B, HR10, CD4IgG2, CD4 (Table 3.3 & Figure 3.8). As a background control for bulk refraction, to subtract effects caused by pH fluctuations or high analyte concentrations, a second empty sensor chip was activated and deactivated. The same solutions as before were flowed over this second sensor chip and the reading was subtracted from the test result.

Epitope	Antibody	Reference:
CD4bs	B12	(Burton et al., 1991; Burton et al., 1994; Zhou et al., 2007)
	HJ16	(Corti et al., 2010)
CD4I	X5	(Moulard et al., 2002)
	412D	(Choe et al., 2003)
	4.8D	(Thali et al., 1993)
	17B	(Thali et al., 1993)
C1-C5	8B	(Robinson)
C1-C4	A32	(Moore et al., 1994)
Gp41	4E10	(Stiegler et al., 2001)
V2	HGP68	(Corti et al., 2010)
V3	19B	(Gorny et al., 1992; Moore et al., 1995; Scott et al., 1990)
	HR10	(Corti et al., 2010)
CD4	CD4IgG2	(Allaway et al., 1995)
	sCD4	

Table 3.3. Range of antibodies that were studied in SPR assays: epitope, mAb, references.

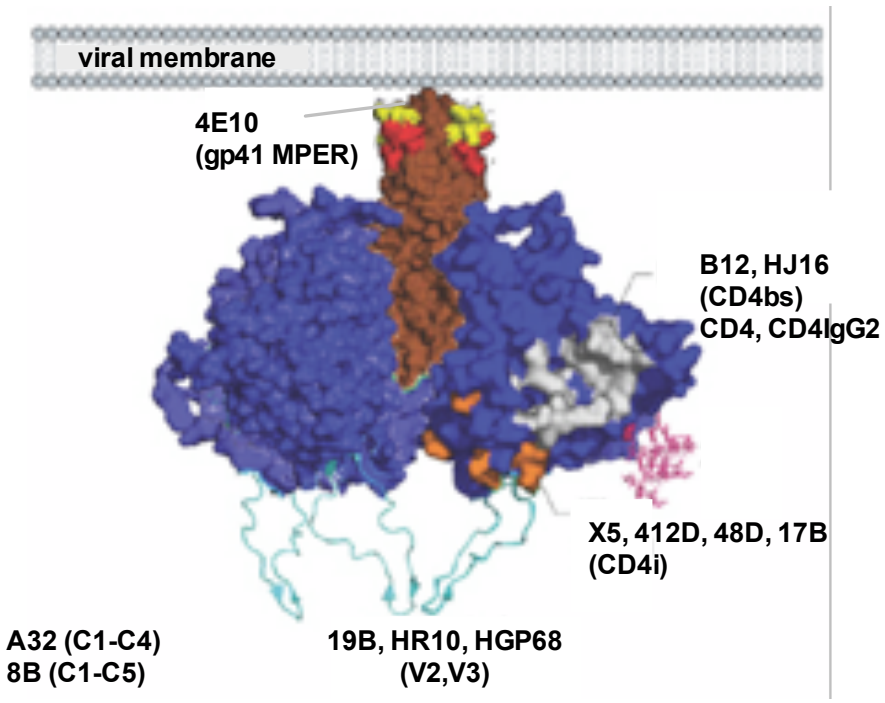


Figure 3.8. Human mAbs and their epitope specificity on Env gp120 and gp41. Since the V2 loop is not visible in this drawing the V2-loop Abs are depicted next to the V3 loop, which is approximately where the V2 loop is located. Adapted from (Karlsson Hedestam et al., 2008).

To explore a possible correlation of PEI–mAb interaction with charge and pI of the human mAb, where possible, the theoretical pIs of the mAb Fab fragments were calculated using the Protein Data Bank and the ProtParam tool of ExPASy. The observed interaction with immobilised PEI expressed in response units (RU) for each of the antibodies in solution, as well as the theoretical pI of the Fab fragments are provided in Table 3.4.

No binding to immobilised PEI was observed with the negative control HEL (pI 9.36) in solution. The positive control BSA (pI 5.38, Pan et al., 2009) was observed to bind to immobilised PEI (~1000 RU).

No detectable binding was observed for mAb B12 (gp120 CD4bs), 8B (gp120 C1-C5), A32 (gp120 C1-C4), or 4E10 (gp41 MPER). By contrast, the remaining ligands interacted directly with immobilised PEI: HJ16 (gp120 CD4bs, ~600 RU), X5 (gp120 CD4i, ~900 RU), 412D (gp120 CD4i, ~300 RU), 4.8D (gp120 CD4i, ~4500 RU), 17B (gp120 CD4i, ~1000 RU), HGP68 (gp120 V2 loop, ~700 RU), 19B (gp120 V3 loop, ~RU), HR10 (gp120 V3 loop, ~100 RU), CD4IgG2 (~75 RU), and sCD4 (~200 RU)(Figure 3.9 & Table3.4).

Highest PEI binding (~4500 RU) was observed for 4.8D mAb that was calculated to have the lowest Fab fragment pI (5.93), while lowest binding (~-50 RU) was observed for B12 mAb that had the highest theoretical Fab fragment pI (9.06) suggesting that the charge of the antibody Fab fragment at pH 7.4 might play a role in the interaction with PEI. Indeed, a negative correlation was found between mAb Fab fragment pI and PEI binding in RU ($P< 0.05$, Pearson r -0.7175, r^2 0.5149, Figure 3.10).

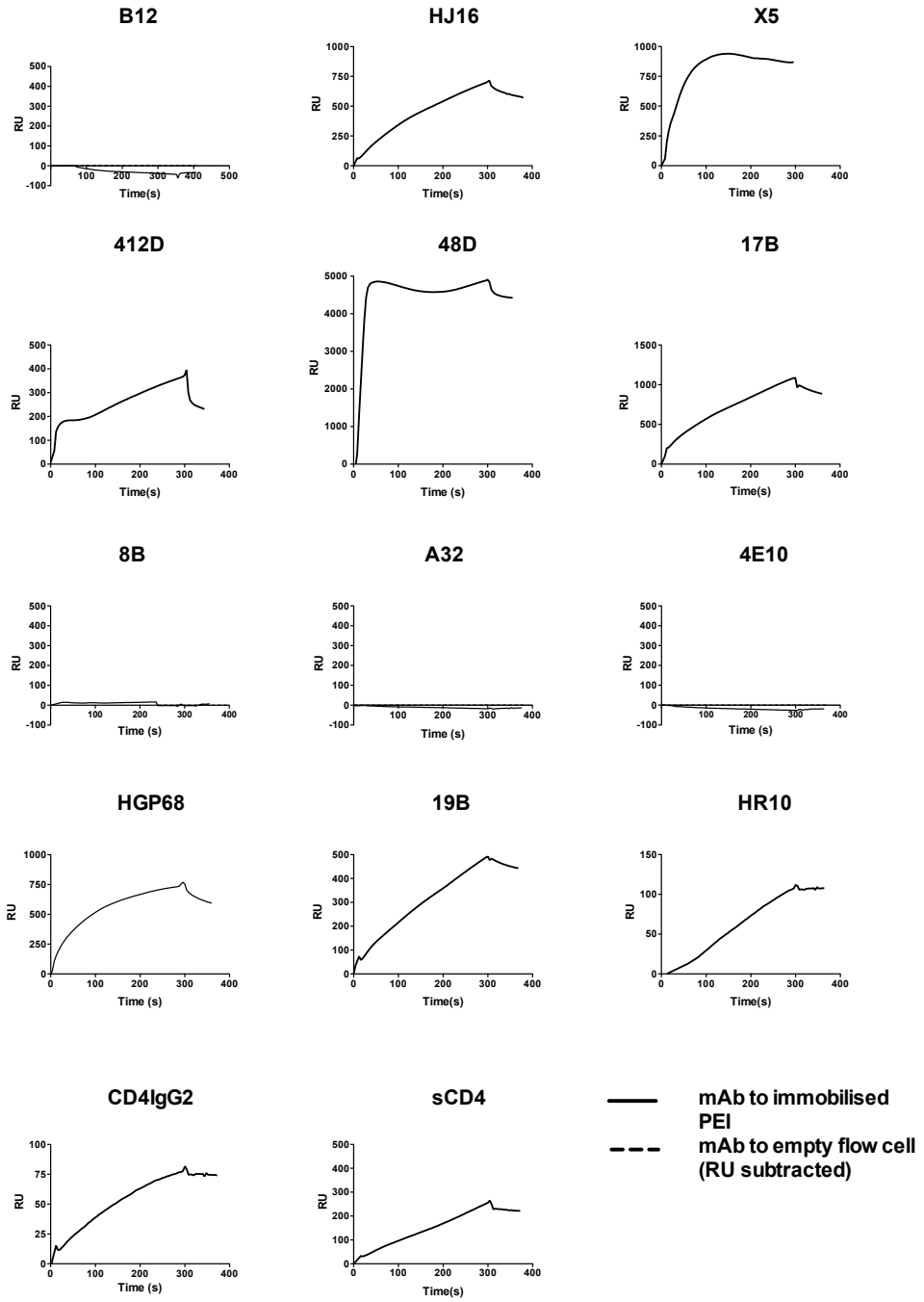


Figure 3.9. SPR binding interactions between human mAbs and PEI. PEI was surface immobilised and 25 µg/mL of human mAb B12 (CD4bs), HJ16 (CD4bs), X5 (CD4i), 412D (CD4i), 4.8D (CD4i), 17B (CD4i), 8B (C1-C5), A32 (C1-C4), 4E10 (gp41 MPER), HGP68 (V2), 19B (V3), HR10 (V3), CD4IgG2 (CD4bs) or sCD4 were flowed over at pH 7.4. Data are representative of >2 independent experiments. BIAcore 3000.

Epitope	Antibody	Fab fragment pI	PEI binding (RU)	No PEI binding (RU)
CD4bs	B12	9.06		-50 (0)
	HJ16	8.82	600	
	CD4IgG2		75	
CD4I	X5	6.61	900	
	412D	6.26	300	
	4.8D	5.93	4500	
	17B	7.22	1000	
C1-C5	8B			0
C1-C4	A32	8.96		0
Gp41	4E10			0
V2	HGP68		700	
V3	19B		400	
	HR10	6.36	100	
sCD4			200	

Table 3.4. Immobilised PEI-mAb interaction in SPR and calculated pI for human mAb Fab fragments. PEI was coupled to the sensor chip and the following human mAbs were flowed over at pH 7.4: B12, HJ16, X5, 412D, 4.8D, 17B, 8B, A32, 4E10, HGP68, 19B, HR10, CD4IgG2, CD4. As a background control a second empty sensor chip was activated and deactivated and the reading was subtracted from the test result. Binding is expressed in RU. Theoretical pIs of the human mAb Fab fragments were calculated, where possible, using the Protein Data Bank and the ProtParam tool of ExPASy. Binding results are representative of 2-3 independent experimental repeats.

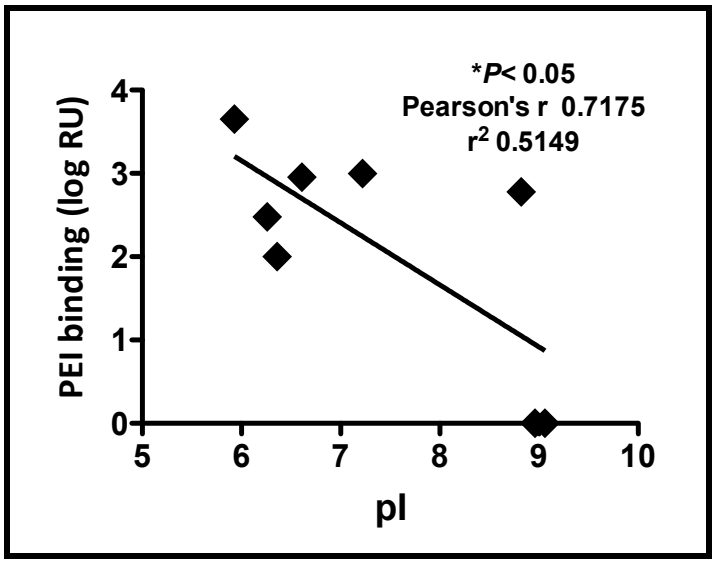


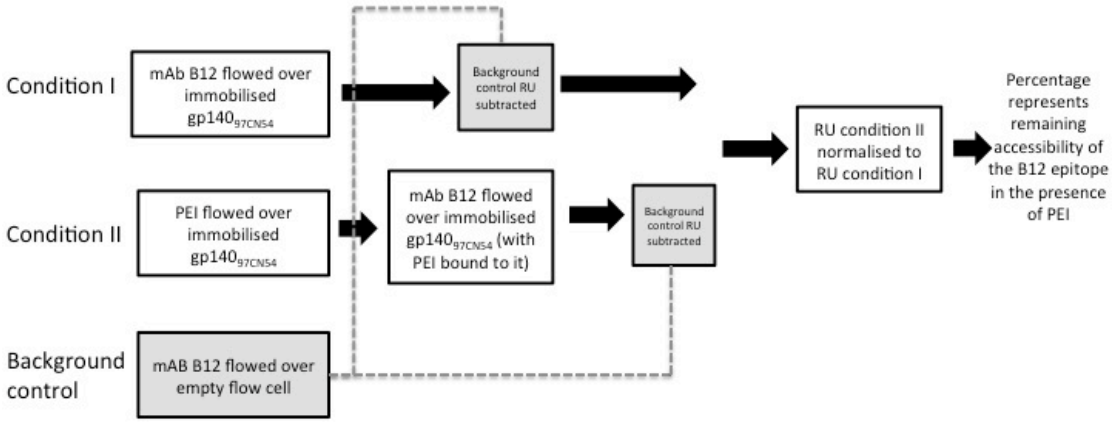
Figure 3.10. Correlation between immobilised PEI binding and theoretical pI of human mAb Fab fragment. *P< 0.05, Pearson's r 0.7175, r^2 0.5249.

3.2.5. Effects of PEI-gp140$_{97CN54}$ interaction on gp140$_{97CN54}$ antigenicity

3.2.5.1. Effect of PEI-gp140$_{97CN54}$ combination on accessibility of gp140$_{97CN54}$ epitopes

In the previous section it was shown that PEI adjuvantation can possibly shift IgG responses away from certain epitopes on gp140$_{97C54}$ towards other epitopes. Although not statistically significant, a trend was shown indicating that PEI possibly shifts the induced IgG response away from the mAb B12 epitope. To investigate whether PEI has occluded this epitope region, the accessibility of the mAb B12 epitope when gp140$_{97CN54}$ is in complex with PEI was investigated. Since gp140$_{97CN54}$ is terminated before the 4E10 epitope and because mAb A32 does not adequately bind to this Env antigen (personal communisation with dr. Simon Jeffs), these two other non-PEI binding mAbs could not be included in this assay. This study was

therefore focused solely on assessing the accessibility of the mAb B12 epitope. To investigate this, $gp140_{97CN54}$ was coupled to a SPR sensor chip and PEI and mAb B12 were flowed over under two experimental conditions (Flow chart 3.2). Similar negative controls as before where included: a) BSA was immobilised on a second flow cell in equivalent molar amounts compared to the immobilised $gp140_{97CN54}$ and b) a third empty flow cell was activated and deactivated and the reading was subtracted from the test results. In the first experimental condition mAb B12 was flowed over each flow cell without prior PEI incubation. In the second condition, PEI was first flowed over each flow cell before mAb B12 was flowed over. The observed binding responses in RU revealed reduced binding to $gp140_{97CN54}$ in the presence of PEI and are shown in Figure 3.11A.



Flow chart 3.2 Studying the accessibility of the B12 epitope on $gp140_{97CN54}$ in the presence of PEI.

No binding of mAb B12 to either the immobilised BSA or the empty flow cell was observed. In the absence of prior PEI incubation, mAb B12 interacted with immobilised HIV-1 $gp140_{97CN54}$

attaining ~3500 RU. When PEI was flowed over immobilised $gp140_{97CN54}$ before mAb B12 was flowed over, B12-$gp140_{97CN54}$ interaction was still observed but decreased to ~2200 RU, indicating that PEI binding to $gp140_{97CN54}$ reduced accessibility of the B12 CD4bs epitope on gp120 but left the epitope mostly available for binding.

To confirm these results in another format, an ELISA assay was carried out in which ELISA plates were coated with HIV-1 $gp140_{97CN54}$ and incubated with mAb B12 under two conditions: 1) in the absence of prior PEI incubation and 2) following 2-h incubation with a saturating amount of PEI (0.354 mg/mL at pH ~7.4). The pH was measured and confirmed to be around pH 7.4 to ensure that the effect observed would not be due to PEI's buffering capacity and any resulting pH changes. The observed binding curves were normalised to the results in the first experimental condition and are shown in Figure 3.11B.

In accordance with the SPR results, in ELISA maximum mAb B12 binding to $gp140_{97CN54}$ was reduced in the presence of PEI, although this was not statistically significant. In ELISA the mean observed reduction was lower than observed in the SPR assay (~12 % in ELISA versus ~37% in SPR).

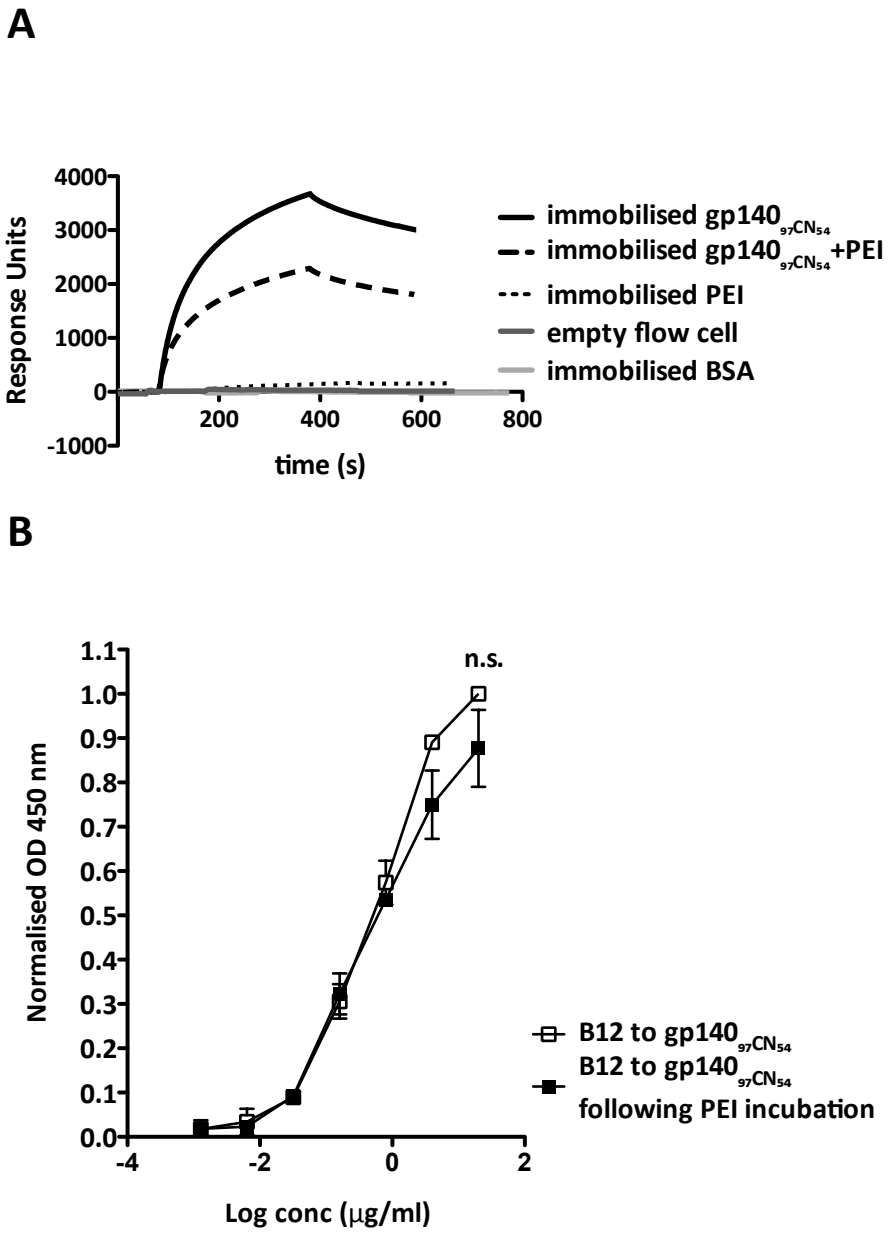


Figure 3.11. The effect of PEI binding to gp140$_{97CN54}$ on the accessibility of the mAb B12 epitope. A. SPR analysis. HIV-1 gp140$_{97CN54}$ was coupled to a SPR sensor chip and PEI and mAb B12 were flowed over under two experimental conditions (Flow chart 3.2). Negative control: immobilised BSA. Background control: an empty flow cell was activated and deactivated and the reading was subtracted from the test results. **B. ELISA.** ELISA plates were coated with 0.5 μg/mL gp140$_{97CN54}$ and mAb B12 was titrated and incubated for 1 h in the presence of absence of a preceding 2-h incubation with a saturating amount of PEI at pH ~7.4 (0.354 mg/mL). Detection was carried out with an anti-human detection Ab. Means ± SD of 3 independent experimental repeats are depicted.Mann-Whitney test, n.s.

3.2.5.2. Investigating potential denaturation of gp140$_{97CN54}$ during PEI-adjuvanted gp140$_{97CN54}$ immunisation

Serum of the previously described rabbit immunisation study (Figure 3.3) was further analysed in an ELISA assay with native and heat-denatured forms of gp140$_{97CN54}$ to examine whether PEI-gp140$_{97CN54}$ interaction had resulted in denaturation of gp140$_{97CN54}$ compared to the Imject®Alum and FCA/FIA adjuvants. If the antigen was partially or completely denatured after administration *in vivo* then immune sera would be expected to recognise denatured antigen more strongly than native antigen. By contrast, if the antigen remained native then the sera should recognise the native antigen better. For this purpose, serum samples collected at 3 weeks post boost (week 9) were used. As a control for native gp140$_{97CN54}$ binding B12 mAb was used, since this mAb is directed to a conformation-dependent epitope (Ho et al., 1991) and therefore is expected not to bind to denatured gp140$_{97CN54}$. As a control for denatured gp140$_{97CN54}$ binding, polyclonal serum raised against denatured IIIB gp120 was used, which is expected to maintain binding to both native and heat-denatured gp140$_{97CN54}$ forms. The EC_{50} titres were calculated for both native gp140$_{97CN54}$- and denatured gp140$_{97CN54}$- specific IgG binding and the ratio of the two was determined and presented in Figure 3.12.

B12 mAb completely failed to bind denatured gp140$_{97CN54}$ forms, which confirmed the success of the heat-denaturing process in prompting loss of glycoprotein conformation. In contrast, the polyclonal serum fully bound to both gp140$_{97CN54}$ forms in line with the expected presence of both linear and conformational epitope-targeting antibodies in polyclonal serum.

Although variation was observed between independently repeated experiments, no clear differences were observed between the distinct adjuvant groups. The EC_{50} titre for native

$gp140_{97CN54}$-specific binding was for all three adjuvants on average two-fold greater (between one and ten-fold) than the EC_{50}-titre for denatured $gp140_{97CN54}$-specific binding, suggesting that during immunisation most of HIV-1 $gp140_{97CN54}$ antigen remained in its native form allowing antibodies against conformational epitopes to be formed.

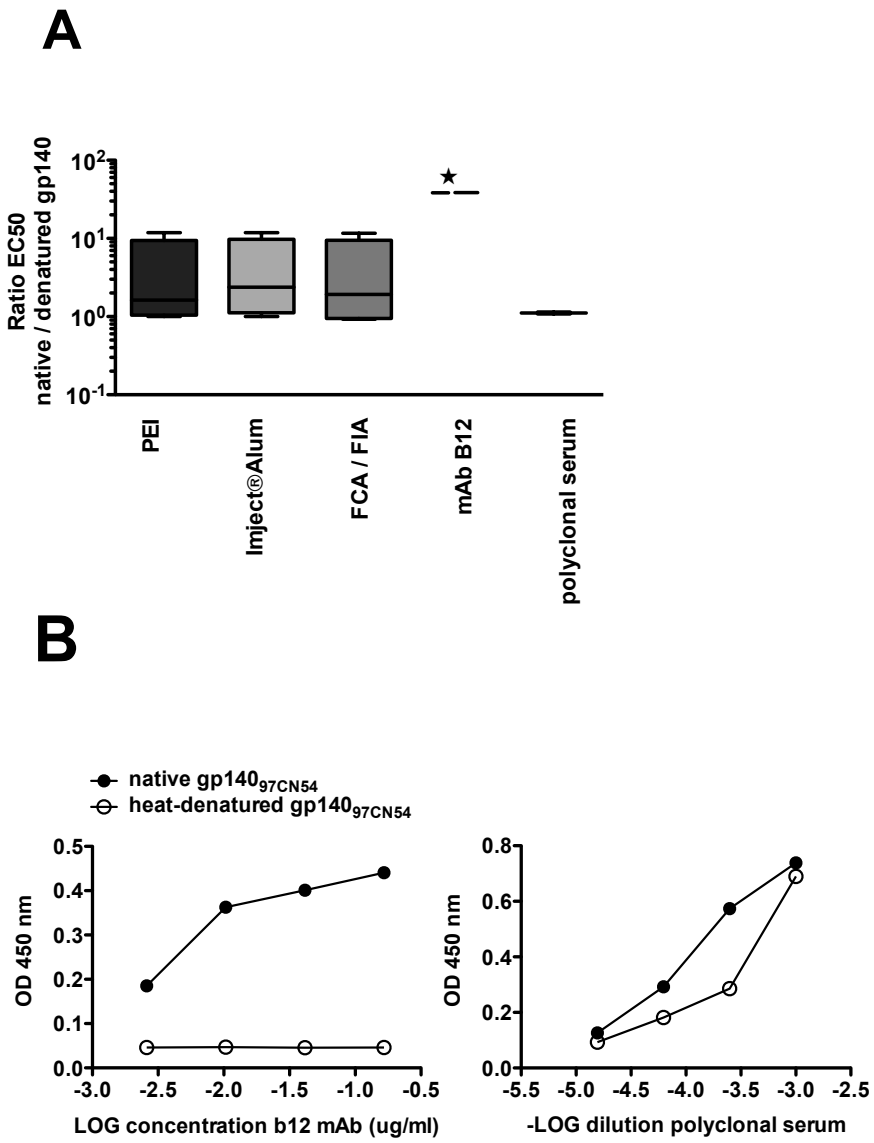


Figure 3.12. The ratio in which PEI-induced serum gp140$_{97CN54}$-specific IgG antibodies recognise native HIV-1 gp140$_{97CN54}$ antigen versus denatured gp140$_{97CN54}$ antigen in comparison to standard adjuvants Imject®Alum and FCA/FIA in a rabbit immunisation model. Rabbits were prime and boost immunised with 50 µg HIV-1 gp140$_{97CN54}$ antigen mixed with 202.52 µg PEI, antigen mixed 1:1 with Imject® Alum, or antigen emulsified 1:1 in FCA/FIA adjuvant at week 0. At week 3 rabbits received boost immunisations with 20 µg gp140$_{97CN54}$ antigen in equivalent formulations. At week 6 and 9 rabbits received 20 µg unadjuvanted gp140$_{97CN54}$. Serum was collected at week 9 and used for ELISA with native and heat-denatured gp140$_{97CN54}$. Human mAb b12 that is directed to a highly conformation-dependent epitope and polyclonal serum raised against denatured IIIB gp120 were used as controls. **A. Ratios of native glycoprotein-specific IgG EC$_{50}$ titres over denatured glycoprotein-specific IgG EC$_{50}$ titres are shown.** Means ± SD of 3 independent experimental repeats (each conducted in triplicate) are depicted. A star was used to indicate that a reliable ratio could not be calculated for mAb b12 binding since denatured gp140$_{97CN54}$–B12 binding was completely absent. **B. B12 mAb and polyclonal serum controls.**

3.3. Chapter discussion

Here it was shown that the gold standard transfection agent PEI is able to act as a robust adjuvant in protein immunisation in rabbits. To the best of my knowledge, this is the first time that PEI is shown to have *in vivo* adjuvant activity in the context of prophylactic protein subunit immunisation.

PEI adjuvantation in the rabbit HIV-1 $gp140_{97CN54}$ immunisation model resulted in significantly enhanced serum $gp140_{97CN54}$-specific antibody titres as early as 1 week post prime (Figure 3.2 & 3.3). The observed adjuvant effect was indistinguishable from the effect observed with Alum, a strong T_h2-adjuvant successfully incorporated into a wide range of human vaccines that rely on antibody-based protection (Mbow et al., 2011). The Env-specific responses were sustained over 20 weeks, which was the total duration of the experiment, suggesting that PEI-induced $gp140_{97CN54}$-specific antibody responses can be long lasting in a prime-boost protocol. The induction of durable antibody responses is crucial for effective immunisation (Amanna et al., 2007). The trimeric, clade C, GMP, HIV-1 $gp140_{97CN54}$ antigen was chosen because of reasons outlined in the chapter introduction. Whether the results obtained on PEI adjuvant activity can be extrapolated to other HIV-1 Env immunogens would need to be elucidated by further investigation.

PEI-induced Env-specific antibodies in the chosen immunisation model were capable of binding a wide range of HIV-1 Envs comprising the three main circulating HIV-1 clades and distinct isolates within each clade, suggesting that PEI adjuvantation induced antibodies to conserved epitopes that are present on the majority of divergent circulating HIV-1 isolates (Figure 3.4). Whether the observed broad HIV-1 Env recognition by serum antibodies can translate into broad protection against HIV-1 isolates in a PEI-adjuvanted immunisation

strategy would need to be investigated at a later stage in HIV-1/SIV challenge models. Furthermore, the observations could represent an antigen-intrinsic effect rather than a property of the adjuvants. However, the observed results suggest that PEI is capable of eliciting HIV-1 Env-specific antibodies that allow broad viral strain recognition and therefore warrant further investigation of PEI adjuvanticity.

The observed trend for recognition of Envs was clade C ≥ A > B, of which only BaL gp120 deviated. It was expected to see greater differences between clades than within each clade, since viral genome diversity is known to be higher inter- than intra-clade (25-35% and 15-20% respectively for Env sequences), which in general corresponded to the observations, but the BaL deviation could not be explained through sequence homology (Korber et al., 2001)(Table 3.2). No differences were found between BaL gp120 and other clade B Envs in terms of overall sequence homology or homology around specific epitopes. It is of interest to mention that the HIV-1_{97CN54} isolate lacks a mAb 2F5 epitope and the $gp140_{97CN54}$ construct is terminated just before the mAb 4E10 epitope, which means that these epitopes could not be included in the sequence analysis (Sheppard et al., 2007). Despite the fact that the BaL gp120 exception cannot be explained through sequence homology, it is still possible that genome diversity around specific epitopes or differences in tertiary/quaternary structure or gp120 glycosylation account for the different results obtained with BaL gp120 compared to the other clade B isolates. Also, a possible difference in efficacy of ELISA coating due to Env differences cannot be ruled out.

Through cross-competition ELISA with human mAbs of known specificity an attempt was made to approximately identify the gp120 regions that are targeted by PEI-induced serum antibodies (Figure 3.5). The results indicated that although not statistically significant, PEI adjuvantation in the $gp140_{97CN54}$ rabbit immunisation model results in the induction of serum

IgG with relatively higher mAb b12 inhibition potency than in the FCA/FIA group and lower than the Alum group. At the same time, PEI induced IgG with relatively weaker inhibition potency to the V3 loop compared to experimental adjuvant FCA/FIA, but with statistically significantly stronger inhibition potency than the licensed Alum. The ability to modestly bias antibodies away from certain antigenic surfaces to other more desirable epitope surfaces, that are involved in virus neutralisation or ADCC, is desirable for immunisation (Pantophlet et al., 2003). Although originally regarded as an inappropriate target for a vaccine, because of its diversity and occlusion by glycan structures, the V3 loop has recently regained popularity (Bonsignori et al., 2011; Hioe et al., 2010; McCaffrey et al., 2004). Since the V3 loop determines CCR5 or CXCR4 coreceptor usage, the V3 region must comprise conserved elements and must at least in part be exposed to enable coreceptor binding (Cardozo et al., 2007; Cocchi et al., 1996; Nolan et al., 2009; Shioda et al., 1992). Indeed, recently-identified V3-loop mAbs were found to achieve significant degrees of broad cross-neutralisation among divergent HIV-1 isolates and recent NMR and crystallographic studies have demonstrated conserved features of the V3 loop (Bonsignori et al., 2011; Hioe et al., 2010; Stanfield et al., 2004; Stanfield et al., 2006; Walker et al., 2009). Alltogether, these most recent findings have replaced the V3 epitope back in the list of desired vaccine targets. However, in recent years the number of identified nAbs has generally been greatly extended, not only including antibodies exclusively targeted to the V3 loop, but also to other loops and the CD4bs, including PG9, PG16, CH01-CH04, HJ16, VRC01 and VRC02, (Bonsignori et al., 2011; Corti et al., 2010; Walker et al., 2009; Wu et al., 2010). The most potent of these, VRC01, as well as HJ16, bind to the CD4bs, reconfirming the importance of this epitope region, which suggests that it is probably not desirable for a vaccine candidate to shift the IgG response away from this epitope (Corti et al., 2010; Wu et al., 2010; Zhou et al., 2010). In this respect, it is promising that

compared to potent FCA/FIA adjuvant PEI-induced serum IgG showed higher relative inhibition of mAb B12. For a complete up-to-date screen of PEI-induced serum antibodies and their epitope regions on gp120, these antibodies should be included in future analyses.

PEI was demonstrated to directly interact with $gp140_{97CN54}$ in both studied SPR conditions and also showed binding to the glycoprotein OVA (Figure 3.6). A later study confirmed the hypothesis that PEI–OVA binding occurs through electrostatic interactions (Chen et al., 2011). Because of PEI's multivalency it is expected that the immobilisation of PEI will have a smaller effect on the observed interaction with $gp140_{97CN54}$, than immobilisation of $gp140_{97CN54}$ would have. Therefore it is expected that the the interaction between PEI and $gp140_{97CN54}$ in solution, as in a vaccine formulation, although the behaviour of both molecules in solution is unknown, will more closely mimic the second studied condition in which $gp140_{97CN54}$ was in solution rather than being arrayed on a solid-phase matrix, and will be most in line with the observed binding in this situation, therefore being present, but relatively weak. Despite this assumption, it is possible that the involvement of PEI amine groups in the immobilised PEI condition rendered fewer amine groups available for interaction with soluble $gp140_{97CN54}$, which might have resulted in a weaker observed interaction in this SPR condition than would be representative of the PEI-$gp140_{97CN54}$ interactions in vaccine formulation. Homology modelling showed that $gp140_{97CN54}$ has a large electronegative surface patch which, in addition to the glycan sialic acid residues, provides a potential site for interaction with cationic PEI (Figure 3.7).

The observed direct interaction between PEI and $gp140_{97CN54,}$ which could be further investigated through the use of Isothermal Titration Calorimetry, might play an important role in PEI-mediated adjuvant effects and possibly adds to its robust activity, for example through

antigen targeting and possible protection against denaturation. Nevertheless, this direct interaction could simultaneously result in adverse effects on $gp140_{97CN54}$ antigenicity and urged an investigation into potential denaturation or loss of accessible epitopes following PEI-$gp140_{97CN54}$ binding.

The first assay to investigate this was limited by the fact that the majority of tested human mAbs were found to bind directly to PEI, which excluded them from use in the assay (Figure 3.9, Table 3.4). Nevertheless the absence of PEI binding with nAb B12 allowed investigation of a potential PEI-mediated effect on the accessibility of the CD4bs epitope, which is one of the main neutralising epitopes on gp120 and because of its conformation-dependence is a very suitable epitope for the purpose of this study. Through two different assay methods it was shown that, although binding was reduced with 12-37% (in ELISA and SPR respectively), one of the most important epitopes, the CD4bs, remained accessible for mAb binding in the presence of PEI (Figure 3.11). No obvious reduction in binding avidity was observed in either format, implying that PEI completely obstructed a proportion of the B12 binding sites rather than partially limiting B12 binding to all sites. These data suggest that the SPR format is more sensitive to changes in mAb binding avidity induced by PEI-$gp140_{97CN54}$ association than the ELISA format, probably as a result of real-time binding dynamics measured by SPR. It is possible that the 1 h B12 incubation time in ELISA allowed more bonds to be formed than the 15 sec injection time in SPR and it is possible that this or other differences in experimental conditions accounted for the difference in observed PEI-induced reduction of B12 binding to $gp140_{97CN54}$.

The second assay to investigate potential PEI-mediated denaturation of $gp140_{97CN54}$ during PEI-adjuvated immunisation was limited by the lack of availability of an unadjuvanted serum

sample for analysis (Figure 3.12). Yet, the mAb B12 and polyclonal serum controls provided a measure of conformation-dependent and conformation-independent binding and the Imject®Alum- and FCA/FIA-adjuvanted serum samples allowed a comparison of PEI to other adjuvants, one of which is licensed for use in man. Indeed mAb B12 binding was completely absent in the case of heat-denatured $gp140_{97CN54}$, while polyclonal serum control binding remained completely intact. The adjuvanted rabbit serum samples showed binding to a level falling in between these two controls, since binding to heat-denatured $gp140_{97CN54}$ was impaired for all adjuvants compared to native $gp140_{97CN54}$ binding, but not completely absent. Some variation was observed between independent repeats of the experiments, possibly due to differences in the extent of $gp140_{97CN54}$ denaturation during the heating step. The respective EC_{50} titre ratios of native: denatured $gp140_{97CN54}$ binding observed for the distinct adjuvants were indistinguishable, suggesting that PEI does not differ from FCA/FIA and Alum adjuvants in this respect. It can therefore be concluded that the event of direct interaction between PEI and $gp140_{97CN54}$ does not pose a threat on $gp140_{97CN54}$ structure conservation, not more than in the case of other adjuvants, and that the majority of present conformation-dependent epitopes remain intact during PEI-adjuvanted immunisation.

The magnitude of PEI-mAb binding was found to correlate with pI of the respective mAb fab fragment, suggesting that electrostatic interactions might play a role (Figure 3.10). This is in line with previous observations that PEI binds to, at neutral pH, negatively charged molecules serum albumin, DNA and sialic-acid containing sulphated proteoglycans, but fails to bind positively charged HEL protein, and with my hypothesis that it is through interaction with negatively charged surface patches and sialic acid residues on $gp140_{97CN54}$ that PEI binds to this Env antigen. Unfortunately, not all pIs could be calculated since the sequences of some of the

used mAbs were not publically available, but the included number of Abs was sufficient to find a statistically significant correlation. The high degree of interaction between PEI and the CD4i mAbs (412D, 17B and 4.8D) was expected since these mAbs are sulphated, with the sulphate groups contributing negative charge, and are assumed to also interact with gp120 in part via charge interactions (Huang et al., 2005). For future studies, it might be worth including more recently discovered mAbs and more informative results could be obtained by doing kinetic BIAcore studies or Isothermal Titration Calorimetry.

The demonstrated binding between PEI and mAbs has important implications for future studies with PEI and stresses the need for isotype controls in assays where PEI is used. In general, studies with PEI are complicated by several unique factors of this polymer, such as its binding to a wide range of (mainly anionic) molecules, its robust pH buffering capacity, and its intrinsic fluorescence that can make certain methods unfeasible or confound assay interpretation (Boussif et al., 1995; Pastor-Pérez, et a., 2007). Furthermore, there is no reliable available method for quantification of PEI other than weighing (Bertschinger et al., 2004). Bertschinger's group introduced a method for detection of PEI in DNA-PEI polyplexes, but this method only works at small concentrations of PEI (0.05-4µg/mL). Ninhydrin assays allow the determination of the amount of primary amine groups present in a sample, but do not reveal the total amount of PEI present (Leane et al., 2004; Tian et al., 2007). I have done a brief exploration of the use of spectrophotometry for PEI quantification and found that PEI was absorbed at 220 nm and the OD_{220} correlated with the used PEI concentration (Pearson's r: 0.7572-0.8403, r^2: 0.57). However, this correlation was only found for pure highly concentrated (>~10 mg/mL) PEI samples and disappeared at lower concentrations or upon the addition of conjugates or glycoproteins (data not shown), suggesting that this quantification by absorbtion does not

provide an adequate quantification method for PEI at concentrations used in experimental conditions.

Key chapter findings

Taken together, PEI was demonstrated in this chapter to:

- **Have robust adjuvant activity in s.c. gp140$_{97CN54}$ immunisation in rabbits**
- **Elicit gp140$_{97CN54}$-specific antibodies that recognise both homologous and heterologous divergent Env glycoproteins**
- **Induce a modest gp140$_{97CN54}$-specific antibody bias to the CD4bs compared to licensed adjuvant Alum**
- **Directly interact with gp140$_{97CN54}$ and OVA antigens, possibly via electrostatic interactions**
- **Directly interact with a range of human mAbs, the extent of which correlates with the pI of the human mAb fab fragment**
- **Not have an additional denaturing effect on gp140$_{97CN54}$ compared to gold standard FCA/FIA and Alum adjuvants and to maintain the accessibility of the B12 CD4bs epitope during interaction with gp140$_{97CN54}$**

CHAPTER 4 [▽]

Mucosal PEI adjuvant activity

&

The effect of PEI structure and biodegradability on adjuvant activity

[▽] Portions of this work have been submitted for publication

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4.1. Chapter introduction

4.1.1. General introduction

In the first thesis chapter, 25 kDa bPEI was shown to be a potent adjuvant in systemic immunisation. Here, I investigated whether 25 kDa bPEI is also able to induce a mucosal immune response via intranasal (i.n.) administration. PEI exists in various forms, high and low molecular weight, linear and branched, biodegradable and standard (non-biodegradable). In this chapter, I aimed to investigate the potential impact of PEI structure on adjuvant activity by analysing a range of selected PEI forms in the $gp140_{97CN54}$ immunisation model in mice. Finally, since toxicity is a major concern in adjuvant use, I investigated this in the context of different PEI forms.

The predominant contribution of sexual transmission to the spread of the HIV-1 pandemic and the crucial role of the mucosa in HIV-1 infection emphasise the need for HIV-1 mucosal immunisation. Furthermore, the generally low immunogenicity of (glycoprotein) antigens via this route urges the development of a safe and effective mucosal adjuvant to enhance mucosal immunisation (Neutra and Kozlowski, 2006; Ryan et al., 2001). In this chapter, I compared the elicitation of mucosal and systemic $gp140_{75CN54}$-specific immune responses of PEI to the gold standard mucosal adjuvant CTB, which is known for its mixed T_h1 and T_h2 responses and induction of strong mucosal IgA responses (Imaoka et al., 1998; Sanchez et al., 2004; Su et al., 2004; Williams et al., 1999). Since mixed T_h1 and T_h2 responses would be desired for HIV-1 immunisation, potential PEI-induced T_h polarisation was investigated by analysis of PEI-induced serum IgG1 and IgG2a responses and the release of cytokines by T cells isolated from local LN and spleen following mucosal immunisation with PEI.

The mucosal route of choice was the nasal route since the current literature states that i.n. immunisation is a particularly potent inducer of both systemic and mucosal immune responses (Table 4.1). Immunisation via the nasal route can induce robust immune responses, including strong IgA antibody responses, in the genital tract, the rectum, and the respiratory tract in mice, NHP and humans (Imaoka et al., 1998; Kozlowski et al., 2002; Kunkel and Butcher, 2003; Staats et al., 1997) as well as cellular responses, including CTL responses, in some of these distal mucosal sites such as the genital tract (Gallichan and Rosenthal, 1998). Also, the nasal route of immunisation has been shown to be capable of inducing higher systemic antibody titres and as a result to require a smaller amount of antigen than other mucosal routes, possibly because antigen and APCs can be easily trafficked to the NALT and draining cervical LNs after i.n. administration (Gallichan and Rosenthal, 1998; Kozlowski et al., 2002; Medaglini et al., 2006; Staats et al., 1997).

Route	Specific serum IgG	Specific IgA antibodies				
		Small intestine	Large intestine	Cervix/Vagina	Salivary glands	Nasal cavity
Nasal	+++++	ND	++	+++	-	+++
Oral	+++	++++	++	+/-	+	-
Rectal	+++	ND	+++++	-	+/-	ND
Vaginal	++	ND	+/-	++++	+/-	ND

Table 4.1 The effect of mucosal immunisation route on local and distal antibody responses to CTB, gold standard mucosal adjuvant/immunogen). Geometric mean of post-immunisation increases in specific antibody levels: +++++ > 50-fold; ++++, 25-49.9-fold, +++, 10-24-fold, ++,5-9.9-fold; +, 2.5-4.9-fold; +/-, > 2.5 fold in a minority of vaccine recipients;-, less than 2.5-fold in all vaccine recipients; ND, not determined. Adapted from: (Neutra and Kozlowski, 2006).

4.1.2. Structural impacts on PEI adjuvant activity: lPEI and bPEI forms of various MW

In this section a brief background will be provided on the existing distinct structural forms of PEI, in particular the forms that were selected for my studies, to provide a context for my research on the potential impact of PEI structure on adjuvant activity in the $gp140_{97CN54}$ immunisation model in mice.

The first patents for polymerisation of ethyleneimine (aziridine) arose in the 1930's and various ways of catalysed synthesis of bPEI have been described as early as the 1940's up until the early 1970's by different groups (Dick, 1970; Jones, 1944; Zhuk, 1944). bPEI forms are most commonly synthesised through acid-catalysed polymerisation of ethyleneimine (aziridine) (Figure 4.1). Briefly, protonation of the aziridine monomer results in the formation of an immonium cation that in turn reacts with another aziridine monomer for propagation or with

an already formed polymer chain to form branches (von Harpe et al., 2000). The synthesis of high MW forms can be achieved by polymerisation of anhydrous aziridine upon addition of initiator. By contrast, when polymerisation is carried out in aqueous solution, the reaction occurs more slowly and can be controlled by variation of reaction temperature or amount of initiator added, resulting in PEI products of various MW (Jones et al., 1943).

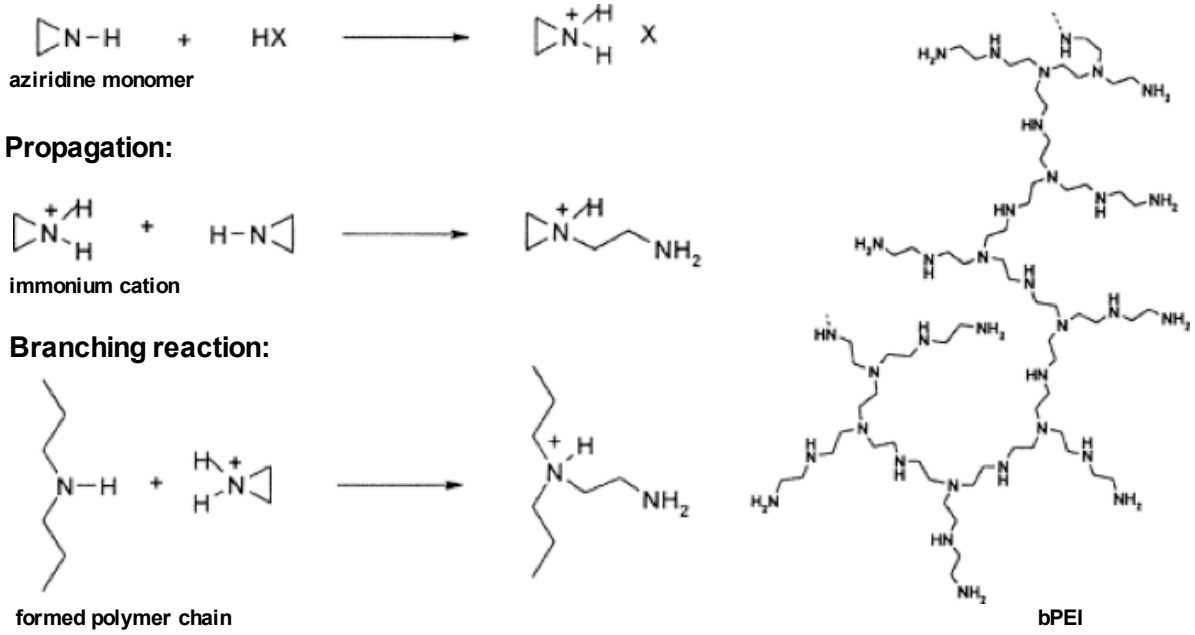

Figure 4.1. Acid-catalysed polymerisation of ethyleneimine (aziridine) during synthesis of bPEI. Adapted from: (Lungwitz et al., 2005), original souce: (Dick and Ham, 1970).

More recently, various ways to synthesise lPEI forms have been described (Brissault et al., 2003; Chemin et al., 1998; Chemin, Caba et al 2004). The most commonly used method involves cationic ring-opening polymerisation of either *N*(2-tetrahydroppyranyl)azidirine or 2-oxazolines followed by acid hydrolysis of the respective *N*-substituted compound (Figure 4.2) (Oh et al., 2002; Weyts and Goethals 1988, Saegusa et al., 1972a, Saugusa et al., 1972b).

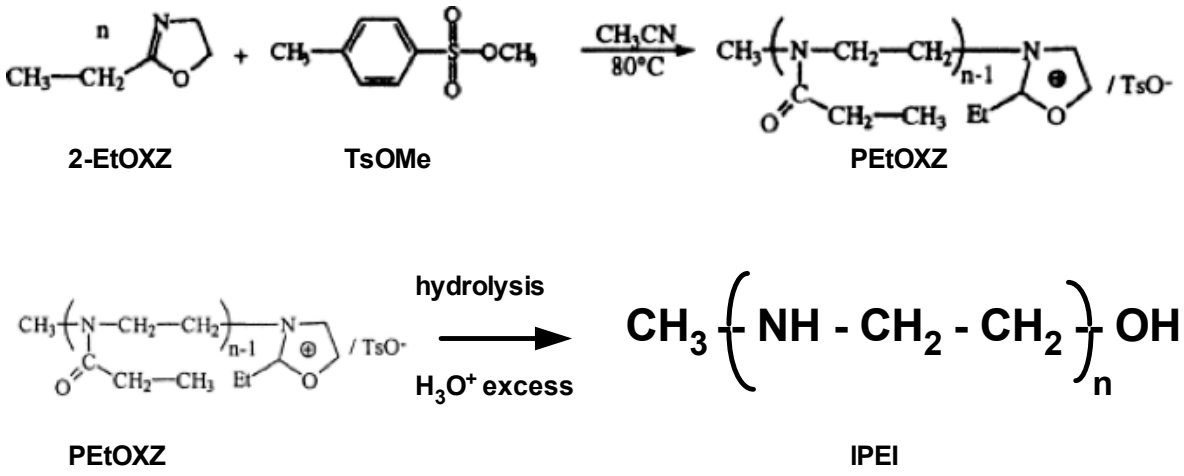

Figure 4.2. Synthesis of lPEI. The first step involves cationic ring-polymerisation of 2-ethyl-2-oxazoline (2-EtOXZ) that is initiated by methyl-p-toluenesulfonate (TsOMe) and yields poly(2-ethyl-2-oxazoline) (pOXZ) with oxazolinium tosylate ends (PEtOXZ). The second step involves acid hydrolysis of PEtOXZ using an excess of hydrochloric acid in aqueous media to produce linear PEI. Adapted from: (Lungwitz et al., 2005).

4.1.2.1. Structural impacts on transfection efficiency

The transfection activity and gene delivery potential of PEI applies to both lPEI and bPEI forms, as was shown in the first study on PEI transfection by Boussif et al. (Boussif et al., 1995). Later studies, however, pointed out differences in transfection efficiency between various PEI forms. Structural differences in PEI, including degree of branching and MW, but also protonation/cationic charge, were shown to affect polyplex characteristics and impact on transfection efficiency and toxicity (Lungwitz et al., 2005; Neu et al., 2005; Sun et al., 2011; Yue et al., 2011).

The degree of PEI **branching** was found to influence transfection efficiency with more highly branched forms having higher transfection efficiency than less branched or linear forms, albeit with higher toxicity (Gharwan et al., 2003; Lungwitz et al., 2005; Sun et al., 2011; Wightman

et al., 2001; Wiseman et al., 2003). This effect is thought to be partially due to the formation of more compact PEI/DNA complexes with highly branched PEI forms, resulting in smaller and more stable particle sizes compared to less branched or lPEI forms, as well as to direct effects of PEI branching on cell entry and nuclear uptake (Boussif et al., 1995; Brunner et al., 2000; Sun et al., 2011; Yue et al., 2011). In contrast with these findings, the Jet-PEI 22 kDa lPEI form has later been proven to be particularly effective for *in vitro* transfection and *in vivo* gene delivery, being superior to the gold standard bPEI 25 kDa form, whilst causing few or no toxic side effects (Bonnet et al., 2008; Bragonzi et al., 1999; Chemin et al., 1998; Ferrari et al., 1999; Jeong et al., 2007; Tracey et al., 1987; Wightman et al., 2001; Wiseman et al., 2003). The Jet-PEI 22 kDa lPEI form was first investigated by Behr and Boussif and later developed for commercial supply by Thermo Scientific (Exgene 500) and Polyplus (*In vivo* Jet-PEI) (Boussif et al., 1995; Behr et al., 1997).

MW has an effect on PEI transfection efficiency, although results on the effect of MW have been controversial at times, possibly due to distinct experimental conditions (Morimoto et al., 2003). Generally however, a higher MW correlates with a higher transfection efficiency *in vitro* in the human endothelial cell-derived cell line EA.hy 926 and *in vivo*, but again correlates with a higher cytotoxicity (Breunig et al., 2008; Fischer et al., 1999; Godbey et al., 1999; Kunath et al., 2003; Sun et al., 2011; Turunen et al., 1999; von Harpe et al., 2000). Similar to branching effects this MW effect is probably mediated through MW effects on PEI/DNA particle size. High MW bPEI forms, for instance, have a superior capacity to form compact and stable DNA/PEI complexes compared to lower MW forms (Godbey et al., 1999) and polyplex size therefore decreases with increasing MW (Lungwitz et al., 2005). Furthermore, a recent study showed that PEI forms with long branches are efficient transfection reagents due to their higher

capacity to disrupt and cross cell membranes (Yue et al., 2011). Similar transfection results can be achieved with lower MW bPEI forms but this requires substantially higher PEI concentrations and higher N/P ratios. The bPEI 25 KDa form first emerged as the gold standard nonviral transfection agent, and later the lPEI 22 kDa as well, because of their combination of high transfection efficiency with relatively low toxicity (Boussif et al., 1995; Fortune et al., 2011).

A recent study by Sun et al (Sun et al., 2011) confirmed the effect of PEI **protonation** state on transfection efficiency. At physiological pH every fifth or sixth nitrogen atom in PEI is thought to be protonated (Suh et al., 1994). Only these positively charged protonated groups can electrostatically interact with negatively charged DNA (Sun et al., 2011). Significantly higher transfection was achieved with a 46% protonated PEI form compared to a 32% protonated PEI form and this effect was shown to dominate over the effect of branching (Sun et al., 2011). It is probably also because of the importance of protonation that pH and the presence of ions in the transfection medium, as well as N/P ratio and resulting ζ-potential, are known to affect transfection results (Boussif et al., 1995; Fukumoto et al., 2010).

In summary, studies have shown a relationship between transfection efficiency and both the extent of branching and MW of PEI, with bPEI structures generally having a higher transfection efficiency than lPEI forms, except for lPEI 22 (also referred to as Jet-PEI), and higher MW forms being more potent than lower MW forms, but in both cases higher efficiency was found to be associated with higher toxicity. I performed a brief exploration into the potential adjuvant activity of selected bPEI and lPEI forms (Table 4.1) in $gp140_{97CN54}$ immunisation in mice to see whether similar trends in structure-function relationships could be found in the context of adjuvant activity.

Name in this chapter	linear/branched (l/b)	MW (kDa)	Source	Original name
bPEI 25	b	25	Sigma Aldrich	Branched polyethylenimine
Jet-PEI	l	22	Polyplus	*In vivo* Jet-PEI
lPEI 4	l	4	Polymer Chemistry Solutions	PEI salt 5
lPEI 40	l	40	Polymer Chemistry Solutions	PEI salt 50
lPEI 160	l	160	Polymer Chemistry Solutions	PEI salt 200
lPEI 400	l	400	Polymer Chemistry Solutions	PEI salt 500
bPEI 2	b	2	Sigma Aldrich	PEI solution 408700
bPEI 600-1000	b	600-1000 (Mr)	Sigma Aldrich	PEI solution 03880
bPEI 750	b	750	Sigma Aldrich	PEI solution P3143

Table 4.2. Overview of lPEI and bPEI forms used in this chapter.

4.1.2.2. Biodegradable forms of PEI

In an attempt to mitigate the noted correlation between toxicity and transfection efficiency, and to better comply with regulatory guidelines of non-persistence *in vivo* of biomolecules, degradable forms of PEI have been developed by others for *in vivo* transfection purposes. These

forms of PEI are of adequate MW to achieve effective gene transfer, but are designed to decompose into smaller non-toxic ethyleneimine units with time or upon a specific stimulus after *in vivo* administration, to achieve higher biocompatibility. To explore a potential way to improve the biocompatibility and safety of PEI-adjuvanted immunisation, I evaluated selected biodegradable forms in $gp140_{97CN54}$ immunisation in mice for adjuvant activity body weight loss, as a measure of adjuvant toxicity.

Researchers have used diverse degradable linker functions to link smaller oligoethyleneimine (OEI) units into larger polymers, including disulphide bonds (Christensen et al., 2006; Gosselin et al., 2001; Kloeckner et al., 2006; Lee et al., 2007; Read et al., 2005), ester bonds (Forrest et al., 2003; Kloeckner et al., 2006; Russ et al., 2008a; Thomas et al., 2005), imines (Kim et al., 2005), phosphoesters (Wang et al., 2001; Zhao et al., 2003) and acid cleavable acetal functions (Knorr et al., 2008). For my studies, a few of these forms were selected based on high transfection results with low toxicity profile: Jet-PEI SS (disulfide linker), G2-HD-OEI (ester bond), and OEI-BAA-OEI (acid cleavable acetal function)(Figure 4.3).

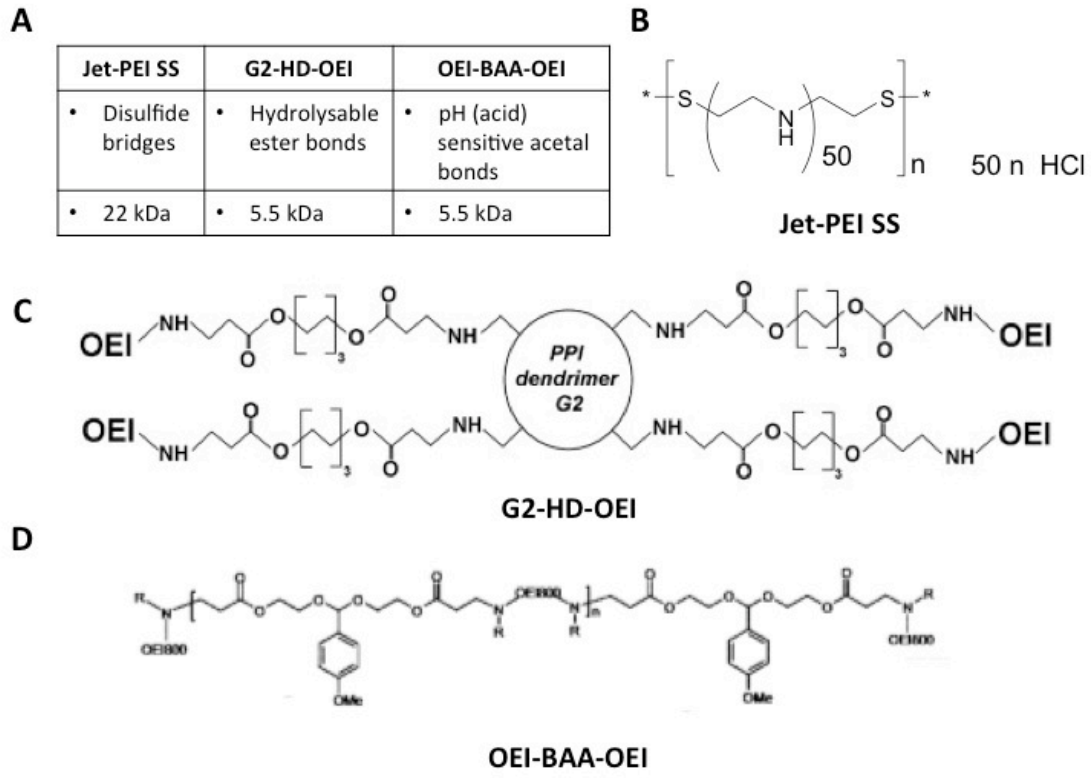

Figure 4.3. Overview of degradable PEI forms used in this chapter. A) Overview of degradable PEI forms and characteristics. B) Molecular structure of Jet-PEI SS. C) Molecular structure of G2-HD-OEI. G2=OEI dendrimer, HD=linker with 6 Carbon groups, OEI=OEI$_{800}$. D). Molecular structure of OEI-BAA-OEI (Taken from: Knorr et al., 2008; Russ et al., 2008a).

In vivo Jet-PEI SS

The *in vivo* Jet-PEI SS form of Polyplus is similar in size and structure to *in vivo* Jet-PEI, but consists of approximately 100 oligomers of 78 Da ethyleneimine monomers that are linked together via disulfide (SS) bridges, and are approximately 440 Da (Figure 4.3). This is achieved via air/DMSO oxidation of thiol groups on the OEIs leading to larger reducible Jet-PEI SS molecules. The resulting disulphide bonds remain stable in the oxidative extracellular

environment but can be rapidly degraded under reductive intracellular conditions (Lee et al., 2007) due to the intracellular presence of 5mM glutathione, a reductant that has the capacity to reduce SS bonds of intracellular proteins to cysteines (Kim et al., 2000; Lee et al., 2007). Light scattering measurements showed that polydispersity of lPEI-SS complexes was higher compared with standard bPEI 25 kDa, while the hydrodynamic diameter of the complexes decreased with increasing N/P ratio as expected, and the ζ-potential was positive when N/P > 12 (Breunig et al., 2008). A similar form of PEI, linear poly(ethylenimine sulphide), was shown to compact siRNA effectively in a gel retardation assay and had similar transfection efficiency to lPEI in HepG2 and HeLa cell lines, while inducing significantly less cytotoxicity in the same cell lines in a MTT assay (Breunig et al., 2008; Lee et al., 2007). Breunig et al even showed superior cellular uptake of siRNA with lPEI-SS compared to lPEI in CHO-K1 cells (Breunig et al., 2008). Furthermore, although siRNA cellular uptake was lower than that seen with bPEI, the resulting intracellular release of siRNA was observed to be higher for lPEI-SS compared to bPEI suggesting that the disulphide bridge might facilitate intracellular release (Breunig et al., 2008).

G2-HD-OEI

A variation of PEI containing hydrolysable ester bonds called G2-HD-OEI was synthesised by modifying polyprolyleneimine (PPI) dendrimer generation 2 via Michael addition with branched 800 Da OEI using 1.6-hexandioldiacrylate (Russ et al., 2008b) (Figure 4.3C). The resulting polymers contain hydrolysable ester bonds that can be degraded under physiological conditions (37°C, pH 7.4, aquous solution) (Russ et al., 2008b). G2-HD-OEI was shown to have the capacity to compact DNA into PEI-DNA complexes of 100-200 nm using ethidium bromide exclusion, photon correlation spectroscopy and light scattering assays (Russ et al.,

2008b). G2-HD-OEI transfection efficiency was increased compared to the OEI monomers and equivalent to standard lPEI and bPEI forms in a luciferase reporter assay (Russ et al., 2008a; Russ et al., 2008b). G2-HD-OEI degraded rapidly under physiological conditions (t½ = 3 days) (Russ et al., 2008b). Similar results have been obtained with similar poly(ester amine)s by other groups (Russ et al., 2008a; Zhong et al., 2005). *In vitro* cytoxicity was shown to be 3-fold lower than that of standard lPEI Jet-PEI 22 kDa and 30-fold lower than that observed with standard bPEI 25 in a metabolic activity assay in Neuro2A cells (Russ et al., 2008b). Furthermore, similar to standard bPEI, but in contrast to standard lPEI, G2-HD-OEI showed very low haemolytic activity in an *in vitro* erythrocyte lysis assay (Russ et al., 2008b). In addition, G2-HD-OEI did not trigger any detectable *in vitro* erythrocyte aggregation, in contrast to both standard PEI forms (Russ et al., 2008b). Also, no increase in liver enzymes indicative of toxicity or liver histopathology were detected with the degradable form (Russ et al., 2008b).

OEI-BAA-OEI

A biodegradable variant of PEI called OEI-BAA-OEI that contains pH-sensitive acetal bonds was synthesised by polymerisation of 800 Da OEI with the 4-methoxybenzaldehyde bisacrylate acetal cross-linker 1,1-bis-(2-acryloyloxy ethoxy)-[4-methoxy-phenyl]methane) (BAA) (Knorr et al., 2008). The idea behind the introduction of acetal groups into polymers was to create polymers that are sufficiently stable at physiological pH (7.4) to deliver DNA to the cells but upon endocytosis and exposure to the lower pH environment in the endosome, these polymers are degraded resulting in small decomposition products (Knorr et al., 2008). The half-life of OEI-BAA-OEI, determined by measuring the increase of UV-absorbance for the released

aromatic benzaldehyde group, was 3 min at pH 5 with completed hydrolysis in less than 30 min, while at pH 7.4 the half-life was about 3.5 h (Knorr et al., 2008). As measured in an ethidium bromide exclusion assay, DNA binding capacity of OEI-BAA-OEI was slightly reduced compared to the OEI monomers (Knorr et al., 2008). OEI-BAA-OEI formed complexes with DNA of 150–300 nm in salt-free conditions (HBG). In luciferase reporter assays in B16F10 and Neuro2A cells OEI-BAA-OEI showed higher transfection efficiency than standard lPEI 22 while inducing significantly lower toxicity (Knorr et al., 2008). Following i.v. injection in mice OEI-BAA-OEI was shown to be well tolerated at both 100µg/20g and 50µg/20g body weight and did not cause any changes in liver tissue histology, by contrast with standard bPEI 25 that was lethal at the 50µg/20g dose and caused necrotic changes in liver tissue.

Principal aims of this chapter:

1) **Investigate PEI mucosal adjuvant activity**
2) **Study the effect of PEI structure (degree of branching, MW) on adjuvant activity via the systemic and mucosal routes**
3) **Analyse whether selected biodegradable forms can act as adjuvants**
4) **Examine *in vivo* toxicity following administration of standard and biodegradable PEI forms**

Chapter hypotheses

- PEI maintains adjuvant activity via the mucosal route and induces local and systemic $gp140_{97CN54}$-specific immune responses

Justification: PEI has successfully been used for DNA immunisation via the mucosal route (references see Section 1.14). PEI might protect antigens, like DNA, to degradation, possibly also facilitating penetration of mucosal barriers and protection against mucosal proteases and low pH (references see Chapter 3).

-Structural differences of distinct PEI forms, such as degree of branching and MW will have an effect on adjuvant activity

Justification: These structural differences have been shown to impact on transfection efficiency (references see chapter introduction), which is likely to partially underly PEI adjuvant activity.

- Biodegradable PEI forms can act as adjuvants in the context of $gp140_{97CN54}$ immunisation in mice

Justification: Biodegradable forms have been shown to be efficient transfection agents and it is likely that transfection partially underlies PEI adjuvant activity (references see chapter introduction).

- Biodegradable and low MW PEI forms may be less toxic than non-degradable PEI forms during $gp140_{97CN54}$ immunisation in mice

Justification: Biodegradable forms have been shown to be less toxic in various *in vitro* and some *in vivo* assays (references see chapter introduction).

4.2. Results

In the following mice studies boost immunisations were done, similar to the previously descrived rabbit immunisation schedules on 3-4 week intervals, pilot studies in my hands have shown that variation within this interval does not substantially affect the study results.

4.2.1. Mucosal adjuvant activity of PEI with gp140$_{97CN54}$

To test mucosal adjuvant activity of PEI, mice were primed via the i.n. route with gp140$_{97CN54}$ antigen alone, antigen mixed with PEI or antigen mixed with the mucosal adjuvant CTB. At week 3.5 post-prime mice were boosted with equivalent formulations. Serum and vaginal lavage gp140$_{97CN54}$-specific IgA and IgG responses measured at 4 weeks post boost are shown in Figure 4.4.

Via the i.n. route, gp140$_{97CN54}$ was non-immunogenic on its own, even following boost immunisation. PEI adjuvantation yielded serum gp140$_{97CN54}$-specific IgG titres of ~10^4 after boosting that were higher than the gp140$_{97CN54}$-specific IgG titres of ~$10^{3.1}$ in the CTB-adjuvanted group. Adjuvanted serum IgG titres were statistically significant for both PEI and CTB adjuvantation compared to unadjuvanted titres (P<0.01, P<0.05 respectively), with PEI yielding the highest titres. At the vaginal mucosa, PEI yielded normalised gp140$_{97CN54}$-specific IgA titres of 800 that were significantly higher than the unadjuvanted group (P<0.01). Although CTB also induced significantly higher normalised gp140$_{97CN54}$-specific IgA responses than the unadjuvanted group (P<0.05), the observed effect was smaller than the PEI group. A similar trend was observed for vaginal gp140$_{97CN54}$-specific IgG responses, although the IgG

responses were generally lower than the IgA responses at the vaginal mucosa. PEI yielded normalised $gp140_{97CN54}$-specific IgG titres of 20 that were significantly higher than the unadjuvanted group (P<0.05). By contrast, CTB induced $gp140_{97CN54}$-specific titres that were statistically indistinguishable from the unadjuvanted group.

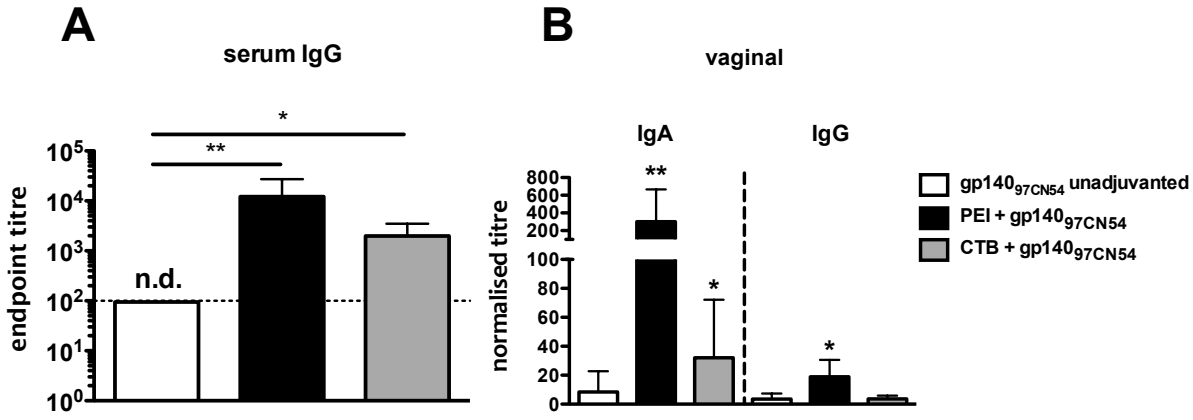

Figure 4.4. Mucosal PEI adjuvant activity following i.n. $gp140_{97CN54}$ immunisation in mice: Ab responses. 6-10 week female BALB/c mice (n=6 per immunisation group) received prime (10 µg $gp140_{97CN54}$ in PBS or adjuvanted with 10 µg PEI or 10 µg CTB) at week 0 and boost (as prime) at week 3.5. Bleeds and vaginal lavages were collected at week 0 and 7.5. Sera were analysed for $gp140_{97CN54}$-specific IgG levels by ELISA. Vaginal lavages were analysed for $gp140_{97CN54}$-specific and total IgA and IgG using ELISA and Luminex™. A) Serum total $gp140_{97CN54}$-specific endpoint titer at 4 weeks post boost. B) Vaginal normalised $gp140_{97CN54}$-specific IgA and IgG responses (normalised for total IgA and IgG) at 4 weeks post boost. Results are representative of 2 repeats. Means +/- SD of biological replicates (mice) within an individual experiment are shown. n.d. = undetectable. Kruskal-Wallis test (all groups), Dunn's post-test (all groups), *P<0.05, **P<0.01.

In order to investigate the T_h-polarising effect of PEI adjuvant, serum samples from the week 7.5 (4 weeks post boost) timepoint were analysed for $gp140_{97CN54}$-specific IgG1 (canonical T_h2-associated isotype) and IgG2a (canonical T_h1-associated isotype) endpoint titres. The results are depicted in Figure 4.5.

PEI-induced serum $gp140_{97CN54}$-specific IgG1 titres were not statistically different from the titres observed following CTB adjuvantation (Figure 4.5A), as were the serum $gp140_{97CN54}$-specific IgG2a titres (Figure 4.5B). Although the IgG1/IgG2a ratio for PEI adjuvant seemed slightly higher than for CTB, the ratios were again statistically indistinguishable, suggesting that similar to T_h1 + T_h2 mixed CTB adjuvant (Imaoka et al., 1998), PEI induces a T_h1 + T_h2 mixed serum IgG response (Figure 4.5C).

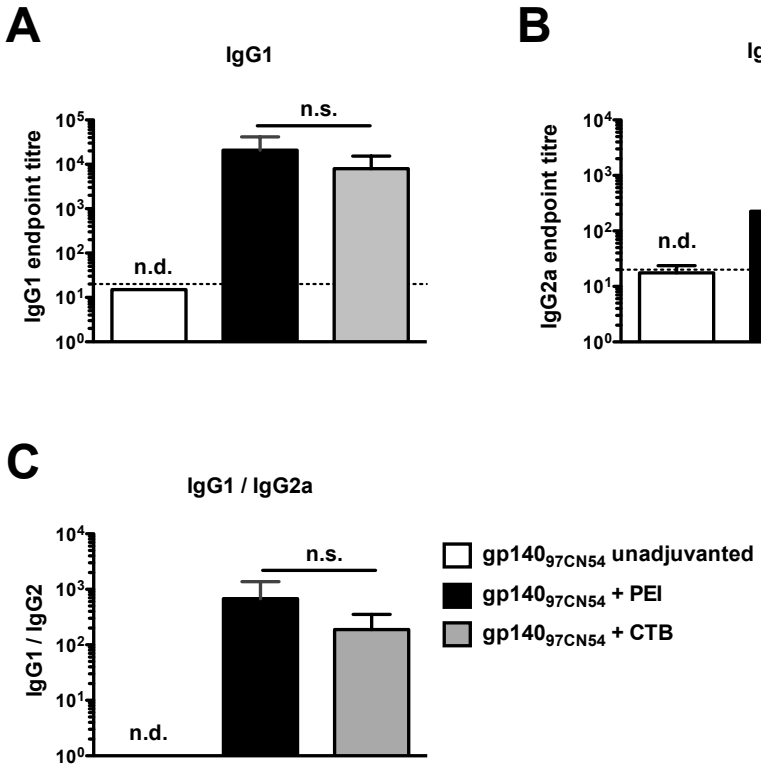

Figure 4.5. Mucosal PEI adjuvant activity following i.n. gp140$_{97CN54}$ immunisation in mice: antibody isotypes IgG1 and IgG2a. 6-10 week female BALB/c mice (n=6 p immunisation group) received prime (10 µg gp14097CN54 in PBS or adjuvanted with 10 µg PEI or CTB) at week 0 and boost (as prime) at week 3.5. Bleeds were collected at week 7.5. Sera were analysed for gp140$_{97CN54}$-specific IgG1 and IgG2a levels by ELISA. **A) Serum total gp140$_{97CN54}$-specific IgG1 endpoint titer at 4 weeks post boost. B) Serum total gp140$_{97CN54}$-specific IgG2a endpoint titer. C) IgG1/IgG2a ratio.** Results are representative of 2 repeats. Means +/- SD of biological replicates (mice) within an individual experiment are shown. Kruskal-Wallis, Dunn's post-test, n.s.

To characterise PEI-induced T cell responses during mucosal gp140$_{97CN54}$ immunisation, mice were i.n. immunised as above followed by an additional boost with equivalent formulations 2.5 weeks before tissue collection. CLN and spleens were collected, T cells harvested and restimulated with gp140$_{97CN54}$ antigen for 3 days. T cell proliferation was analysed by a ^{3}H-

thymidine incorporation assay and supernatant cytokine production was assayed by multiplex bead array (Luminex™) analysis. T cell proliferation and selected cytokines are shown in Figure 4.6.

HIV-1 $gp140_{97CN54}$ antigen alone induced T cell responses both in the CLN and in the spleens, but PEI adjuvantation significantly enhanced these responses ($P<0.01$ in spleen, and $P<0.001$ in CLN respectively) to ~4 (spleen) and ~5 (CLN) $\log_{10}$ counts per minute (cpm) that was statistically indistinguishable from the CTB group. Interestingly, calculation of the ratio of local to systemic T cell responses (CLN vs. spleen) revealed that PEI adjuvantation resulted in a more localised T cell response than the CTB group (Figure 4.6B). PEI and CTB both elicited higher titres of cytokines than unadjuvanted $gp140_{97CN54}$ in spleen (Figure 5.6C) and CLN (Figure 4.6D). Statistically significant increases in the PEI group compared to the unadjuvanted group were observed for the T_h2 cytokine IL-4 in the CLN and for the T_h2 and T_h17 cytokines IL-4, IL-5, IL-13, and IL-17 in the spleens (for all $P<0.05$ and for spleen IL-4 $P<0.01$). The trend was seen for all cytokines, including T_h1 (IFN-**γ**, TNF-**α**) T_h2 (IL-4, IL-5, IL-13) and T_h17 (IL-17) cytokines. Calculation of the ratio of IFN-**γ** to IL-4 (canonical T_h1 and T_h2 cytokines respectively) revealed that a more T_h2-biased response was observed for PEI compared to the CTB and unadjuvanted groups in the CLN cells (Figure 4.6E), whereas no significant difference in T_h polarity was observed for the splenocytes (Figure 4.6F). When the ratio was calculated between the defining T_h17 cytokine IL-17 and T_h2 cytokine IL-4, significantly higher T_h17 bias was observed in the presence of CTB compared to the presence of PEI for both splenocyte and CLN cell supernatants (Figure 4.6 G & H).

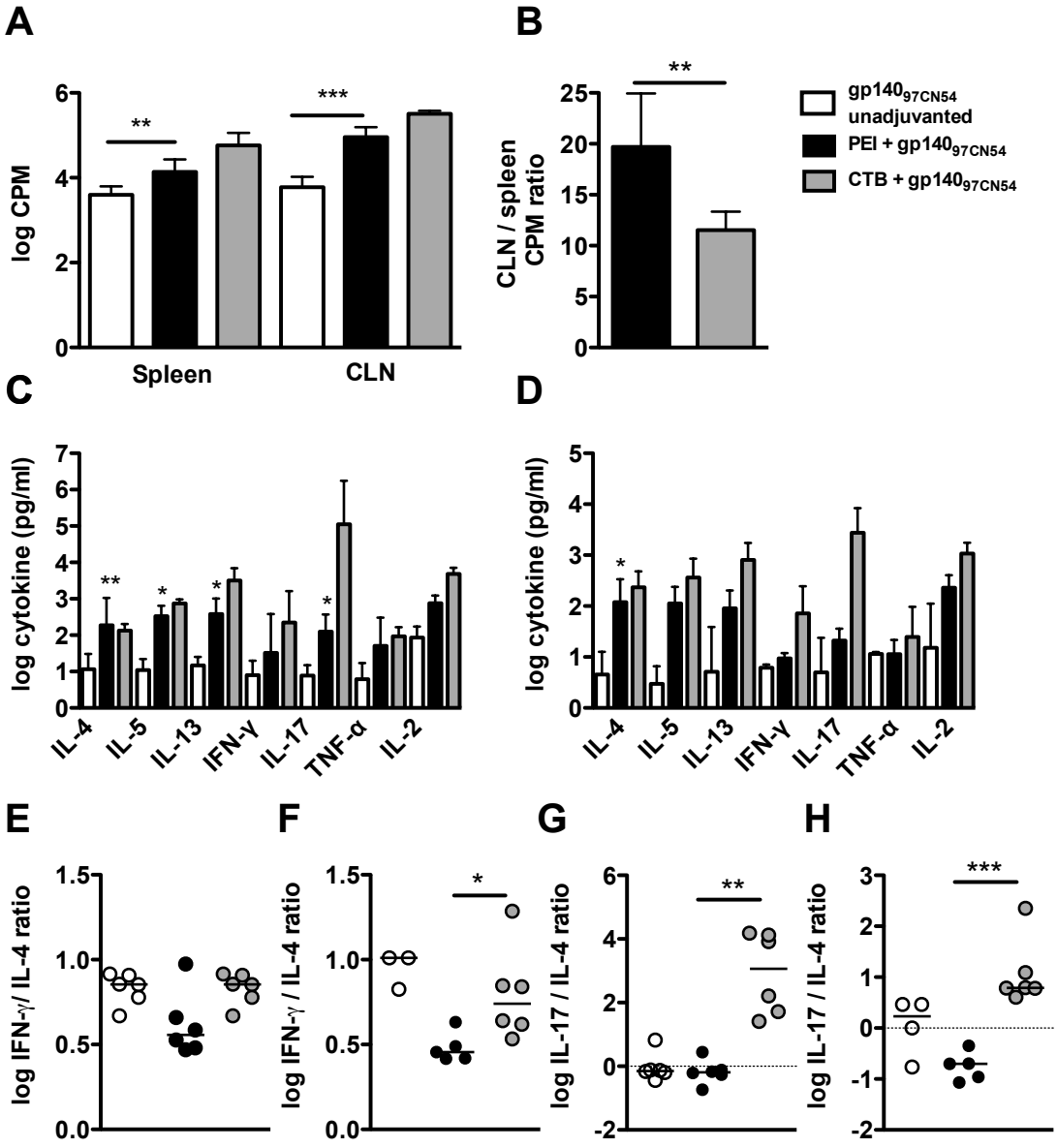

Figure 4.6. Mucosal PEI adjuvant activity following i.n. gp140$_{97CN54}$ immunisation in mice: T cell responses. 6-10 week female BALB/c mice (*n*=6 per immunisation group) received prime (10 ug gp140$_{97CN54}$ in PBS or adjuvanted with 10 µg PEI or 20 µg CTB) at week 0 and boost (as prime) at week 3.5 and week 20. Spleens and CLN cells were collected 2.5 weeks after the final boost. A) Proliferation of gp140$_{97CN54}$-restimulated splenocytes or CLN cells from immunised mice shown by ^{3}H-thymidine incorporation at d 3. B) Ratio of local (CLN) proliferation versus systemic (spleen) proliferation (unpaired *t*-test) at d 3. C) Cytokine production in Luminex^{TM} of gp140$_{97CN54}$-restimulated splenocytes D) Cytokine production of gp140$_{97CN54}$-restimulated CLN cells. E) IFN-γ/IL-4 ratio splenocytes. F) IFN-γ/IL-4 ratio CLN cells. G) IL-17/IL-4 ratio splenocytes. H) IL-17/IL-4 ratio CLN cells**. A-D) Means +/- SD of biological repeats. E-H) Medians of biological repeats (mice). Kruskal-Wallis (all groups), Dunn's post-test (PEI group vs PBS group), *P< 0.05, P< 0.01, P< 0.001.**

4.2.2. Conservation of PEI adjuvant activity with various MW forms of lPEI and bPEI

To investigate the adjuvant activity of the various forms of PEI, we chose initially to use the subcutaneous route of immunisation. The systemic route was chosen because these studies were started before we had discovered that PEI was a potent mucosal adjuvant via the intranasal route. In an initial investigation of the effects of degree of branching and MW on PEI adjuvant activity, mice (*n*=3) were s.c. immunised with 10 or 2 µg $gp140_{97CN54}$ antigen in PBS alone, or antigen formulated with 80 µg PEI (see Table 4.2; lPEI 4, lPEI 40, lPEI 160, lPEI 400, bPEI 2, bPEI 25 (standard), bPEI 600-1000, or bPEI 750). The antigen dose was titrated in an attempt to better observe differences between distinct PEI forms. At week 3, mice received a boost immunisation with equivalent formulations. Serum samples were collected at week 0, 3, and 6 and were analysed for $gp140_{97CN54}$-specific IgG responses using ELISA. Serum $gp140_{97CN54}$-specific IgG endpoint titres ranged from ~10^3 to ~10^5 and although slight differences between distinct PEI groups were observed, no statistically significant difference between the different PEI forms was measured (Figure 4.7A & B).

To further investigate the structural impact on PEI adjuvant activity in the same model with larger group sizes allowing for a more precise statistical comparison, the four PEI forms that appeared most effective in the previous pilot study were selected for further analysis: lPEI 40, lPEI 160, bPEI 750, and bPEI 25. Mice (*n*=6) were immunised subcutaneously as before with 1 µg $gp140_{97CN54}$ in PBS or formulated with 80 µg PEI (lPEI 40, lPEI 160, bPEI 25, or bPEI 750). Equal weights of adjuvant were used rather than molar equivalents, with the considerations that the number of active groups would be similar with equivalent weights, and

that activity could retrospectively be normalised to molarity if appropriate. At week 4, 6, and 9 mice received boost immunisations with equivalent $gp140_{97CN54}$ in PBS or in PEI formulations. Serum was collected at week 3, 6, 9, 12, 20 and 24. Serum $gp140_{97CN54}$-specific total IgG endpoint titres are shown in Figure 4.7C.

HIV-1 $gp140_{97CN54}$ alone was as before in s.c. immunisation, only weakly immunogenic with no detectable $gp140_{97CN54}$-specific serum IgG responses after the prime and only weak ($\sim10^2$–$\sim10^3$) $gp140_{97CN54}$-specific IgG endpoint titres after the first and second boost immunisatiions. This low response to antigen alone reflected a decision to use a low dose (1 μg $gp140_{97CN54}$) to allow greater differentiation between differentially adjuvanted (and unadjuvanted) responses. However, after the third boost $gp140_{97CN54}$-specific serum IgG titres in the unadjuvanted group started approaching the levels of responses seen in the PEI-adjuvanted groups with an endpoint titre of $\sim10^{4.5}$.

bPEI 25 had an immune enhancing effect as expected and induced $gp140_{97CN54}$-specific serum IgG responses after the prime with a reciprocal endpoint titre of $\sim10^3$ that further increased after each boost immunisation and peaked around 12 weeks post prime immunisation with $\sim10^{5.5}$ endpoint titre.

Remarkably, all PEI forms induced very similar $gp140_{97CN54}$-specific serum IgG responses that were significantly increased over the unadjuvanted group (P<0.01) but statistically indistinguishable between the various PEI-adjuvanted groups (see Figure 4.7B. and 4.7C), indicating that PEI adjuvant activity was a shared attribute of lPEI and bPEI forms of various MW in the context of s.c. $gp140_{97CN54}$ immunisation in mice.

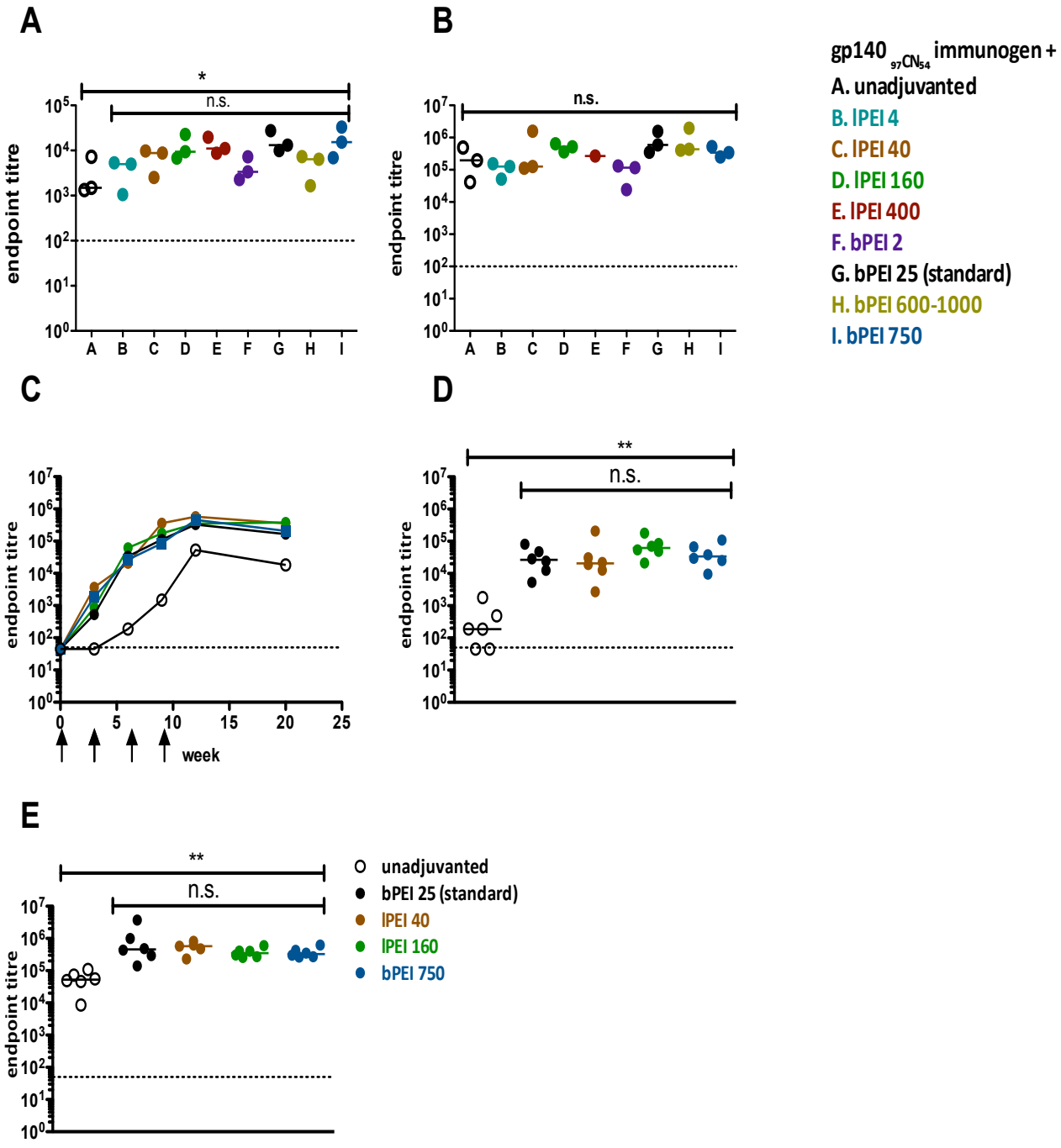

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Figure 4.7. S.c. immunisation with gp140$_{97CN54}$ antigen and lPEI or bPEI forms of varying MW. 6-10 week female BALB/c mice were used. A & B) Pilot experiment with *n*=3 per immunisation group. Mice were immunised with 10 µg (A) or 2 µg (B) gp140$_{97CN54}$ and 80 µg of lPEI 4, lPEI 40, lPEI 160, lPEI 400, bPEI 2, bPEI 25 (standard), bPEI 600-1000, or bPEI 750 at week 0, 3, and 4.5 (A) or week 0, 4, 6, and 9 (B). **Bleeds were collected at week 10.5 (A) and week 12 (B) and analysed for serum gp140$_{97CN54}$ total IgG endpoint titres using ELISA**. Four PEI forms were selected for further studies. C-E) Mice (*n*=6 p immunisation group) received prime (1 µg gp140$_{97CN54}$, 80 µg PEI (pH neutralised)(lPEI 40, lPEI 160, bPEI 25, or bPEI 750) or PBS) at week 0 and boost (as prime) at week 4,6 and 9 (arrows). Bleeds were collected at 3 weeks post prime as well as week 6,9,12,20 and 24. **C) Timecourse total gp140$_{97CN54}$-specific IgG endpoint titres D) Total gp140$_{97CN54}$-specific IgG endpoint titre 2 weeks post second boost. E) Total gp140$_{97CN54}$-specific IgG endpoint titer 3 weeks post third boost** Sera were analysed for gp140$_{97CN54}$ -specific IgG levels by ELISA. Data are representative of 3 independent experiments. Medians of replicates within individual experiment are depicted. Kruskal-Wallis test (all groups) and Dunn's post-test (PEI groups), $^{**}P< 0.01$.

4.2.3. PEI adjuvant activity of distinct structural forms is maintained via the mucosal route

Since mucosal adjuvant activity is particularly desired for a newly developed adjuvant, I decided to investigate potential effects of degree of branching and MW on mucosal PEI adjuvant activity, mice (*n*=6) were i.n. immunised with 10 µg gp140$_{97CN54}$ antigen in PBS or antigen mixed with either 10 µg CTB, or antigen mixed with 10 µg PEI (lPEI 40, lPEI 160, bPEI 25, or bPEI 750). At week 3.5 mice received boost immunisations with immune formulations, as before. Bleeds and vaginal lavages were collected pre-prime and at week 2.5, 3.5, 7.5, 9.5 and 17.5. Serum gp140$_{97CN54}$-specific IgG responses and vaginal gp140$_{97CN54}$-specific IgG and IgA responses are shown in Figure 4.8.

HIV-1 gp140$_{97CN54}$ antigen alone was, as before, only weakly immunogenic via the mucosal route with the complete absence of detectable serum gp140$_{97CN54}$-specific IgG responses or vaginal gp140$_{97CN54}$-specific IgG responses. Vaginal gp140$_{97CN54}$-specific IgA responses for the unadjuvanted group were also below detection at all timepoints except for 14 weeks after

boosting, but even at this timepoint vaginal $gp140_{97CN54}$-specific IgA endpoint titre was below 10^2.

In line with previous results, PEI induced $gp140_{97CN54}$-specific antibody responses after boost immunisation both systemically and at the vaginal mucosa. At 4 weeks post boost antibody responses in both systemic and mucosal compartments reached statistical significance compared to the unadjuvanted group (>10^3-fold difference, P<0.01). Titres also peaked at this timepoint and although serum $gp140_{97CN54}$-specific IgG responses remained at the same level until the final timepoint 14 weeks post boost, both vaginal $gp140_{97CN54}$-specific IgA and IgG responses dropped to below 10^2 at the final timepoint 14 weeks post boost. Interestingly, PEI-induced vaginal $gp140_{97CN54}$-specific IgA levels decreased more slowly than PEI-induced vaginal $gp140_{97CN54}$-specific IgG levels. PEI-induced $gp140_{97CN54}$-specific antibody titres were consistently equivalent or superior to CTB-induced $gp140_{97CN54}$-specific antibody responses.

As with the systemic immunisation route, all selected PEI forms showed increased $gp140_{97CN54}$-specific IgG responses both systemically and mucosally compared to antigen alone, and differences between the distinct PEI forms did not reach statistical significance. Although a trend towards higher $gp140_{97CN54}$-specific IgG responses in the vagina was observed with bPEI 750 compared to the other PEI groups, overall no statistically significant effects were found. Similarly, although there was a trend for $gp140_{97CN54}$-specific IgG responses in the vagina to lPEI 40 to drop to a lower level towards the end of the experiment, this was not statistically significant in the current experimental conditions. Also, mucosal $gp140_{97CN54}$-specific IgA responses were statistically indistinguishable for the different PEI-adjuvanted groups, although bPEI 750-induced mucosal $gp140_{97CN54}$-specific IgG responses were found to be higher than the

other groups. These results suggest that mucosal PEI adjuvant activity is also a shared trait of PEI of different molecular structure and MW.

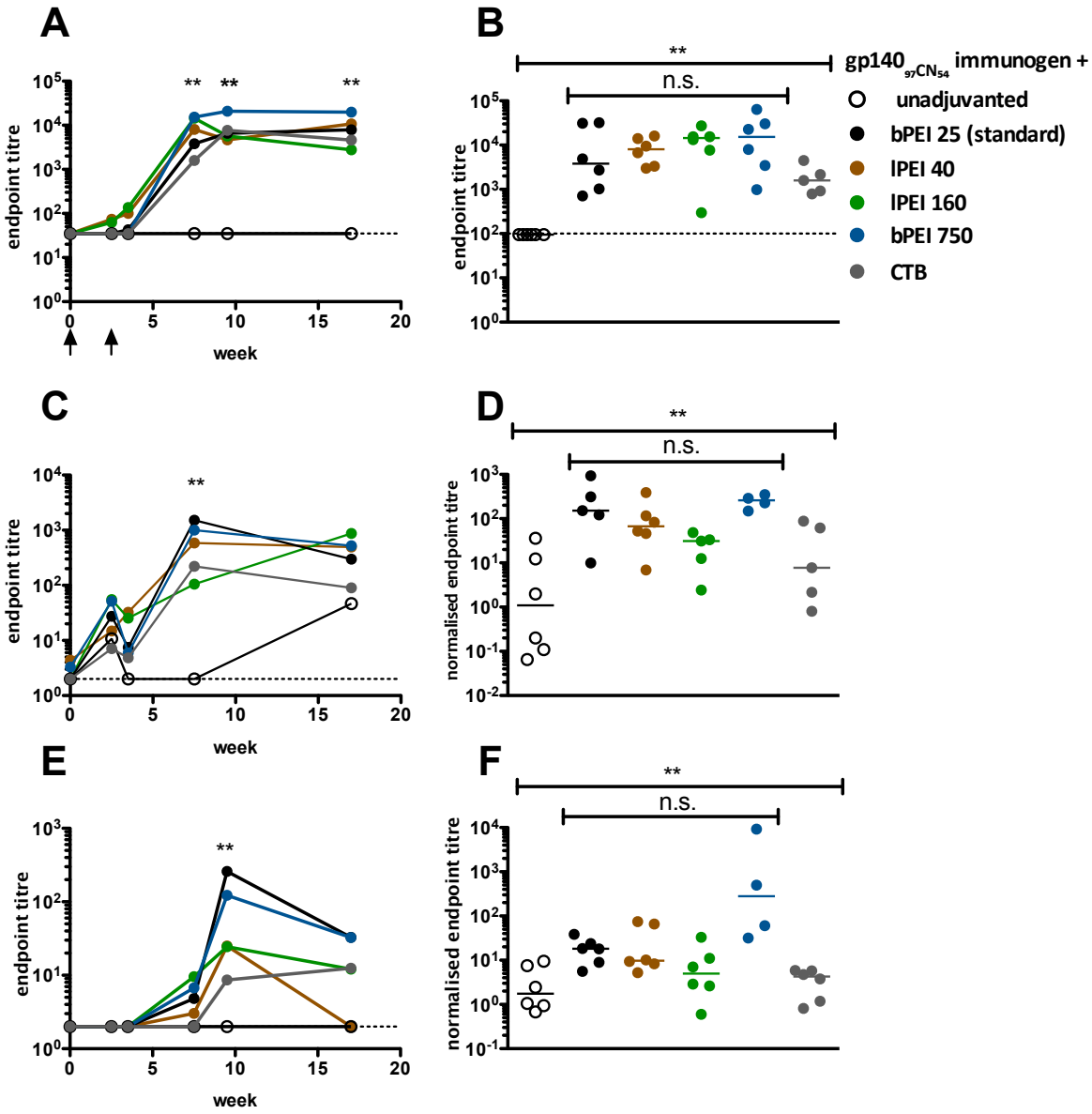

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Figure 4.8. I.n. gp140$_{97CN54}$ immunisation with lPEI and bPEI forms of varying MW. 6-10 week female BALB/c mice *(n*=6 per immunisation group) received prime (10 µg gp140$_{97CN54}$ antigen in PBS, antigen mixed with 10 µg PEI (lPEI 40, lPEI 25, bPEI 25, or bPEI 750) or antigen mixed with 10 µg CTB) at week 0 and boost (as prime) at week 3.5 (arrows). Bleeds and vaginal lavages were collected at 2.5 and 3.5 weeks post prime and 4, 6, and 14 weeks post boost. Sera were analysed for gp140$_{97CN54}$-specific IgG levels using ELISA. Vaginal lavages were analysed for gp140$_{97CN54}$-specific and total IgA and IgG using ELISA and LUMINEX. **A) Serum total gp140-specific IgG endpoint titer timecourse B) Serum total gp140$_{97CN54}$-specific IgG endpoint titer at 4 weeks post boost. C) Vaginal total gp140-specific IgA endpoint titer timecourse. D) Vaginal normalised gp140$_{97CN54}$-specific IgA endpoint titer (normalised for total IgA) at 4 weeks post boost. E) Vaginal total gp140$_{97CN54}$-specific IgG endpoint titer timecourse F) Vaginal normalised gp140-specific IgG endpoint titer (normalised for total IgG) at 4 weeks post boost.** Medians are depicted. Kruskal-Wallis test (all groups) and Dunn's post-test (PEI groups), $^{**}P< 0.01$.

4.2.4. Biodegradable PEI forms show varying systemic adjuvant activity

Adjuvant activity of a selection of degradable PEI forms, including Jet-PEI SS that contains disulfide bridges, G2-HD-OEI with hydrolysable ester bonds and OEI-BAA-OEI that consists of monomers linked via pH-sensitive acetal bonds, was investigated by immunisation of mice using 2 µg gp140$_{97CN54}$ antigen in PBS alone or gp140$_{97CN54}$ antigen formulated with 80 µg PEI (standard bPEI 25 or one of the above biodegradable forms). Mice were boosted with equivalent immune formulations at week 3. Serum samples were collected pre-prime and at week 3, 7.5, and 9.5. Serum gp140$_{97CN54}$-specific IgG antibody responses are depicted in Figure 4.9.

In accordance with previous results, gp140$_{97CN54}$ antigen only induced detectable gp140$_{97CN54}$-specifc serum IgG responses after s.c. boosting (endpoint titres ~10^4). For both adjuvanted and unadjuvanted groups, serum IgG responses peaked around 4.5 weeks post-boost and had fallen substantially at 6.5 weeks post boost.

Standard bPEI 25 induced $gp140_{97CN54}$-specific IgG endpoint titres that were significantly higher than the unadjuvanted group at all timepoints (10^3 at 3 weeks post-prime, $P< 0.05$; ~$10^{5.5}$ peak titres at 4.5 weeks post boost, $P<0.01$). Jet-PEI SS and G2-HD-OEI adjuvantation resulted in serum $gp140_{97CN54}$-specific IgG titres that were higher than the unadjuvanted group but lower than the standard bPEI adjuvanted group with peak titres of ~$10^{4.3}$, but the observed effects were statistically indistinguishable from the standard bPEI group. By contrast, OEI-BAA-OEI adjuvantation showed a trend towards an inhibitory effect on serum $gp140_{97CN54}$-specific IgG responses, as observed titres were lower than the unadjuvanted group, although not statistically significant, and statistically significantly lower than the standard bPEI 25 group (~$10^{3.1}$ at 4.5 weeks post boost, $P<0.01$). These results indicate that degradable PEI forms Jet-PEI SS and G2-HD-OEI have adjuvant activity in the context of $gp140_{97CN54}$ s.c. immunisation in mice that is statistically indistinguishable from, although trendwise being lower than, standard bPEI 25, whereas the pH-sensitive acetal-linked OEI-BAA-OEI did not have any adjuvant effect in this context.

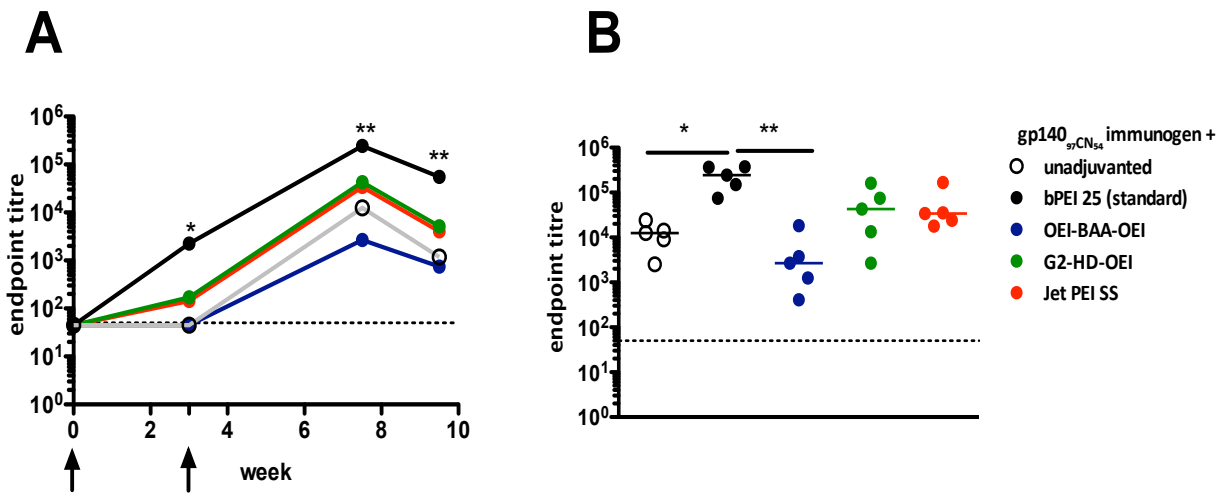

Figure 4.9. S.c. gp140$_{97CN54}$ immunisation with standard bPEI 25 and selected biodegradable PEI forms. 6-10 weeks female BALB/c mice (*n*=5 per immunisation group) received prime (2µg gp140$_{97CN54}$ in PBS or adjuvanted with 80µg PEI (bPEI 25, OEI-BAA-OEI, G2-HD-OEI, or Jet-PEI SS) at week 0 and boost (as prime) at week 3 (arrows). Bleeds were collected 3 weeks post prime and 4.5 and 6.5 weeks post boost. Sera were analysed for gp140$_{97CN54}$-specific IgG levels by ELISA. **A) Serum total gp140$_{97CN54}$-specific IgG endpoint titre timecourse. B) Serum total gp140$_{97CN54}$-specific endpoint titre at 4.5 weeks post boost.** Medians are depicted. Kruskal-Wallis test (all groups) and Dunn's post-test (PEI groups), **P*<0.05, ***P*< 0.01.

4.2.5. Biodegradable PEI forms show mucosal adjuvant activity

To investigate adjuvant activity of biodegradable forms via the mucosal route, mice were immunised with 10 µg $gp140_{97CN54}$ in PBS or adjuvanted with 20 µg PEI (bPEI 25 (standard), Jet-PEI (standard), G2-HD-OEI, OEI-BAA-OEI, or CTB at week 0 and week 12 and bleed and lavages were collected at week 0, 4, 8, 12, and 15. HIV-1 $gp140_{97CN54}$–specific serum IgG and mucosal IgA and IgG responses are depicted in Figure 4.10.

Detectable $gp140_{97CN54}$-specific serum IgG responses were only observed after the boost immunisation, when except for one mouse, all PEI-adjuvanted mice showed detectable serum $gp140_{97CN54}$-specific endpoint point titres, compared to only one out of five mice in the unadjuvanted group. While adjuvantation had significantly increased the serum antibody responses ($P<0.01$), no statistical significance was reached between the distinct PEI groups, but a more extensive study would be needed to confirm this result. At the mucosa $gp140_{97CN54}$-specific IgA and IgG responses were for most mice, still undetectable. This was also seen in the PEI-adjuvanted groups, although a trend was visible towards increased responses in these groups compared to the unadjuvanted group. Again, a more extensive study would be required to investigate mucosal IgG and IgA responses following mucosal biodegradable PEI immunisation, but these initial results indicate that all studied biodegradable PEI forms were able to induce detectable serum $gp140_{97CN54}$-specific responses in mucosal immunisation in mice.

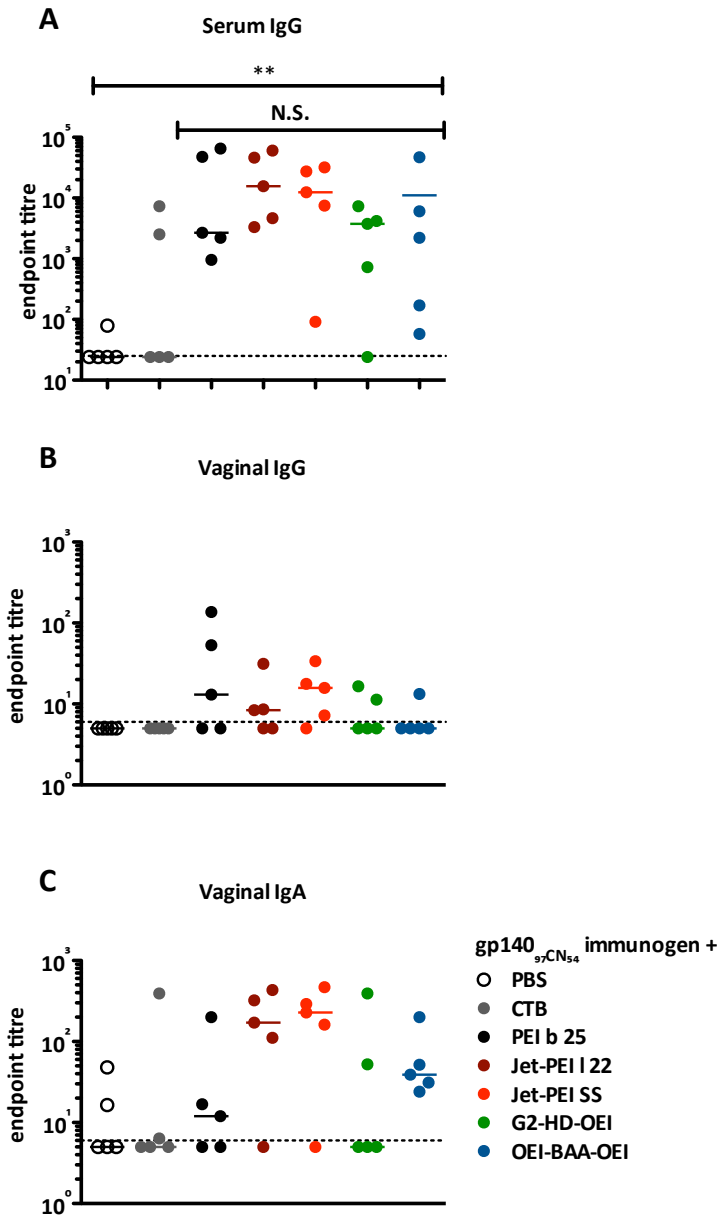

Figure 4.10. I.n. gp140$_{97CN54}$ immunisation with standard bPEI 25, Jet-PEI and biodegradable PEI forms. 6-10 week female BALB/c mice (*n*=6 per immunisation group) received prime (10 µg gp140$_{97CN54}$ in PBS, antigen mixed with 20 µg PEI (bPEI 25, Jet-PEI, Jet-PEI SS, OEI-BAA-OEI, G2-HD-OEI) or antigen mixed with 20 µg CTB) at week 0 and boost (as prime) at week 8. Bleeds and vaginal lavages were collected at 4 weeks post boost. Sera were analysed for gp140$_{97CN54}$-specific IgG levels by ELISA. Vaginal lavages were analysed for gp140$_{97CN54}$-specific IgA and IgG only using ELISA, since previous assays had shown no substantial differences in vaginal total IgA or IgG between mice (data not shown). **A) Serum gp140$_{97CN54}$-specific IgG endpoint titres at 4 weeks post boost. B) Vaginal gp140$_{97CN54}$-specific IgG endpoint titres at 4 weeks post boost. C) Vaginal gp140$_{97CN54}$-specific IgA endpoint titres at 4 weeks post boost.** Medians of biological replicates (mice) within individual experiment are depicted. Kruskal-Wallis test (all groups) and Dunn's post-test (PEI groups),**P<0.01.

In the same i.n. immunisation study body weight loss was recorded at 0, 8, 12, 24, 48, and 72 h following prime immunisation and expressed as a percentage of original body weight (at 0 h) in Figure 4.11. No body weight loss was observed in the unadjuvanted group. Similarly no substantial body weight loss was observed in the CTB group. The PEI forms induced body weight loss, which peaked around 24 h post injection (~4-10%), and had fully recovered at 48 h post injection (Figure 4.11D & E). Unexpectedly, bPEI 25 standard form induced the smallest effect on body weight, while other forms, including Jet-PEI standard lPEI and the selected biodegradable forms showed higher body weight loss, although statistical significance was not reached between the distinct PEI forms. It is interesting to note that despite the strong body weight effects of biodegadable PEI forms compared to bPEI 25, there was a slight indication of an improved body weight loss profile in the degradable form of standard lPEI Jet-PEI SS over nondegradable Jet-PEI (ns), although Jet-PEI–adjuvanted mice recovered from body weight loss more speedily than Jet-PEI SS-adjuvanted mice. Similar patterns of body weight loss were found when the experiment was repeated.

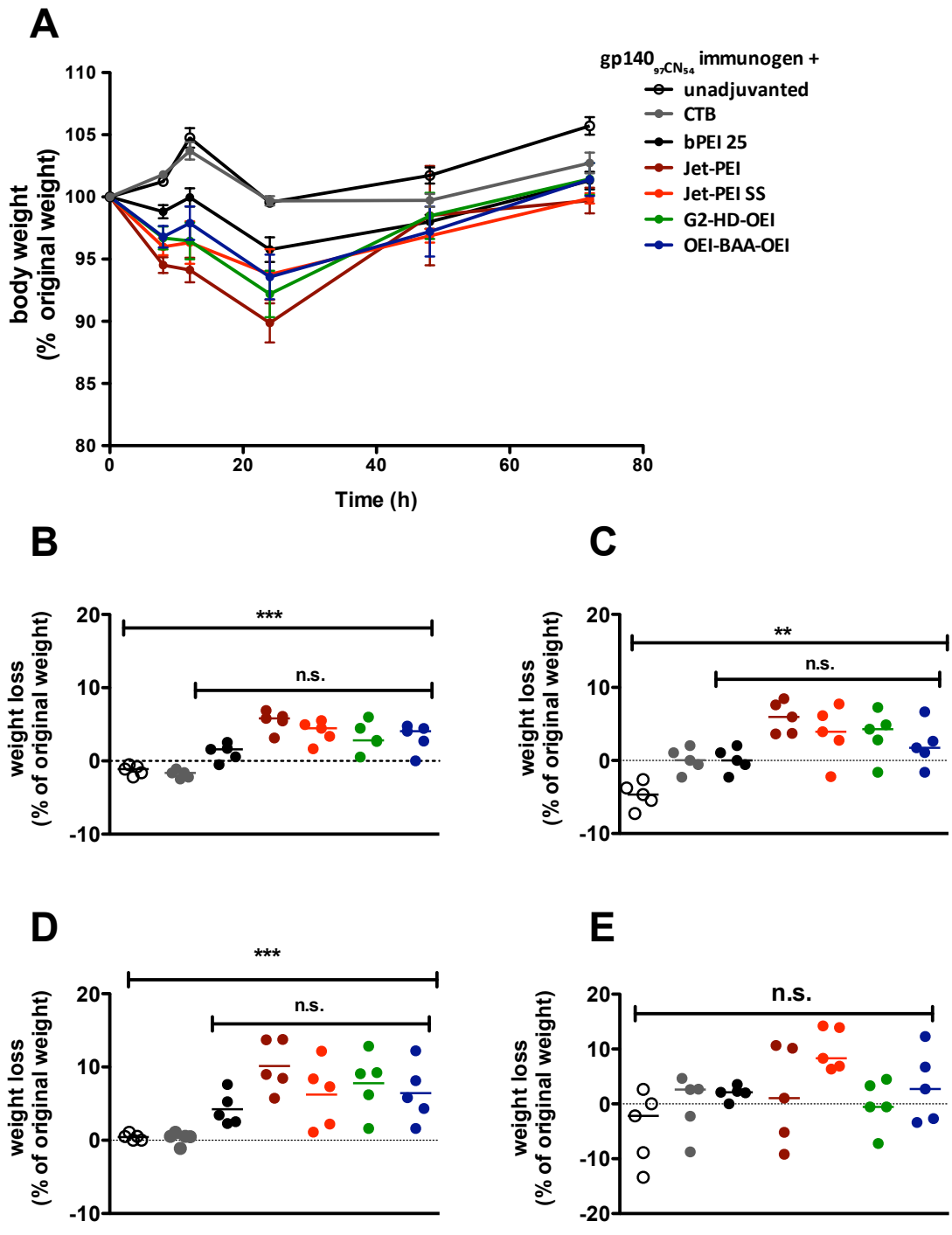

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Figure 4.11. Body weight loss following gp140$_{97CN54}$ immunisation with bPEI 25, CTB, or degradable PEI forms. 6-10 week female BALB/c mice (*n*=6 per immunisation group) received prime (10 µg gp140$_{97CN54}$ in PBS , antigen mixed with 20 µg PEI (bPEI 25, Jet-PEI, Jet-PEI SS, OEI-BAA-OEI, G2-HD-OEI) or antigen mixed with 20 µg CTB) at week 0 and boost (as prime) at week 8. Body weights were recorded at 0, 8 **(A)**, 12 **(B)**, 24 **(C)**, 48 **(D)**, and 72 h post prime injection and depicted as % of original body weight (at 0 h). Medians of biological replicates (mice) within an individual experiment are depicted. Kruskal-Wallis test (all groups) and Dunn's post-test (PEI groups).***P*<0.01, ***P*<0.001. The study format was repeated once.

4.2.6. Jet-PEI SS had equivalent adjuvant activity but induced less body weight loss than standard Jet-PEI after peritoneal gp140$_{97CN54}$ immunisation

To further analyse Jet-PEI SS adjuvant activity and body weight loss via an additional administration route and to compare observed body weight loss following Alhydrogel administration, mice (*n*=5) were i.p. immunised with 10 µg gp140$_{97CN54}$ alone, immunigen mixed with 70 µg Jet-PEI (standard lPEI), antigen mixed with 70 µg Jet-PEI-SS, or antigen mixed with 1 mg Alhydrogel at week 0. Bleeds were collected at week 2 and week 6 and were analysed for gp140$_{97CN54}$-specific total IgG endpoint titres using ELISA (Figure 4.12A & B). The week 2 timepoint was chosen, which is earlier than the previously discussed general 3-4 week timepoint, and boost immunisations were not performed, because ip immunisations are known to generally be more effective in induction of antibody responses that are generally characterised by faster kinetics, than other immunisation routes, including s.c. and i.n. administration. Body weight loss was recorded at 12 h post injection and expressed as a percentage of original body weight (at 0 h) in Figure 4.12C.

All PEI forms statistically significantly enhanced serum IgG titres compared to the unadjuvanted group. At 2 weeks post prime, Jet-PEI SS-induced titres were indistinguishable from Jet-PEI induced titres, while body weight loss at 12 h post immunisation were statistically

significantly lower for Jet-PEI SS. The induced titres to the degradable form of Jet-PEI did not increase further between 2 and 6 weeks following immunisation, whereas serum IgG titres induced by both Jet-PEI SS and Alhydrogel did.

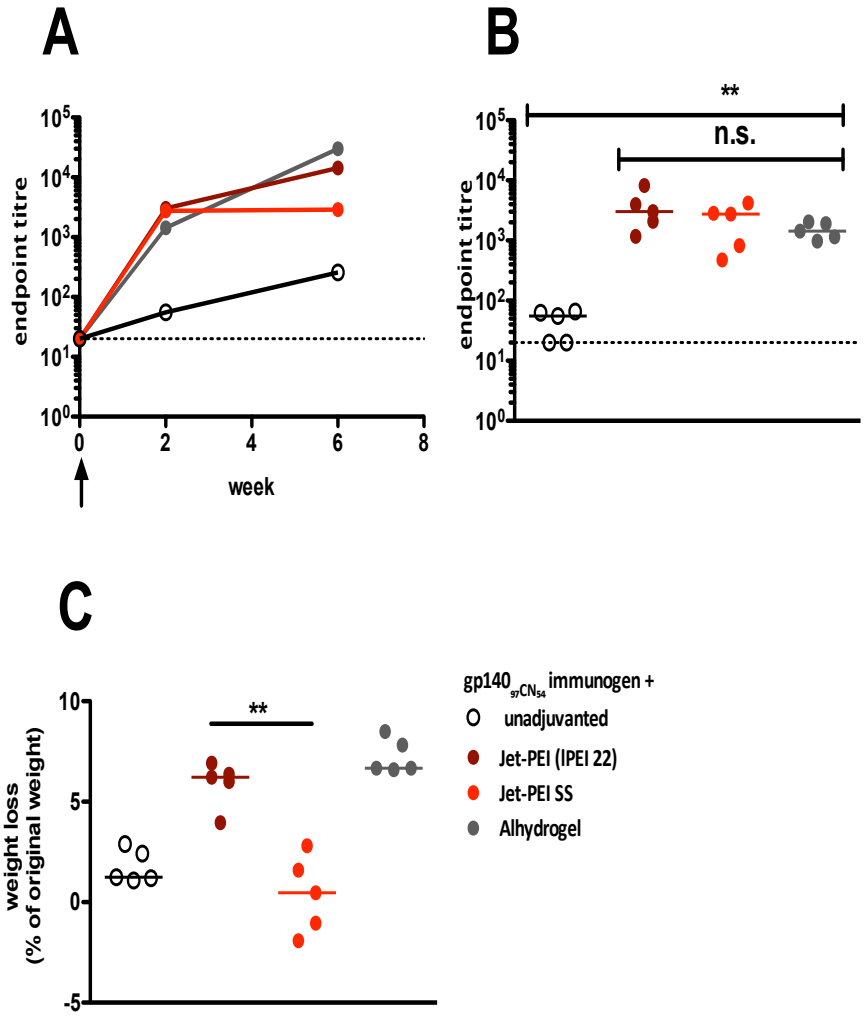

Figure 4.12 .I.p. gp140$_{97CN54}$ immunisation with degradable and standard lPEI forms. 6-10 week female BALB/c mice (*n*=5 p immunisation group) received prime (10 µg gp140$_{97CN54}$ in PBS or adjuvanted with 70 µg PEI (Jet-PEI or Jet-PEI SS) or 1 mg Alhydrogel) at week 0 (arrow). Body weights were recorded before and 12 h post prime immunisation and were expressed as a percentage of original weight (at 0h). Bleeds were collected at 2 and 6 week post prime. Sera were analysed for gp140$_{97CN54}$-specific IgG levels by ELISA. **A) Serum total gp140$_{97CN54}$-specific IgG endpoint titer timecourse. B) Serum total gp140$_{97CN54}$-specific endpoint titre at 6 week post prime. C) Body weight loss as a percentage of original body weight at 12 h post prime immunisation.** Medians of replicates (mice) within an individual experiment are depicted. Kruskal-Wallis test (all groups) and Dunn's post-test (Jet-PEI vs Jet-PEI SS), ***P*< 0.01.

4.3. Chapter discussion

PEI was demonstrated to be a particularly potent adjuvant via the mucosal route, eliciting superior $gp140_{97CN54}$-specific antibody responses and approximately equivalent, but more localised, $gp140_{97CN54}$-specific T cell responses than the gold standard mucosal adjuvant CTB (Figure 4.4). The observed loss of antibody-inducing capacity of $gp140_{97CN54}$ alone when administered mucosally compared to previous s.c. administration is in line with the expected challenges of mucosal immunisation and re-emphasises the need for development of an effective mucosal adjuvant to enable successful mucosal immunisation with protein subunit antigens, including HIV-1 Env antigens (Neutra et al., 2001; Ryan et al., 2001). The strong PEI-induced enhancement of $gp140_{97CN54}$ immunogenicity included robust mucosal $gp140_{97CN54}$-specific IgA responses that were superior to IgA-responses induced by CTB, which is known for its exceptionally strong mucosal IgA responses (Imaoka et al., 1998; Sanchez et al., 2004; Su et al., 2004; Williams et al., 1999). This finding, together with the more localised nature of PEI adjuvanticity compared to CTB adjuvanticity, is desirable for mucosal HIV-1 vaccination to achieve effective responses in the desired compartment and to limit potential unwanted side-effects (Figure 4.5)(Ryan et al., 2001). Furthermore, the balanced PEI-induced T_h1 + T_h2 antibody and cytokine response, similar to balanced T_h1 + T_h2 adjuvant CTB, is another favourable aspect for HIV-1 immunisation (Figure 4.5 & 4.6)(Imaoka et al., 1998). Various PEI-related factors could underly this potent mucosal adjuvanticity. Firstly, the current literature suggests that PEI protects nucleic acids against DNase-mediated degradation following *in vivo* administration in the context of DNA transfection (Peng et al., 2008; Boussif et al., 1995). It is possible that similar PEI-induced protection of protein antigen against the

harsh mucosal conditions partially underlies the robust mucosal adjuvant activity of PEI. Secondly, particulate vaccines are generally more potent via the mucosal route, so PEI-induced particle formation, which was investigated in the next chapter, Chapter 5, might also contribute to the strong observed mucosal activity (Ryan et al., 2001). Furthermore, enhanced PEI-induced permeabilisation of the mucosal membranes or increased targeting of antigen to M-cells, for instance via interaction with sulphated proteoglycans located on M cells or enhanced interaction with M cells due to particle form, could underlie the mucosal activity, but further investigation would be required to determine the role of any of these factors (Ryan et al., 2001).

The selected non-biodegradable lPEI and bPEI forms were all found to have adjuvant activity in the systemic and mucosal $gp140_{97CN54}$ immunisation models (Figure 4.7 & Figure 4.8), indicating that adjuvanticity is a shared attribute of PEI forms. Despite the observation of occasional differences between $gp140_{97CN54}$-antibody responses induced by distinct PEI forms no overall, or statistically significant, trend was observed. In contrast with the impact of PEI structure on transfection efficiency that is elaborately described in the literature, no significant effect of either degree of branching or MW on adjuvant activity could be drawn from these immunisation studies. Since differences in transfection efficiency have been observed between these different forms of PEI, this suggests that different mechanisms may underly PEI transfection and PEI adjuvant activity. PEI-induced processes that are thought to be involved in transfection, such as endocytosis, DNA complexing, and endosome-rupture, might still be involved in adjuvant activity, but my studies suggest that there are at least other factors involved, and that transfection-related processes are not necessarily limiting for glycoprotein immune enhancement.

The finding that adjuvant activity is a shared attribute of distinct lPEI and bPEI forms of various MW is a positive observation as it paves the way for the optimisation of PEI structure so that forms can be designed that combine limited toxicity with sufficient adjuvant activity.

To investigate potential reduction of toxicity while maintaining adjuvant activity, a selected range of biodegradable PEI forms was studied in $gp140_{97CN54}$ immunisation. Body weight loss was chosen as a measure of toxicity, since it is the most well studied measure in mice. By contrast with my starting hypothesis, no reduction in toxicity as measured by reduced body weight loss was observed with the disulphide-linked, acetal-linked, or ester-linked biodegradable forms over standard bPEI 25. In fact, bPEI 25 showed less induction of body weight loss than the selected biodegradable forms, although none of the differences between PEI forms reached statistical significance (Figure 4.9). The form bPEI 25 has emerged as a gold standard nonviral transfection agent due to its combined characteristics of strong transfection efficiency with relatively limited toxicity, so perhaps similarly exceptional and optimal characteristics of this PEI form are reflected here when studying the combination of adjuvant activity and body weight loss. In previous *in vitro* studies in the literature however, including metabolic activity and erythrocyte aggregation assays, the selected biodegradable forms ester-linked G2-HD-OEI and acetal-linked OEI-BAA-OEI were shown to be less toxic than standard bPEI and lPEI (Knorr et al., 2008; Russ et al., 2008a), indicating that *in vitro* toxicity assays are not always a good predictor of *in vivo* toxicity. However, one of these groups observed high toxicity also *in vivo* with bPEI compared to some other PEI forms including G2-HD-OEI, which is discrepant with my studies (Russ et al., 2008b). This discrepancy suggests that in future studies a range of additional measuresof of *in vivo* toxicity might need to be investigated, such as body temperature or serum acute phase protein levels. The linear standard PEI form, lPEI Jet-PEI,

induced substantial body weight loss, as expected, and although statistical significance was not reached in the mucosal immunisation model, the biodegradable version of this form, Jet-PEI SS, seemed to induce less body weight loss. This potential difference was further investigated in the i.p. immunisation model, where indeed a statistically significant reduction in induced body weight loss was observed for Jet-PEI SS over Jet-PEI (P<0.01)(Figure 4.11). Jet-PEI SS–induced serum $gp140_{97CN54}$-specific IgG responses were equivalent to Jet-PEI, suggesting that the addition of disulphide linkers in Jet-PEI had successfully decreased toxicity in mice immunisation, but not at the expense of its adjuvant activity, although the levels of induced serum antibodies seemed to decrease back to baseline more rapidly.

In light of the previously described toxicity of CTB, which hampers its use in humans (Coffman et al., 2010), it is surprising that in my studies little to no body weight loss was observed with this adjuvant, whereas in another immunisation model (i.p.) significant body weight loss was observed following adjuvantation with the widely licensed Alhydrogel adjuvant (Coffman et al., 2010; Ryan et al., 2001). This observation suggests that body weight loss in mice is not always a good predictor of *in vivo* toxicity or safety in humans, and that other measures are required to make inferences about human safety.

Except for OEI-BAA-OEI in the s.c. immunisation model, biodegradable PEI forms showed adjuvant activity under all studied conditions. Via the s.c. route OEI-BAA-OEI was ineffective in inducing $gp140_{97CN54}$-specific serum IgG responses (Figure 4.9). The limited capacity of OEI-BAA-OEI to bind and complex DNA, as well as its very short lifespan compared to the other PEI forms, may have some bearing on this, and may suggest that the ability of the different forms of PEI to complex with negatively charged structures underlies at least some of its activity. Biodegradable PEI forms were shown to induce systemic $gp140_{97CN54}$-specific

antibody titres following i.n. immunisation but further investigation will be neeeded to elucidate their ability to enhance mucosal $gp140_{97CN54}$-specific IgG and IgA responses.

Key chapter findings

- **Distinct structural PEI forms can act as glycoprotein adjuvants via systemic and mucosal administration routes in mice**
- **Biodegradable PEI forms have varying adjuvant activity, but are generally able to act as adjuvants**
- **Biodegradable PEI forms did not always show reduced *in vivo* toxicity in my studies, measured as body weight loss, compared to nondegradable PEI forms**

CHAPTER 5[+]

Cellular mechanisms of PEI-induced immune responses

[+] Portions of this work have been submitted for publication

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5.1. Chapter introduction

Over recent years, the immune potentiating mechanisms of existing adjuvants have increasingly been studied and have appeared to often involve effects on antigen as well as direct adjuvant-mediated immune stimulation. I used a variety of *in vitro* and *in vivo* model systems to study the cellular immune activating properties of PEI, including I) immune cell recruitment and cytokine release; II) complex formation with antigen and effects on particle size; III) enhancement of antigen interaction with target immune cells; IV) induction of T_h polarisation appropriate for the invading pathogen; V) direct activation of the main APCs: DCs. First, the potential PEI-mediated direct immune stimulation was investigated, followed by an analysis of the PEI effects on antigen and antigen delivery. Finally, a return will be made to the exploration of potential direct immune stimulation when studying BMDC activation.

I. One increasingly investigated mechanism in the literature on adjuvants is immune cell recruitment, which can for instance be induced by the activation of DAMP- or PAMP-mediated pathways (Calabro et al., 2011; Kool et al., 2008a; Kool et al., 2008b; Kool et al., 2011). As such the adjuvant effect of MF59 has been shown to depend on ICAM-1 mediated immune cell recruitment (Calabro et al., 2011). To investigate potential PEI-induced immune cell recruitment, the peritoneal administration model was used. Immune cell recruitment could potentially be studied after mucosal PEI administration, which would involve analysis of the NALT and other mucosal lymphoid tissues, but considering the impracticalities that would come with studying these tissue structures, initial studies were performed in the peritoneal cavity. Furthermore, the peritoneal administration model is considered particularly appropriate for the analysis of immune cell populations since the peritoneal cavity in mammals contains different immune cell populations crucial for innate immune responses (Ray and Dittle, 2010). Peritoneal cells are easily accessible and high numbers of cells can be collected via peritoneal lavage. Before employing this model it was confirmed that PEI maintained

adjuvant activity via the i.p. route. The appearance of local immune cells following exposure to PEI in this model was tracked using flow cytometry.

II. Current literature suggests that adjuvants can enhance immunogenicity of antigens through complex formation with antigen and effects on particle size, herewith mimicking pathogen properties and leading to for instance better interaction with APCs and complement proteins (Bachmann and Jennings, 2010; de Veer and Meeusen, 2011; Fifis et al., 2004; Gamvrellis et al., 2004). Furthermore, particulate vaccines have been shown to be more potent inducers of mucosal immunity in mucosal immunisation strategies (Ryan et al., 2001). In chapter three, PEI was demonstrated to bind to $gp140_{97CN54}$ in SPR, possibly via electrostatic interactions that resemble the interactions between DNA and PEI. In the literature PEI has been shown to form PEI–DNA polyplexes that facilitate PEI-mediated gene transfection (Goula et al., 1998; Sun et al., 2011). I therefore hypothesised that analogous to PEI-DNA complex formation, PEI forms complexes with $gp140_{97CN54}$. This hypothesis was studied using Transmission Electron Tomography (TEM), a technique that had been successfully used before to study PEI-DNA and PEI-HSA complexes (Abassi et al., 2011; Goula et al., 1998).

III. Another suggested mechanism for existing adjuvants is the enhancement of antigen interaction with target immune cells, for instance resulting in enhanced antigen presentation and trafficking to draining LNs (Calabro et al., 2011; Kool et al., 2008a; Kool et al., 2008b). Studies have shown that by virtue of its positive charge and binding to negatively charged membrane-associated sulphated proteoglycans followed by endocytosis, PEI induces cell entry of PEI-DNA complexes (Hu et al., 2012; Khalil et al., 2006; Mislick and Baldeschwieler, 1996). Sulphated proteoglycans are present on a wide variety of cells, including myeloid and lymphoid cells (Mislick and Baldeschwieler, 1996). Also, cationic molecules can easily be internalised by macrophages and DCs (Bachman et al., 2010; Yang and Hsu, 2008). Based on this, I hypothesised that highly cationic PEI might facilitate association of antigen with immune cells. To test this hypothesis, a

sequence of *in vitro* and *in vivo* studies were conducted. Prior to these studies, fluorescent labelling of PEI and $gp140_{97CN54}$ was optimised in a range of pilot experiments (Section 2.8). Firstly, OVA antigen and PEI, both fluorescently-labelled, were administered in the peritoneal model and local immune cells were analysed for potential association with OVA or PEI using flow cytometry. As a corollary assay it was confirmed that fluorescent labelling of PEI did not modify PEI peritoneal adjuvant activity. Since macrophages play an important role in immune surveillance and antigen presentation (Metchnikoff, 1982; Takahashi et al., 1996; Van Furth et al., 1972), the association of fluorescently-labelled PEI with peritoneally-derived macrophages was further investigated in an *in vitro* assay using laser scanning confocal microscopy (LSCM). Furthermore, the potential targeting of fluorescently-labeled $gp140_{97CN54}$ to these immune cells by PEI was studied. Since PEI has been shown to enter cells via endocytosis (Hu et al., 2012) and endocytic and phagocytic pathways can potentiate antigen presentation via the MHC class II pathway potentiating immune responses, the potential participation of these pathways in the internalisation of PEI by peritoneally-derived macrophages was studied through co-localisation assays with endosome- and lysosome-specific fluorescent markers.

IV. Since DCs bridge innate and adaptive immunity and have been shown to be the most important APCs by having the capacity to stimulate naïve T cells (Steinman and Cohn, 1973; Steinman et al., 1999), it is not surprising that many exisiting adjuvants have been shown to achieve their activity at least partially through acting on these immune cells, either indirectly or directly via, for example, PRR signalling and cytokine release (Section 1.12)(De Becker et al., 2000; De Smedt et al., 1996; Fujii et al., 2003; Kool et al., 2008b; Shah et al., 2003). To investigate potential direct PEI-induced stimulation of DCs, BMDC were incubated with various concentrations of PEI and the potential upregulation of activation- and maturation- associated surface markers was analysed by flow cytometry.

Principal aims of this chapter:

1. **Analyse the effect of PEI on early innate leukocyte type and number in the peritoneal administration model**
2. **Analyse the interaction between PEI-gp140$_{97CN54}$ and target cells, including APCs, *in vitro* and *in vivo***
3. **Study the effect of PEI on the expression of activation- and maturation- associated surface markers by BMDC**

Chapter hypotheses:

- PEI induces early innate changes in local leukocyte number and type in the peritoneal cavity

Justification: early innate changes in local leukocyte number and type have been found to underlie adjuvant effects of various adjuvants (references see chapter introduction).

- PEI forms particles with $gp140_{97CN54}$

Justification: PEI was shown to bind to $gp140_{97CN54}$ in Chapter 3 and has been shown by others to form particles with HSA and DNA (references see chapter introduction).

- PEI associates with early innate peritoneal immune cells and enhances the cellular association with antigen

Justification: PEI was shown to bind to $gp140_{97CN54}$ in Chapter 3, and has previously been shown to interact with sulphated proteoglycans, resulting amongst other effects in increased interaction of DNA with target cells, when used for transfection purposes. Furthermore, sulphated proteoglycans have been found on myeloid and lymphoid cells (references see chapter introduction).

- PEI induces changes in BMDC expression of activation- and maturation- associated cell surface markers *in vitro*

DCs are crucial for eliciting adaptive immunity and many adjuvants have been found to act directly on DCs resulting in DC activation and maturation (references see chapter introduction).

5.2. Results

5.2.1. Analysis of PEI effects on early innate leukocyte number and type in the peritoneal administration model

5.2.1.1. Confirming peritoneal PEI adjuvant activity

Use of the peritoneal model of leukocyte recruitment requires that the adjuvant maintains adjuvant activity via this route. To investigate this, mice were primed with 10 µg $gp140_{97CN54}$ antigen alone, antigen mixed with 70 µg standard bPEI (25 kDa), antigen mixed with a linear form of PEI called Jet-PEI, antigen mixed with the fluorescently- labelled linear form called Jet-PEI-FluoF®, or antigen adsorbed to 1 mg Alhydrogel (2% $Al(OH)_3$). Serum $gp140_{97CN54}$-specific IgG endpoint titres are shown in Figure 5.1.

HIV-1 $gp140_{97CN54}$ antigen alone was immunogenic via the i.p. route, inducing titres of $10^{3.2}$ at 3 weeks post prime (Figure 5.1A & B). Nevertheless, serum IgG responses to $gp140_{97CN54}$ were significantly higher in all adjuvanted groups compared to the unadjuvanted group ($P<0.01$). Unlabelled and fluorescently-labelled linear PEI forms induced approximately equivalent $gp140_{97CN54}$-specific serum endpoint titres and on average higher peak titres than the branched standard PEI form ($10^{4.2}$ and $10^{4.1}$ vs. 10^4 respectively), implying that fluorescent labelling did not obviously modify peritoneal PEI adjuvant activity. Alhydrogel elicited the highest peak serum $gp140_{97CN54}$-specific responses of ~$10^{4.4}$. However none of the inter-adjuvant differences reached statistical significance (Figure 5.1B).

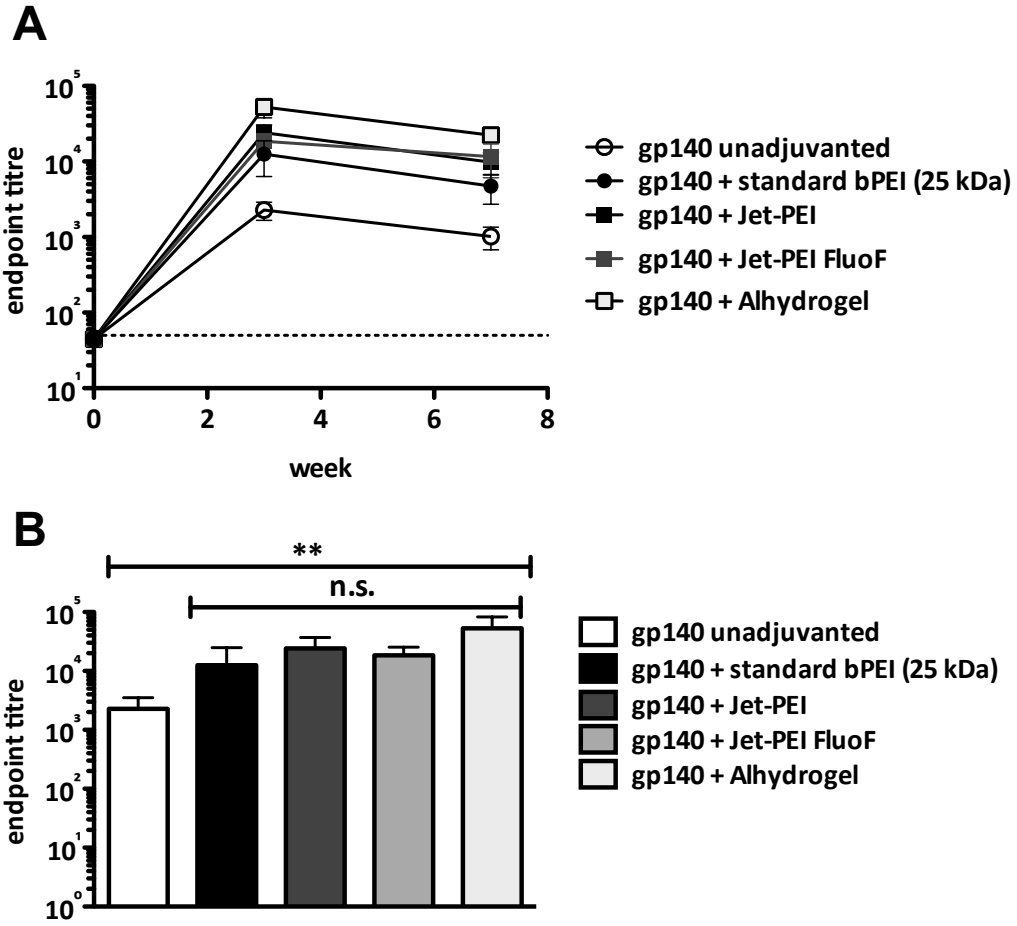

Figure 5.1. Peritoneal adjuvant activity of unlabelled and FITC-labelled PEI forms in gp140$_{97CN54}$ immunisation in mice. 6–10 week old female Balb/C mice (n=4) were i.p. immunised with 10 µg gp140$_{97CN54}$ in PBS or adjuvanted with 70 µg PEI (standard PEI, Jet-PEI, or FITC-labelled Jet-PEI FluoF®) or 1 mg Alhydrogel. Bleeds were collected at week 0, 3, and 7. **A) Serum gp140$_{97CN54}$-specific IgG endpoint titre timecourse.** Medians and range are depicted. **B) Serum gp140$_{97CN54}$-specific IgG responses at week 3 post prime.** Means +/- SD are depicted of biological replicates (mice) within an individual experiment. Data are representative of 2 independent experiments. Kruskal–Wallis, Dunn's post-test, $^{**}P< 0.01$.

5.2.1.2. PEI administration modifies peritoneal leukocyte number and type

A leukocyte gating scheme was designed for flow cytometry of peritoneal lavage samples based on previously published strategies (Kool et al., 2008b). The gating scheme included the relevant resident immune cell types in the peritoneum including macrophages, B cell and T cell resident populations, which are expected to decrease in number upon stimulation, and inflammatory monocytes, neutrophils, dendritic cells and eosinophils, which have been shown to increase upon stimulation and recruitment of which has been implicated previously in adjuvant activity (Auffray et al., 2009; Calabro et al., 2011; Inman et al., 2011; Jenkins et al., 2011; Kool et al., 2008b; Scotland et al., 2011). An overview of the gated populations can be found in Table 5.1. Examples of the gating strategies used are shown in Figure 5.2.

Leukocyte population	**Surface expression**
Monocytes	Ly6C $^{++}$/F480 ^{int}/CD11b^{+}
Macrophages	F4/80 $^{++}$/ Ly6C $^{-}$/CD11b^{+}
Neutrophils	Ly6C $^{+}$/ F480 $^{-}$ /CD11b^{+}
Dendritic cells	F4/80 $^{-/LO}$/CD11C^{+}/ MHCII^{++}
Eosinophils	F4/80^{int}/Ly6C^{LO}/SSC ^{HI}/CD11b^{+}
B cells	CD19 $^{+}$/CD11b^{-}
T cells	CD3 $^{+}$/CD11b^{-}

Table 5.1. Analysed populations and surface markers.
+=positive, -=negative, HI=high, LO=low, int=intermediate.

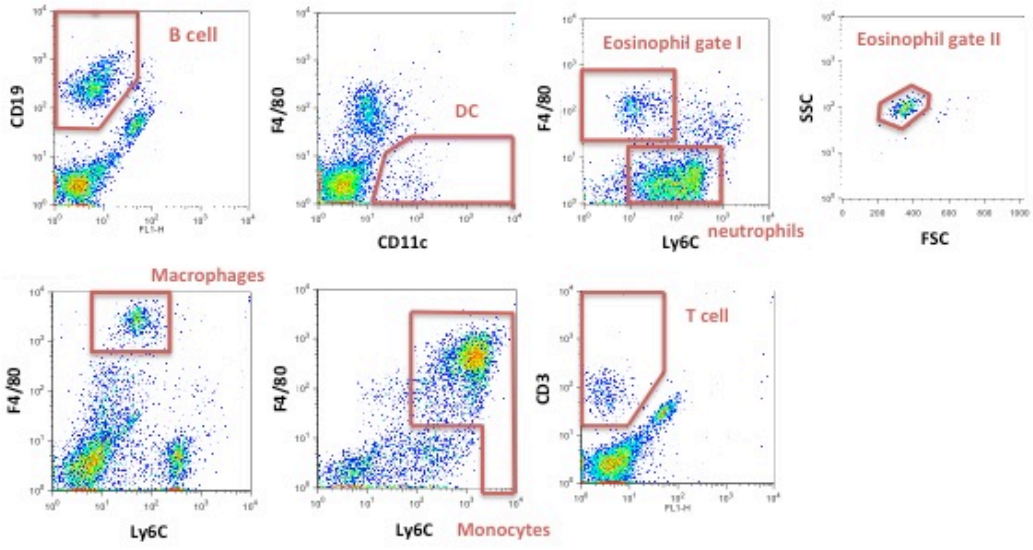

Figure 5.2. Example dot plots to represent gating strategies.

To investigate the effect of PEI on local leukocyte numbers, BALB/c mice were i.p. injected with 70 µg PEI (standard bPEI 25), PBS or 1 mg Alhydrogel (2% $Al(OH)_3$) and peritoneal lavages were analysed for leukocyte populations at 4 and 24 h post-injection, these timepoints being based on pilot assays and the literature. Peritoneal lavages were analysed using antibodies specific for leukocyte surface markers and four-colour flow cytometry (Table 5.1). Absolute numbers of peritoneal leukocytes are depicted in Figure 5.3.

As described in the literature for the naïve female mouse, the total number of peritoneal leukocytes in the animals that were administered PBS alone was ~3.5 x 10^6 cells (Scotland et al., 2011). Low numbers of all leukocytes were present with relatively higher numbers of macrophages (0.2-0.4 x 10^6 cells), B cells (~2 x 10^6 cells), and T cells (~0.2 x 10^6 cells) in line with that previously described in a naïve female mice, although macrophage and T cell numbers were slightly lower, potentially due to a stimulatory effect of the injection method itself or due to mouse strain differences as similar results were observed to the PBS-injected mice in McKee et al, who also used mice with BALB/c genetic background (McKee et al., 2009). The frequency of

individual leukocyte populations was approximately equivalent at 4 and 24 h post PBS injection. Both PEI and Alhydrogel induced changes towards a more inflammatory leukocyte profile in the peritoneum. PEI induced an increase in neutrophils (~4 x 10^6 cells, P<0.05), monocytes (~0.6 x 10^6 cells, P<0.01) and eosinophils (~0.6 x 10^6 cells, P<0.01) at 4 h post injection that was accompanied by a drop in resident B cell numbers (~0.2 x 10^6 cells, P<0.01) and macrophage numbers (~0.1 x 10^6 cells, n.s.). At 24 h post PEI injection neutrophil and eosinophil numbers had dropped to ~0.6 x 10^6 cells and ~0.2 x 10^6 cells respectively, but were still elevated compared to the PBS group (n.s.). By contrast, monocyte numbers had further increased to ~4 x 10^6 cells (P<0.01) while DC populations had also expanded (~0.4 x 10^6 cells, P<0.05). At both time points total peritoneal leukocyte numbers in the PEI group were increased compared to the PBS group (~6 x 10^6 cells compared to ~3-4 x 10^6 cells, P<0.01 at 4 h, n.s. at 24 h). Alhydrogel induced similar changes but the response was dominated by neutrophil increases, as previously described, instead of the predominant monocyte increases seen with PEI (Kool et al., 2008b). At 4 h post Alhydrogel injection leukocyte frequencies were comparable to the PEI group; neutrophil, monocyte and eosinophil numbers had increased to ~3-4 x 10^6 cells, ~0.6 x10^6 cells, and ~0.4 x 10^6 cells respectively, while macrophage and B cell numbers had dropped to ~0.4 x 10^6 cells and ~0.1 x 10^6 cells respectively. Total peritoneal leukocyte numbers were increased compared to the PBS group but were lower than the PEI group (~5 x 10^6 cells compared to ~3 x 10^6 cells in PBS group, ~6 x 10^6 cells in PEI group). In contrast, at 24 h post Alhydrogel injection the leukocyte profile differed substantially from the PEI group. Neutrophil numbers attained ~4 x 10^6 cells and eosinophil numbers had further risen to ~0.9 x 10^6 cells while monocyte numbers (~0.7 x 10^6 cells) were higher than the PBS group but substantially lower than the PEI group, again in accordance with previous studies on Alhydrogel i.p. injection (Kool et al., 2008b). Total peritoneal leukocyte numbers in the Alhydrogel group 24 h post injection were ~7 x 10^6 cells.

Taken together the results show that PEI caused a substantial early innate increase in leukocyte populations in the peritoneum that was predominantly characterised by increases in total cells, neutrophil numbers, eosinophils, DCs, and a distinct delayed increase in monocytes.

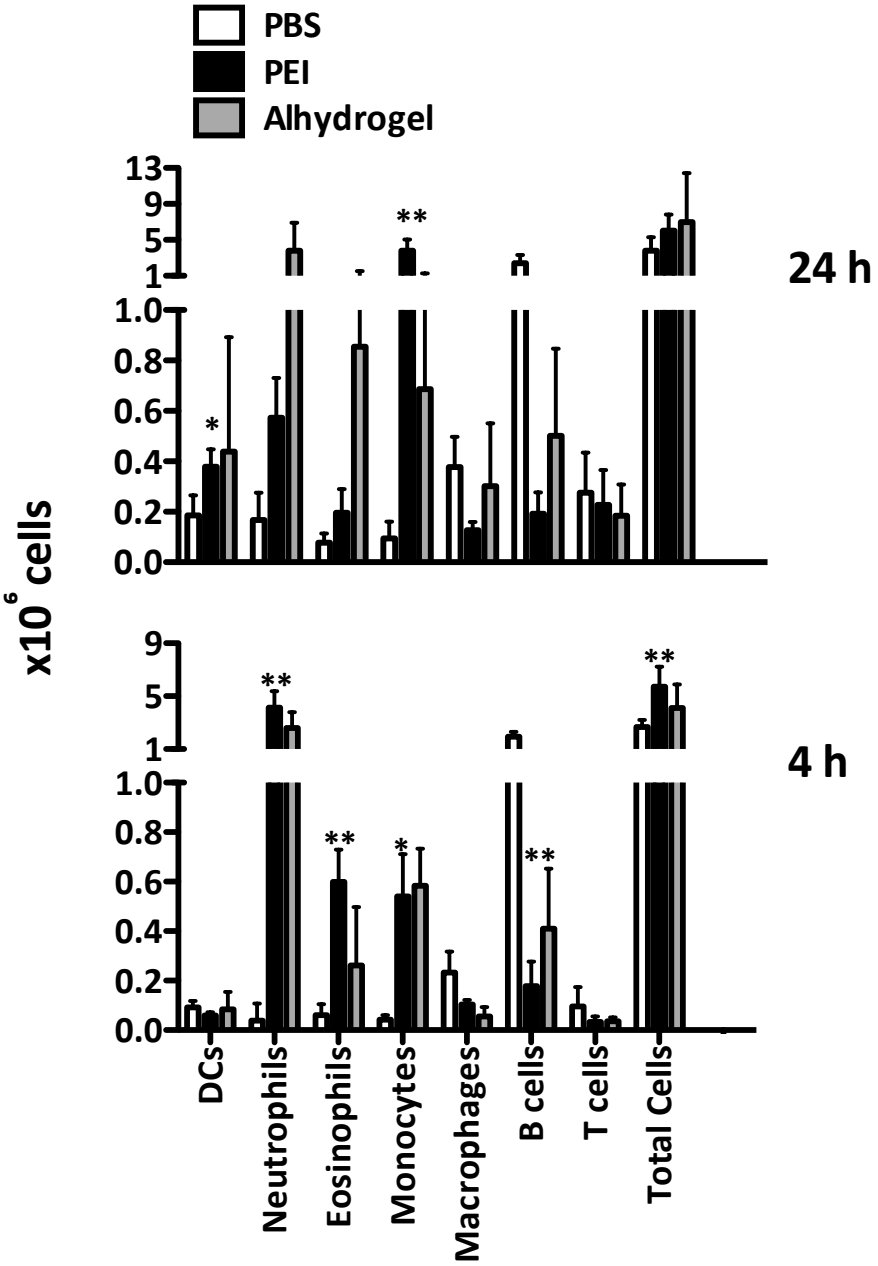

Figure 5.3. Peritoneal early innate leukocyte recruitment 4 or 24 h after i.p. PEI injection. 12 week-old female Balb/C mice (*n*=5) were i.p. injected with 80 µg PEI, PBS only, or 1 mg Alhydrogel. At 4h or 24h mice were sacrificed and peritoneal lavages collected for flow cytometry analysis to determine leukocyte numbers. Means +/- SD are depicted. Kruskal-Wallis (all), Dunn's post-test (PBS vs PEI), **P*<0.05, ***P*<0.01. The results are representative of >4 independent experimental repeats.

In parallel with the cellular recruitment studies, the presence of peritoneal soluble factors was determined by Luminex™ bead-based assay performed by another member in the Sattentau lab (Appendix I). This analysis showed that PEI substantially upregulated a range of soluble factors, including T_h1 cytokine (IFN-**γ**), T_h2 cytokines (IL-5, IL-13), growth factors (G-CSF, GM-CSF), inflammation-associated soluble factors IL-10, TNF-**α**, IL-6, and IL-1**β** and cell-attractant proteins (including KC (CXCL1) (neutrophil-attractant), Eotaxin (CCL11) (eosinophil-attractant), MCP-1 (CCL2) (mainly monocyte and DC attractant), MIP1-**α** (CCL3) (granulocyte-attractant), MIP1-**β** (CCL4) (monocyte and NK cell-attractant), and RANTES (CCL5) (eosinophil-, basophil-, and T cell-attractant).

5.2.2. PEI and antigen interaction with leukocytes

5.2.2.1. PEI and gp140$_{97CN54}$ form complexes

In chapter three it was shown in SPR assays that PEI binds to gp140$_{97CN54}$ antigen. To further characterise PEI-gp140$_{97CN54}$ interactions and the potential formation of PEI-gp140$_{97CN54}$ complexes, a negative stain TEM analysis was conducted. Samples were prepared with 4 µg gp140$_{97CN54}$ alone or mixed with 12 µg PEI (3:1 (w:w) PEI to gp140$_{97CN54}$), PEI alone, 0.55 µg DNA alone or DNA in combination with PEI at N:P= ¼, 2, or 6 as described in the literature (Goula et al., 1998). Representative single image views are shown in Figure 4.4. Quantification per grid area for particle sizes in the PEI, PEI and gp140$_{97CN54}$, or PEI and DNA groups was determined by counting of randomly chosen fields. An overview is represented in Figure 5.4H.

PEI alone appeared as globular structures that were predominantly 20-70 nm in diameter (Figure 5.4B, 4.7H). By contrast, gp140$_{97CN54}$ alone appeared as amorphous staining with occasional denser patches probably corresponding to gp140$_{97CN54}$ aggregates (Figure 5.4A). When PEI was mixed with gp140$_{97CN54}$ antigen, more pleomorphic and bigger particles were observed that ranged between 20 to > 200 nm (Figure

5.4C). In accordance with previous studies, DNA and PEI formed particles that increased in size with decreasing N:P ratio, and that were of similar size and form as previously shown DNA-PEI 'polyplexes' with ~60 nm at N:P=6 and ~100 nm at N:P=2 (Figure 5.4E-G) (Goula et al., 1998).

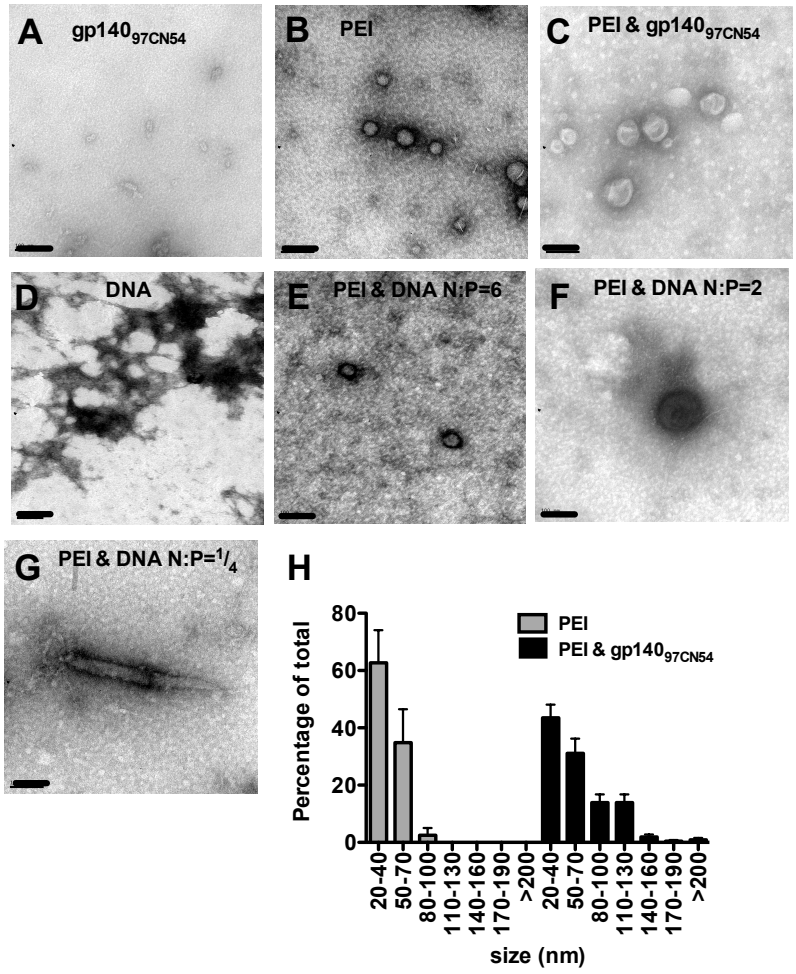

Figure 5.4. TEM study of interactions between PEI and gp140$_{97CN54}$ or PEI and DNA. A) 4 µg gp140$_{97CN54}$ alone; B) 12 µg PEI alone; C) 4 µg gp140$_{97CN54}$ and 12 µg PEI; D) 0.55 µg sheered salmon sperm DNA alone; E) PEI and DNA at N:P=6 (1.39 µg DNA and 12 µg PEI); F) PEI and DNA at N:P=2 (4.18 µg DNA and 12 µg PEI); G) PEI and DNA at N:P = ¼ (33.44 µg DNA and 12 µg PEI); H) Quantification of PEI, PEI-gp140$_{97CN54}$ or PEI-DNA particle sizes per grid area determined by counting of randomly-chosen fields imaged by negative stain transmission electron microscopy. Data are representative of three independent experiments. Depicted are means +/- SD of replicates (randomly chosen fields) within an individual experiment. Scale bar=100 nm.

5.2.2.2. PEI-enhanced Alexa$_{647}$-OVA association with peritoneal leukocytes

To investigate whether PEI adjuvant enhanced interaction of antigen with immune cells, such as peritoneal leukocytes, mice were injected i.p. as before, but this time with 10 µg Alexa$_{647}$-OVA antigen alone, antigen mixed with 70 µg PEI, or antigen mixed with 1 mg Alhydrogel (2% $Al(OH)_3$). OVA antigen was used instead of gp140$_{97CN54}$, because optimisation of fluorescent labelling and analysis in pilot studies had indicated that the commercially available OVA$_{647}$ was most suitable for the assays presented herein (see Section 2.8.1). OVA antigen is a glycoprotein with a low pI (approximately 4.7) that in chapter 3 of this thesis was demonstrated to directly interact with PEI analogously to gp140$_{97CN54}$. It is therefore expected that OVA and gp140$_{97CN54}$ will behave similarly in the current studies. PBS, PEI and Alhydrogel alone were injected as controls to determine the appropriate gating strategies later in the studies. At 4 and 24 h post injection, peritoneal lavages were collected, leukocytes were isolated and incubated with Abs specific for leukocyte surface markers, as before, to prepare for flow cytometry (see Table 5.1). All leukocyte populations were analysed for Alexa$_{647}$-fluorescence indicative of association with Alexa$_{647}$-OVA antigen. Absolute and relative numbers of Alexa$_{647}$-positive peritoneal leukocytes at both time points are depicted in Figure 5.5. Representative images for individual dot plots of Alexa$_{647}$-OVA association are shown in Figure 5.6.

PEI induced substantially increased percentage of Alexa$_{647}$-OVA association of total peritoneal leukocytes from ~35% in the PBS group to ~80% (P<0.001) in the PEI group at both time points while Alhydrogel only modestly increased or even decreased association to ~22% at 4 h and ~50% at 24 h post-immunisation.

In the absence of adjuvant, Alexa$_{647}$-OVA-association was observed for macrophages and B cells (4 h: ~1 x 10^6, 40% B cells; ~0.8 x 10^6, 100% macrophages; 24 h: 0.5 x 10^6, 100% macrophages), which is in accordance with the sentinel and antigen-engulfing role of these leukocyte populations. In the presence of PEI, higher Alexa$_{647}$-positive leukocyte percentages were observed for all populations, suggesting that PEI had

enhanced OVA-association non-specifically for all leukocyte populations (Figure 5.5B). A similar but much smaller effect was seen with Alhydrogel adjuvantation. The largest PEI-induced increase in percentage OVA-association was observed for monocytes, where percentage OVA–association increased from ~20% in the PBS group to ~90-100% (P<0.001) in the PEI group at both time points (Figure 5.5B & 5.6A). The second largest PEI effect on percentage association was observed in the DC population where percentage association rose from ~30% to ~70% at both time points (P<0.001)(Figure 5.5B & 5.6D). Additionally, percentage association was statistically significantly increased for neutrophils at 4 h and B cells at 24 h (P<0.001) in the PEI group compared to the PBS group. The effects with Alhydrogel adjuvantation were substantially smaller with ~40% monocyte and ~58% DC association at 4 h and ~80% monocyte and ~42% DC association at 24 h post injection (vs. 20% for monocytes and 30% for DC in the PBS group at both timepoints).

The total number of OVA-associated cells increased substantially in the PEI group (~4.1 x 10^6 cells) compared to the PBS and Alhydrogel groups (~1.6 x 10^6 cells and ~1.9 x 10^6 cells respectively), which re-emphasises the increased total leukocyte numbers and enhanced association of cellular association with OVA in the presence of PEI. Interestingly, PEI induced particularly high absolute numbers of $Alexa_{647}$-associated monocytes at 24 h post injection (~5 x 10^6 cells, Figure 5.5A & 5.6A), and neutrophils (~2.5 x 10^6 cells, Figure4.5B), monocytes (~0.7 x 10^6 cells) and eosinophils (~0.4 x 10^6 cells) at 4 h post injection compared to the PBS group, in accordance with previously shown PEI-induced enhancement of these populations and increased OVA-association percentages. Similarly in accordance with previously shown PEI–induced reduction of B cell and macrophage numbers, absolute numbers of $Alexa_{647}$- associated cells B cells and macrophages were lower in the PEI group (4 h: ~0.5 x 10^6 macrophages, ~0.1 x 10^6 B cells; 24 h: no macrophages) compared to the PBS group (4 h: ~0.8 x 10^6 macrophages, ~1 x 10^6 B cells; 24 h: ~0.5 x 10^6 macrophages) despite a higher percentage association in the PEI group. Absolute numbers of $Alexa_{647}$-OVA association in the Alhydrogel group also corresponded to previously shown Alhydrogel-induced changes in local leukocyte profile, with

high absolute numbers of $Alexa_{647}$-OVA associated neutrophils (~1 x 10^6 cells) and monocytes (~0.7 x 10^6 cells) at 24 h post injection, representing the substantial size of these populations.

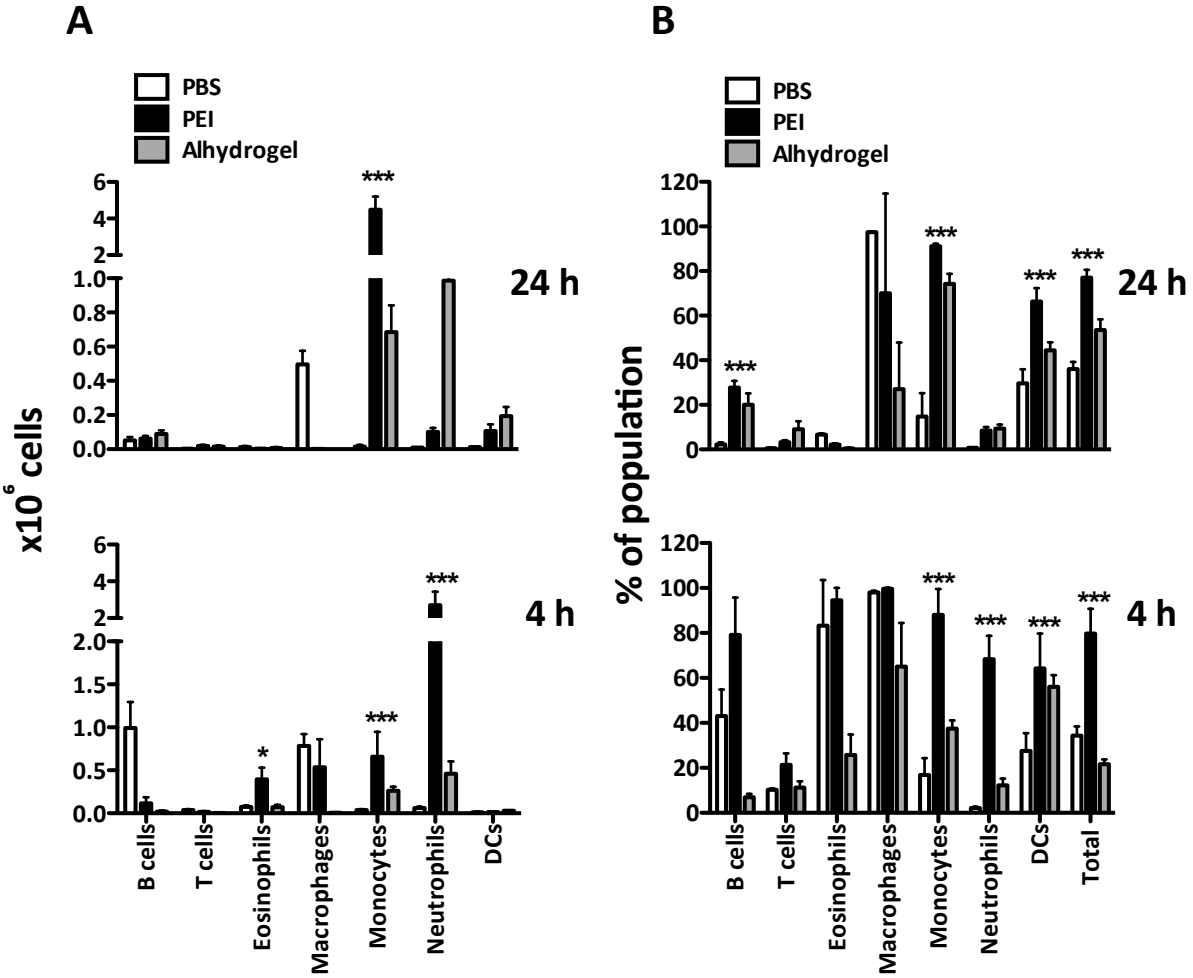

Figure 5.5. $Alexa_{647}$-OVA association with innate peritoneal leukocytes. BALB/c mice (*n*=5) were i.p. injected with 10 µg $Alexa_{647}$-OVA antigen in PBS, antigen mixed with 70 µg PEI or antigen adsorbed with 1 mg Alhydrogel. To determine gating strategies, control groups were injected with PBS alone, 70 µg PEI alone or 1 mg Alhydrogel alone. At 4 h and 24 h post injection, mice were sacrificed and peritioneal lavages collected. Isolated cells were stained with fluorescently-labelled leukocyte markers and analysed using four-colour flow cytometry. **A) Absolute numbers of $Alexa_{647}$-OVA associated leukocytes at 4h and 24h post injection. B) Percentage $Alexa_{647}$-OVA associated leukocytes at 4h and 24 h post injection.** Means +/- SD deviation of biological repeats (mice) within an individual experiment are depicted. Kruskal-Wallis (all), Dunn's post-test (PEI vs. PBS), **P*<0.05, ****P*<0.001.

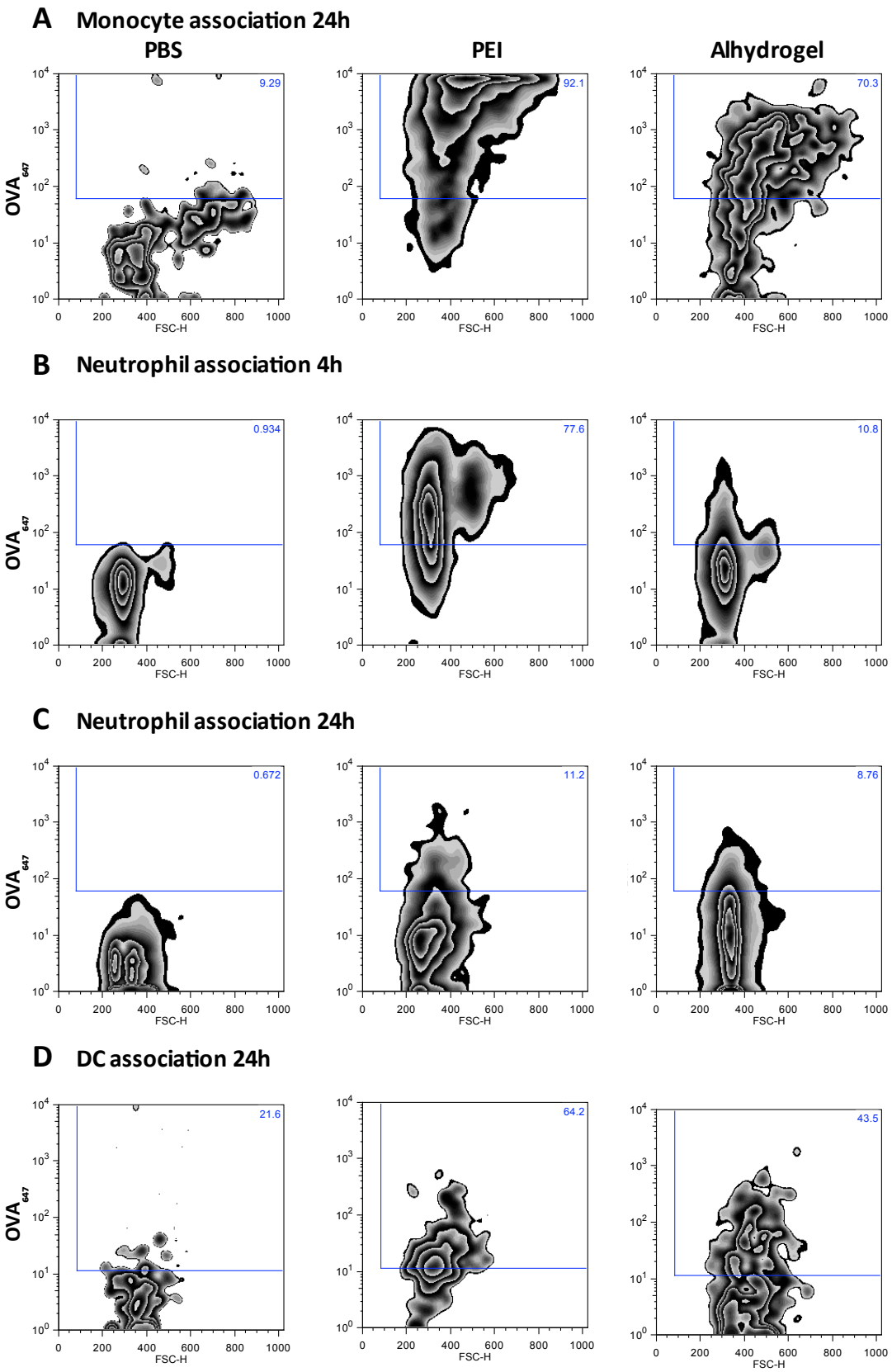

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Figure 5.6. Example of gating strategies: Alexa$_{647}$-OVA association with peritoneal monocytes, neutrophils, and DC. BALB/c mice *(n*=5) were i.p. injected with 10 µg Alexa$_{647}$-OVA antigen in PBS (left panel), antigen mixed with 70 µg PEI (centre panel) or antigen adsorbed on 1 mg Alhydrogel (right panel). At 4 and 24 h post injection, mice were sacrificed and peritoneal lavages collected. Isolated cells were stained with fluorescently- labelled leukocyte markers and analysed using four-colour flow cytometry. **A) Dot plots of Alexa$_{647}$-OVA association of monocytes at 24 h post injection. B) Dot plots of Alexa$_{647}$-association of neutrophils at 4 h post injection. C) Dot plots of Alexa$_{647}$-OVA association of neutrophils at 24 h post injection D) Dot plots of Alexa$_{647}$-OVA association of DCs at 24 h post injection.** Dot plots are representatives from biological repeats (mice).

5.2.2.3. PEI association with innate leukocytes in the peritoneum

In order to investigate whether the PEI-induced increased OVA-association with leukocytes was due to interaction of PEI-antigen complexes with leukocytes, the association of local leukocytes with fluorescently-labelled PEI was investigated.

Mice were injected i.p. as before, but this time with FITC-labelled linear PEI (Jet-PEI-FluoF®), standard linear PEI (Jet-PEI), or PBS. At 24 h post-injection, peritoneal lavages were collected, leukocytes isolated and incubated with leukocyte antibodies specific for surface markers as before to prepare for flow cytometry (see Table 5.1). All leukocyte populations were analysed for FITC-fluorescence that was indicative of association with Jet-PEI-FluoF®. Absolute numbers and percentages of FITC-positive peritoneal leukocytes are depicted in Figure 5.7A & B. Representative images of individual dot plots are presented in Figure 5.7C & D.

Similar to previously investigated Alexa$_{647}$-OVA, Jet-PEI-FluoF® associated with all analysed leukocyte populations and statistically significantly differences in percentage OVA-association between Jet-PEI-FluoF® and Jet-PEI group were observed for all studied cell populations (P<0.05), except for the eosinophil population (n.s.). Percentage association was equivalent to OVA-association at 24 h post-injection for B cells (~30% PEI-association vs. ~30% OVA-association), eosinophils (~5% vs. ~5%), macrophages (~70% vs. ~70%) while slight differences were seen for T cells (~30% PEI association vs. ~5% OVA-association), monocytes (~80% vs. ~90%), neutrophils (~20% vs. ~10%), and DCs (~50% vs. ~70%). Similar to OVA-

association, PEI association at 24 h post-injection was, in absolute numbers, highest for monocytes (~9 x 10^6 cells, P<0.01). In contrast to OVA-association high absolute numbers of FITC-positive neutrophils (~1.2 x 10^6 cells, P<0.05) and DCs (~0.5 x 10^6 cells, P<0.05) were observed at 24 h post-injection for PEI.

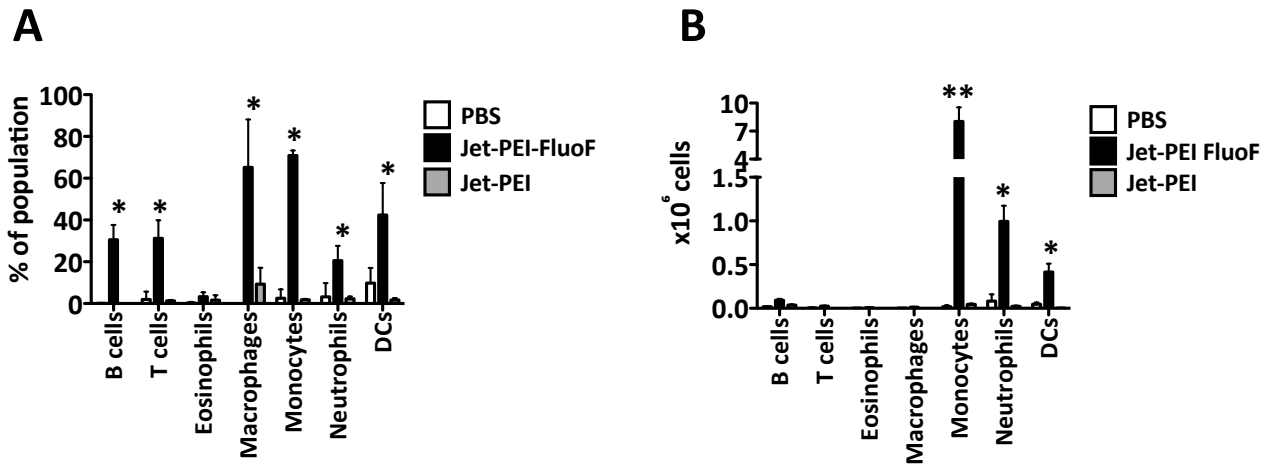
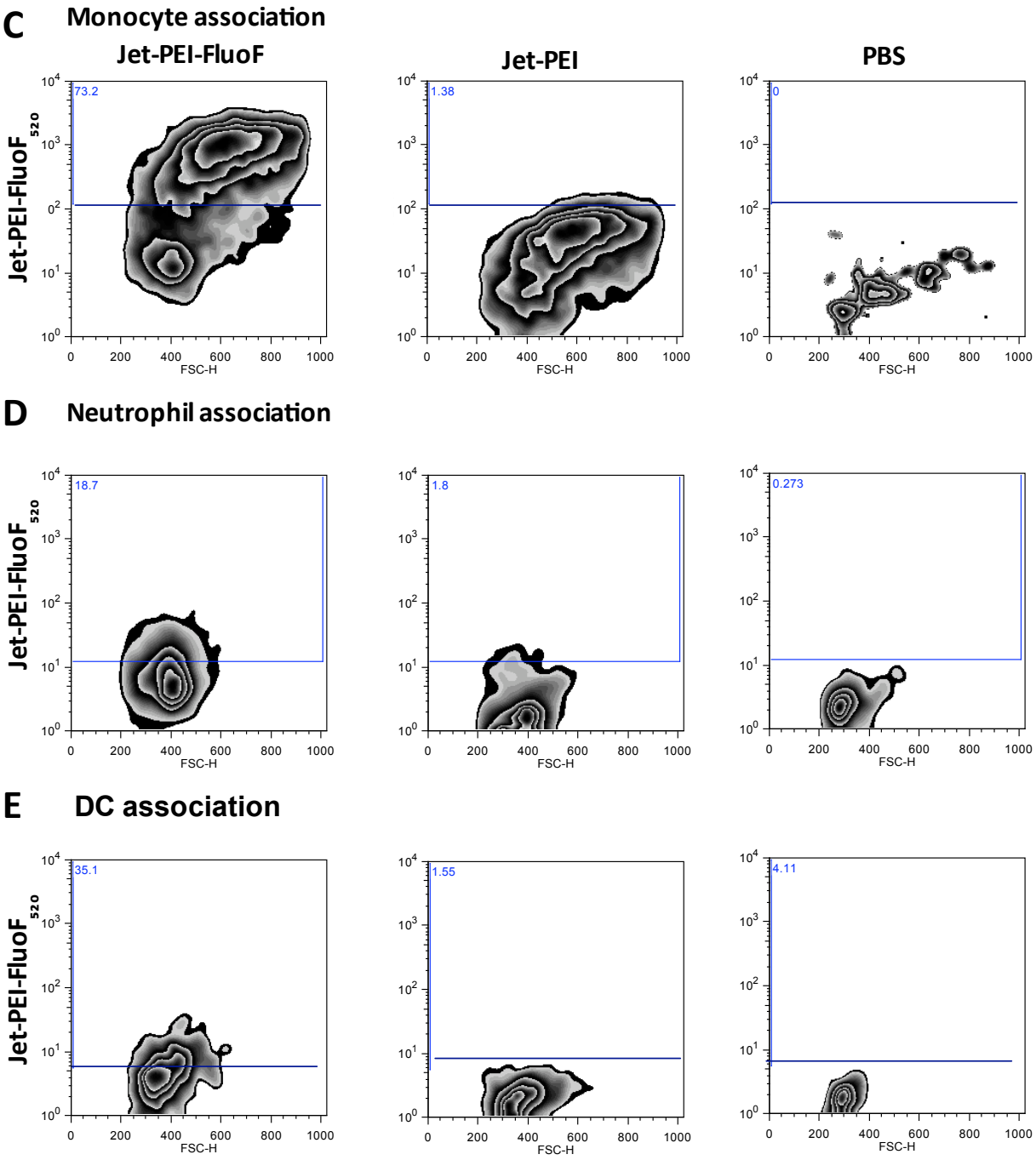


Figure 5.7. Jet-PEI-FluoF® association with peritoneal leukocyte populations. BALB/c mice (*n*=5) were i.p. injected with 70 µg Jet-PEI-FluoF® (left panel), 70 µg Jet-PEI (centre panel), or PBS (right panel). At 24 h post-injection, mice were sacrificed and peritoneal lavages collected. Isolated cells were stained with fluorescently labelled leukocyte markers and analysed using four-colour flow cytometry. **A) Percentages of FITC (Jet-PEI-FluoF®)-associated leukocytes. Kruskal-Wallis (all), Dunn's post-test (Jet-PEI FluoF vs. Jet-PEI), *P< 0.05, **P< 0.01. B) Absolute numbers of FITC (Jet-PEI-FluoF®)-associated leukocytes. A & B) Means +/- SD deviation are depicted. C) (next page) Dot plots of FITC (Jet-PEI-FluoF®)-association of monocytes. D) (next page) Dot plots of FITC (Jet-PEI-FluoF®)-association of neutrophils. E) (next page) Dot plots of FITC (Jet-PEI-FluoF®)-association of DC**. Representative dot plots of biological replicates (mice) within an individual experiment are depicted.


C
Monocyte association
Jet-PEI-FluoF
Jet-PEI
PBS
73.2
1.38
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Neutrophil association
18.7
1.8
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E
DC association
35.1
1.55
4.11
Jet-PEI-FluoF 520
FSC-H

5.2.2.4. In vitro uptake of fluorescently-labelled PEI and gp140$_{97CN54}$ by peritoneally derived macrophages

In the previous section both PEI and OVA were found to associate with various leukocyte populations, including APC. To investigate gp140$_{97CN54}$-association and potential uptake by APC, as well as to investigate whether the phagocytic and endocytic pathways are involved which potentiate antigen presentation via MHC class II, LSCM analysis was carried out with peritoneally-derived macrophages that were incubated *in vitro* with combinations of fluorescently-labelled PEI, fluorescently-labelled gp140$_{97CN54}$, and endosomal/lysosomal markers.

HIV-1 gp140$_{97CN54}$ and standard bPEI 25 kDa were labelled with Alexa$_{488}$-Fluor microscale protein labelling kit (Molecular Probes) (Section 2.8). Naïve mice were sacrificed and peritoneal lavages obtained. Leukocytes were isolated from the lavages and macrophages were selected by cover slip adherence. Subsequently, macrophages were incubated with Alexa$_{488}$-gp140$_{97CN54}$ alone, gp140$_{97CN54}$ mixed with PEI (unlabelled), Alexa$_{488}$-PEI, Alexa$_{488}$-conjugate alone or with PEI, or PBS. After this incubation, macrophages were permeabilised and stained with fluorescent nuclear (DAPI), lysosomal (LAMP-1/Cathepsin D), late endosomal (Cathepsin D) or surface (F4/80) labels and analysed by fluorescence and LSCM. Representative images are shown in Figure 5.8, 5.9, and 5.10.

Incubation of peritoneal macrophages with control unconjugated Alexa$_{488}$-label and PEI alone did not result in detectable Alexa$_{488}$-signal inside the cells. Conjugated Alexa$_{488}$-PEI however visibly associated with the peritoneally-derived macrophages at all concentrations of PEI used (0.1, 1, 10 µg), so assays were conducted with the lowest concentration (Figure 5.8 A & B). The intracellular location of Alexa$_{488}$-PEI was confirmed by a 3D composition of Z-stack images (Figure 5.8C).

Incubation with Alexa$_{488}$-gp140 alone did not result in detectable Alexa$_{488}$-signal (Figure 4.9A). Addition of PEI to the incubation, however, resulted in detectable Alexa$_{488}$-gp140 signal inside the cells at all used PEI concentrations, therefore further assays were performed using the lowest PEI concentration (Figure 5.9B). As before, the location of the Alexa$_{488}$-antigen in intracellular compartments was confirmed by 3D overlay of Z-stack images (Figure 5.9B).

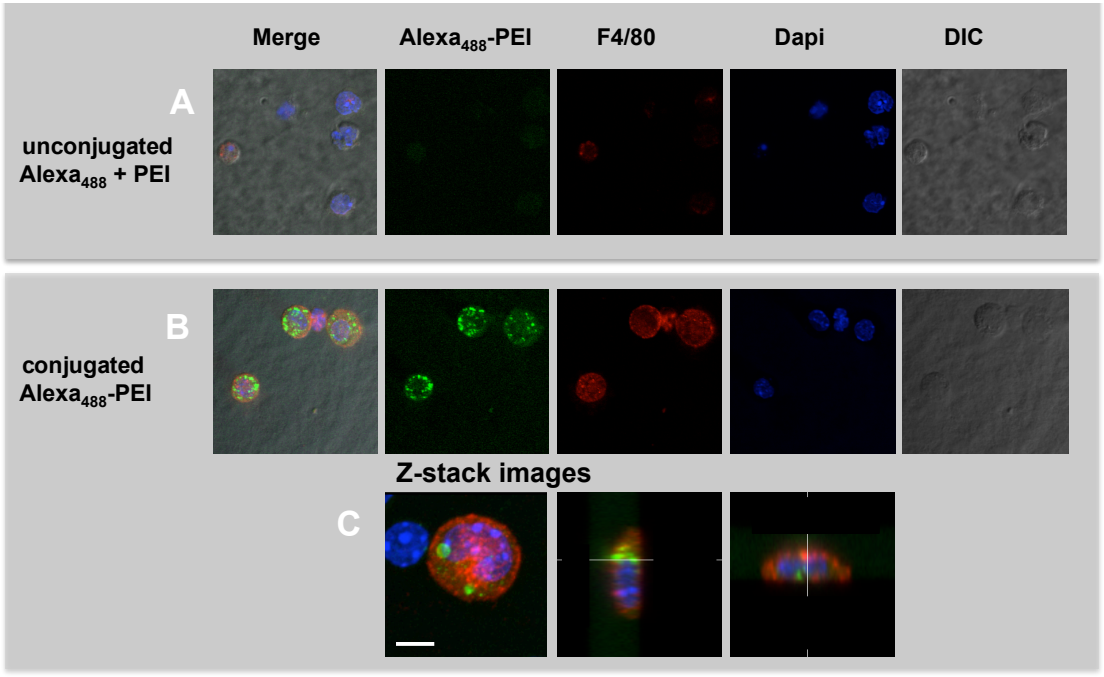


Figure 5.8. ***In vitro*** **peritoneally-derived macrophage uptake of fluorescently-labelled PEI.** Peritoneally-derived macrophages were incubated with Alexa$_{488}$-PEI (0.1 µg/ml), unconjugated Alexa$_{488}$ label and PEI (0.1 µg/ml) or PBS for 2h. Cells were fixed, permeabilised and stained with biotinylated F4/80, streptavidin-TRITC. **A) LSCM images of cells incubated with Alexa$_{488}$ label + PEI. B) LSCM images of cells incubated with Alexa$_{488}$-PEI. C) 3D reconstructions of an individual macrophage incubated with Alexa$_{488}$-PEI.** Scale bar=5 µm. Olympus confocal microscope FV1000 and IX81 (N/A 1.35).

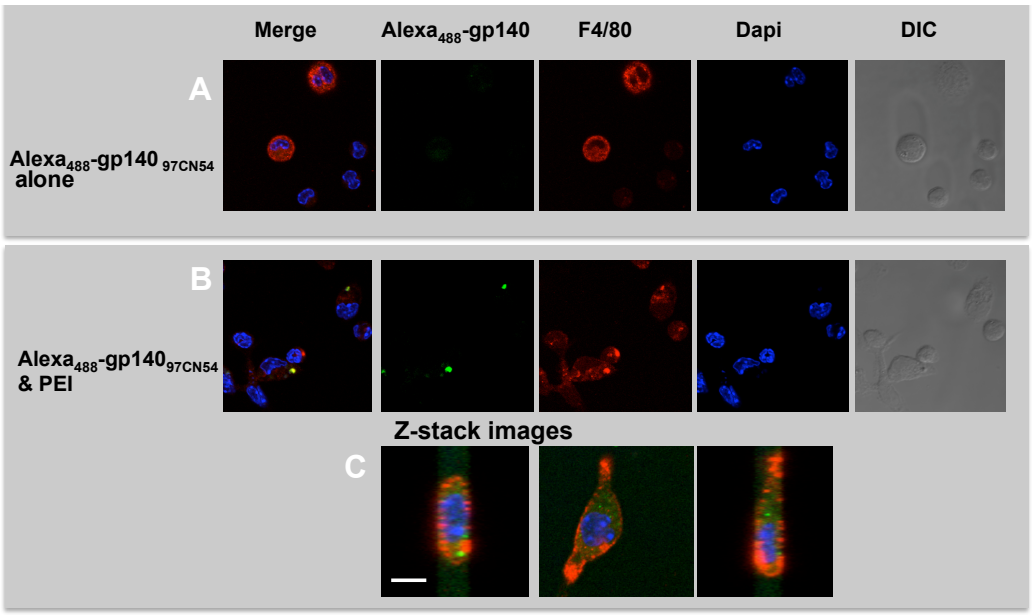


Figure 5.9. ***In vitro*** **peritoneally-derived macrophage uptake of fluorescently-labelled gp140$_{97CN54}$.** Peritoneally-derived macrophages were incubated with Alexa$_{488}$-gp140$_{97CN54}$ (2 µg/ml) alone, Alexa$_{488}$-gp140 mixed with PEI (0.1 µg/ml) or PBS for 2h. Cells were fixed, permeabilised and stained with biotinylated F4/80, streptavidin-TRITC. **A) LSCM images of cells incubated with Alexa$_{488}$–gp140$_{97CN54}$ in the absence of PEI B) LSCM images of cells incubated with Alexa$_{488}$ –gp140$_{97CN54}$. + PEI. C) 3D reconstructions of an individual macrophage incubated with gp140-Alexa$_{488}$ and PEI.** Scale bar=5 µm. Olympus confocal microscope FV1000 and IX81 (N/A 1.35).

To investigate the intracellular location of PEI and $gp140_{97CN54}$ in the peritoneal macrophages, co-localisation analysis with endosomal and lysosomal markers was carried out using ImageJ and JaCoP plugin (using Costes' automatic threshold) (Bolte and Cordelieres, 2006). Cathepsin D is an aspartic protease present in late endosomes and lysosomes. Lysosomal-associated membrane protein 1 *(*LAMP-1*),* also known as CD107a, is a membrane glycoprotein present in lysosomes.

Some co-localisation of $Alexa_{488}$-PEI with the late endosomal/lysosomal marker Cathepsin D was observed, resulting in yellow dots of overlapping signal r=0.498 (M1=0.862, is fraction of Cathepsin D overlapping $Alexa_{488}$-PEI; M2=0.662, is fraction of $Alexa_{488}$-PEI overlapping Cathepsin D)(Figure 5.10A). Similarly, some co-localisation was observed between $Alexa_{488}$-PEI and LAMP-1 r=0.328 (M1=0.828, is fraction of LAMP-1 overlapping $Alexa_{488}$-PEI; M2=0.645, is fraction of $Alexa_{488}$-PEI overlapping LAMP-1)(Figure 5.10B), suggesting that PEI was partially targeted to late endosomes and lysosomes.

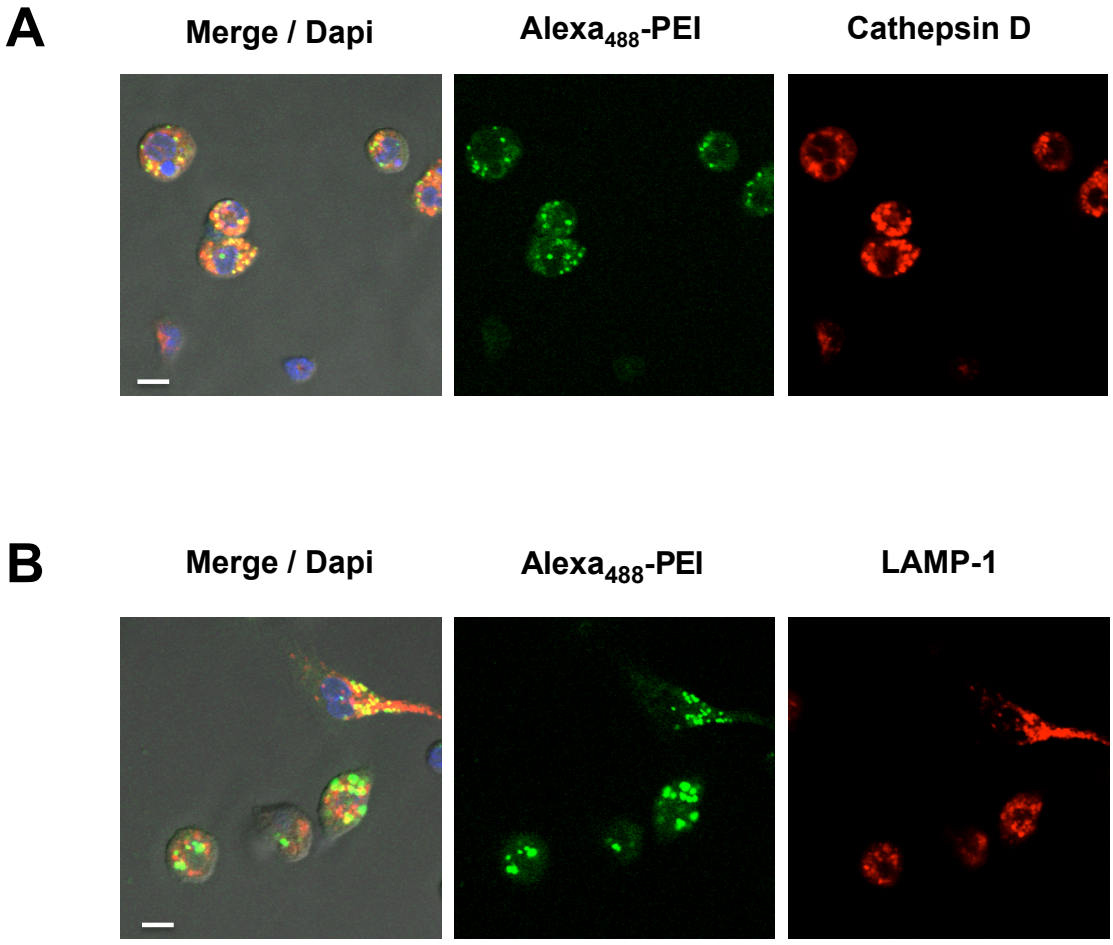


Figure 5.10. ***In vitro*** **peritoneally-derived macrophage uptake of Alexa$_{488}$-PEI and co-localisation with late endosome/lysosomal markers Cathepsin D and LAMP-1.** Peritoneal macrophages were incubated with Alexa$_{488}$-PEI (0.1 µg/ml), Alexa$_{488}$ label and PEI (0.1 µg/ml) or PBS for 2h. Cells were fixed, permeabilised and stained with biotinylated Cathepsin D or biotinylated LAMP-1, streptavidin-TRITC **A) LSCM images of Alexa$_{488}$-PEI co-localisation with late endosomal marker Cathepsin D B) LSCM images of Alexa$_{488}$-PEI co-localisation with lysosomal marker LAMP-1.** Scale bar = 5 µm. Olympus confocal microscope FV1000 and IX81 (N/A 1.35). ImageJ and JaCoP analysis of TRITC (1) and Alexa$_{488}$ (2) with Costes' automatic threshold: r=0.498 for A (M1=0.846, M2= 0.662), r=0.328 for B (M1=0.828, M2=0.645).

5.2.2.5. PEI effect on BMDC expression of activation- and maturation-associated surface markers

Previously it was shown that PEI interacts with local leukocytes in the peritoneum, including DCs. In order to investigate a potential PEI-induced effect on BMDC expression of activation- and maturation- surface markers, naïve mice were sacrificed and femur and tibia were surgically removed. Bone marrow was collected. Cells were isolated and cultured in the presence of GM-CSF for 7 days. At day 6, immature BMDC were stimulated with PEI (0.1 µg/mL, 1 µg/mL, 10 µg/mL), LPS from E.Coli, or culture medium only (positive and negative control respectively). Following overnight incubation, cells were harvested and stained for CD markers CD40, CD80, CD86, MHCII molecules, and isotype controls for each surface marker. Cells were prepared and analysed by flow cytometry. MFI were normalised to the isotype control. The normalised MFI for expression of each surface molecule in medium only, PEI (0.1, 1, 10 µg/mL) and LPS (1 µg/mL) stimulation are depicted in Figure 5.11.

As expected, LPS stimulation clearly increased CD40, CD86 and MHCII expression on BMDC compared to medium only, confirming an LPS-induced maturation effect on BMDC *in vitro,* although statistical significance was only reached for MHCII expression ($P<0.05$) (Figure 5.11 and 5.12). By contrast, the different concentrations of PEI induced equivalent expression levels for all surface markers to levels observed in the medium only condition, implying that PEI does not have a direct effect on BMDC maturation *in vitro*. At the highest PEI concentration (10 µg/mL), the BMDC shifted towards a more apoptotic-like phenotype (decreased FSC) and the number of viable cells decreased from ~24.56 ± 3.89 % in the medium group to 15.35 ± 4.20 % at the highest PEI concentration (both mean ± SD) in line with PEI cytotoxicity findings in the literature, whereas no substantial effect on number of viable cells was observed at the lowest and middle PEI concentration (0.1. µg/mL and 1 µg/mL). A statistically non-significant trend was visible towards lower surface expression with the increasing concentration of PEI, but this is probably due to the observed reduction

in viable mature BMDC numbers and higher isotype control binding at higher concentrations of PEI, which confounds the interpretation of the results (Figure 5.12).

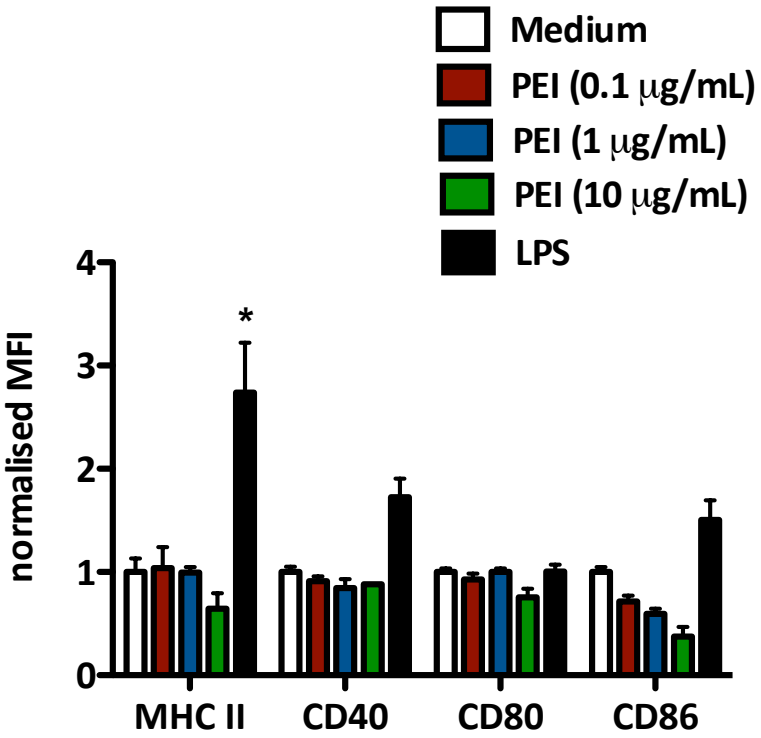


Figure 5.11. ***In vitro*** **PEI effect on BMDC maturation: normalised MFI.** Naïve mice were sacrificed and bone marrow cells were isolated. Cells were cultured in the presence of GM-CSF for 7 days. At day 6, immature BMDC were stimulated with PEI (0.1 μg/mL, 1 μg/mL, 10 μg/mL), or LPS (1 μg/mL) from E.Coli or culture medium only (controls). After 16 h cells were harvested and stained for surface markers CD40, CD80, CD86 and MHCII. Cells were analysed using flow cytometry. MFI were normalised to isotype control and to medium condition. The results are depicted in mean +/- SD or range, and are representative of three independent experimental repeats. Kruskal-Wallis, Dunn's post-test,*$P < 0.05$.

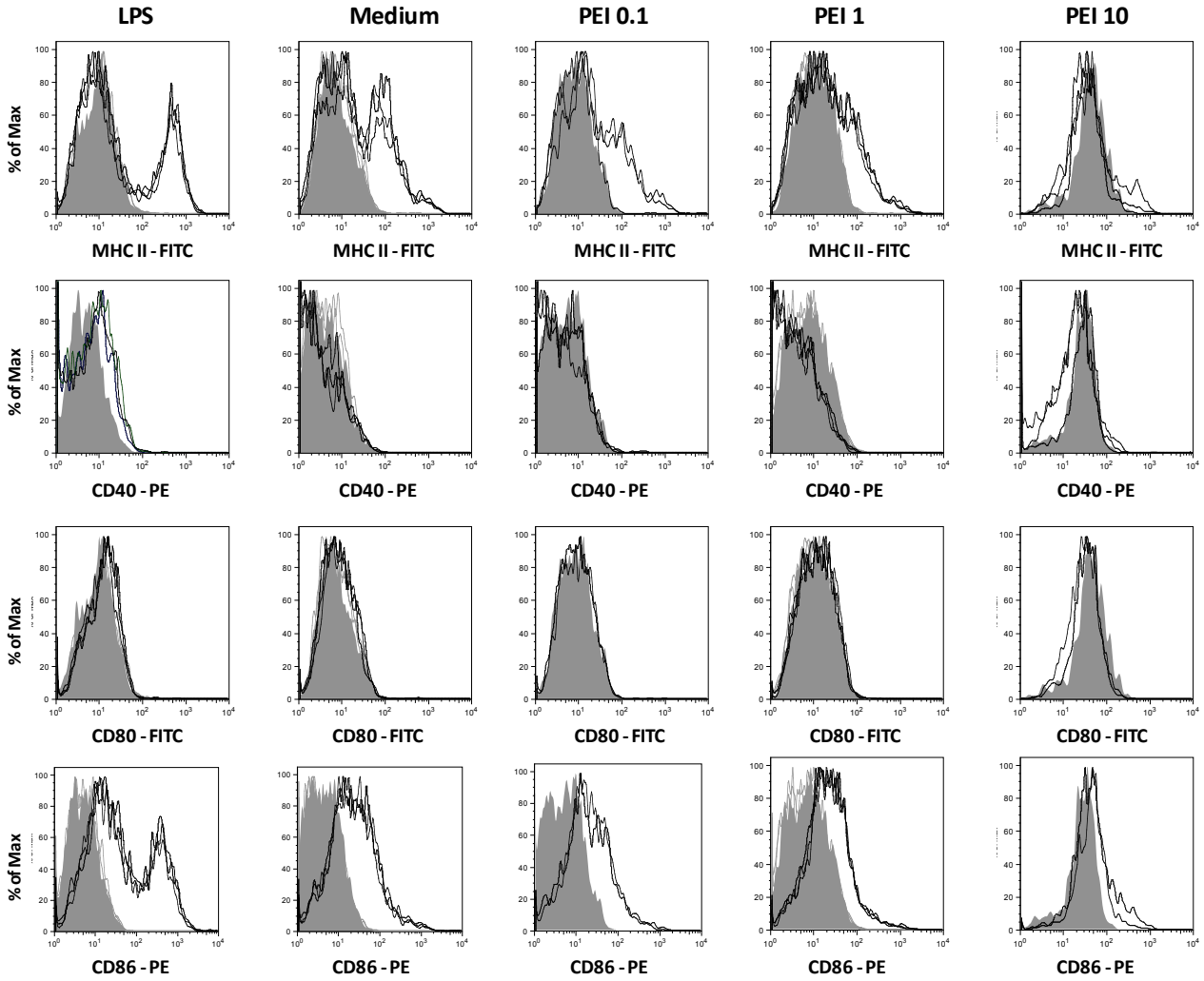


Figure 5.12. ***In vitro*** **PEI has no effect on BMDC expression of activation– and maturation-markers: histograms.** Naïve mice were sacrificed and bone marrow cells were isolated. Cells were cultured in the presence of GM-CSF for 7 days. At day 6, immature BMDC were stimulated with PEI (0.1 µg/mL, 1 µg/mL, 10 µg/mL), or LPS from E.Coli (c) or culture medium only (control). After 16 h cells were harvested and stained for surface markers CD40, CD80, CD86 and MHCII. Cells were analysed using flow cytometry. Black lines: incubating condition LPS, medium, or PEI (0.1, 1, or 10 µg / mL), grey shadow: isotype control.

5.3. Chapter discussion

I.p. immunisation is generally regarded as a systemic immunisation route because antigens are thought to be rapidly resorbed following i.p. administration (Kool et al., 2008b). The high immunogenicity compared to other immunisation routes seen with i.p. administered $gp140_{97CN54}$ is in line with this. Nevertheless, PEI significantly enhanced $gp140_{97CN54}$–specific systemic antibody responses, suggesting that PEI can further enhance immune responses when administered via this administration route (Figure 5.1).

PEI induced a substantial change in leukocyte type and number in the peritoneal cavity as also observed with other adjuvants, including MF59 and Alhydrogel (Calabro et al., 2011; Kool et al., 2008b). The response that was demonstrated following Alhydrogel exposure was in line with previously published results, with particular increases in eosinophils and neutrophils and an inflammation-characteristic drop in resident macrophages and B cells (Kool et al., 2008b). PEI induced a similar typical drop in macrophage and B cell numbers, which could have represented the departure of these immune cells towards the draining LNs, attachment to the peritoneal lining, cell death, and might be in line with their sentinel role (Figure 5.3) (Aderem and Underhill, 1999; Aschoff, 1924; Akazaki et a., 1964, Inman et al., 2011; Metchnikoff, 1892; Van Furth et al., 1972).

PEI-induced local cell number increases were substantially higher than observed with Alhydrogel and were characterised by similar neutrophil and eosinophil increases with an additional robust elevation of inflammatory monocyte levels (Figure 5.3). This characteristic enhancement of inflammatory monocytes seen in the presence of PEI suggests that upon PEI stimulation, these monocytes are recruited to local sites via CCR2 signalling and might here differentiate into macrophages and DCs, which will then be involved in antigen presentation and might mediate PEI adjuvant activity (Gordon and Taylor, 2005),.

The appearance of immune cells in the peritoneal cavity following exposure to PEI was tracked using flow cytometry. While strong immune activation is desired and is often mediated by inflammatory responses, extensive inflammation is undesirable in the context of adjuvant development, as only adjuvant candidates with good safety profiles and limited side effects will be able to progress to clinical development (O'Hagan and De Gregorio, 2009). To examine inflammation following exposure to PEI, the production of soluble factors in the peritoneal cavity, including inflammation-associated cytokines, was monitored (Appendix I). The observed secretion of cell-attractant proteins possibly underlies the observed changes in peritoneal leukocyte profile and in this way may contribute to PEI adjuvant activity. The induction of inflammation-associated soluble factors included anti-inflammatory IL-10, and pro-inflammatory TNF-**α** and IL-6, which have been shown to be involved in *in vivo* toxicity (Borish and Steinke, 2003; Kawakami et al., 2006; Tracey et al., 1987a; Tracey et al., 1987b). However, these cytokines were only transiently increased at a single timepoint and the PEI-induced effect had completely disappeared at 24 h post injection, suggesting that PEI had only transient pro-inflammatory effects, in line with a previous study on a linear form of PEI that demonstrated the absence of detectable levels of these inflammatory cytokines in serum following systemic injection of PEI (Bonnet et al., 2008). In contrast to the other inflammation-associated soluble factors, IL-1**β** levels were consistently elevated in the PEI group, suggesting that the inflammasome pathway could play a role in PEI adjuvant activity. In fact, when this was further tested PEI was observed to induce the release of IL-18, IL1-**β**, and caspase 1 by Bone Marrow-Derived Macrophages (BMM) following LPS stimulation, but no abrogation of PEI adjuvant activity was seen in NLRP3$^{-/-}$ mice (personal communication with Amin Moghaddam and William Hilson in the Sattentau lab). IL-1 **β** has also been implicated before in monocyte recruitment and trafficking (Kool et al., 2008).

This suggests that the inflammasome pathway is probably not involved centrally in PEI adjuvant activity, although it is possible that the inflammasome pathway is involved in PEI-induced IL-1**β** release, but is not

required for adjuvant activity, similar to what has recently been suggested in the literature for Alum (Franchi and Nunez, 2008; Kuroda et al., 2011; Williams et al., 2010).

In line with the demonstrated interaction between PEI and $gp140_{97CN54}$ in chapter three, PEI and $gp140_{97CN54}$ were shown here to form PEI-$gp140_{97CN54}$ complexes that were globular and ranged from 20 nm–200 nm in size (Figure 5.4). This complex formation and the increased size of the resulting immunisation particles, possibly contributes to PEI-mediated adjuvant activity (Bachmann and Jennings, 2010; de Veer and Meeusen, 2011; Fifis et al., 2004; Gamvrellis et al., 2004). In line with my hypothesis that PEI can enhance association of antigen with immune cells, possibly through interaction of PEI-antigen complexes with sulphated surface proteoglycans or other negatively charged structures on immune cells, PEI substantially enhanced OVA association with all leukocyte populations at 4 h and 24 h post-peritioneal administration, by contrast with Alhydrogel that only modestly enhanced antigen association (Figure 5.5 & 5.6). A particular increase was seen for OVA-association with monocytes and DCs that are phagocytic APCs and express high levels of suphated proteoglycans, which can trigger endocytosis (Gordon and Taylor, 2005; Steinman, 1991). Both PEI and OVA were found to substantially interact with local leukocytes, suggesting that PEI-OVA complexes associate with local immune cells following administration (Figure 4.7). It is expected, because of the shared glycoprotein features and low theoretical pI as well as the observed direct interaction with PEI for both $gp140_{97CN54}$ and OVA, that these glycoprotein antigens behave similarly in the current studies, suggesting that the results could be extrapolated to $gp140_{97CN54}$ immunisation , but further investigation is required to confirm this.

I demonstrated that PEI is taken up by peritoneally-derived macrophages *in vitro* and that PEI can enhance the uptake of $gp140_{97CN54}$ antigen by these APCs (5.8 & 5.9). PEI was demonstrated to be present in late endosomes and lysosomes, suggesting that endocytic and phagocytic pathways that can potentiate MHC class II antigen presentation and the induction of T_h2 responses, are involved in the uptake of PEI and possibly

PEI-antigen complexes (Figure 5.10) (Aderem and Underhill, 1999; Khalil et al., 2006). The finding that there was only partial co-localisation with these compartments suggests that internalised PEI was also present in other intracellular compartments or in the cell cytosol, which is in line with PEI's ability to cause endosomal/lysosomal rupture (Section 1.14). This might imply that part of the PEI or PEI-complexed antigen is processed via the MHC class I pathway, resulting in cross-presentation of the glycoprotein antigens. A very recent study has confirmed this suggestion and demonstrated that PEI-induced endosome-disruption was sufficient and effective for protein antigen (OVA) cross-presentation in BMDCs (Chen et al., 2011), which implies that in addition to the PEI-induced glycoprotein antigen-specific Ab responses that I have investigated, PEI has the capacity to induce CTL responses to glycoprotein antigens, possibly including HIV-1 $gp140_{97CN54}$

Despite the fact that most well studied adjuvants have been previously shown to induce BMDC maturation, PEI lacked such an effect in my *in vitro* BMDC assay (Figure 5.11) (De Becker et al., 2000; De Smedt et al., 1996; Fujii et al., 2003; Kool et al., 2008b; Shah et al., 2003). This suggests that PEI is not capable of direct DC activation through PRR on DCs. A study by Cubillos-Ruiz et al however suggested that PEI can bind TLR5, which is also expressed on DCs (Cubillos-Ruiz et al., 2009), but this finding could not be reproduced in our lab in a range of TLR-reporter cell line assays. Nor was any effect on adjuvant activity observed in MYD88- or TRIF-deficient mice (personal communication Frank Wegmann). These assays indicated that PEI immune enhancing effects are not dependent on TLR-mediated pathways. PEI is nevertheless likely to induce DC activation and maturation indirectly in an *in vivo* setting, for example via DAMP- or other PAMP-associated pathways. One possibility is that PEI induces release of intracellular DNA or other intracellular DAMPs through cytotoxic effects, which can trigger DAMP-mediated pathways resulting in immune induction, but this would need to be further investigated. Similar results have been found with

Alhydrogel that also does not act as a TLR-ligand and has been shown to induce *in vivo* DC maturation while lacking an *in vitro* effect on BMDC maturation (Kool et al., 2008b; Marichal et al., 2011).

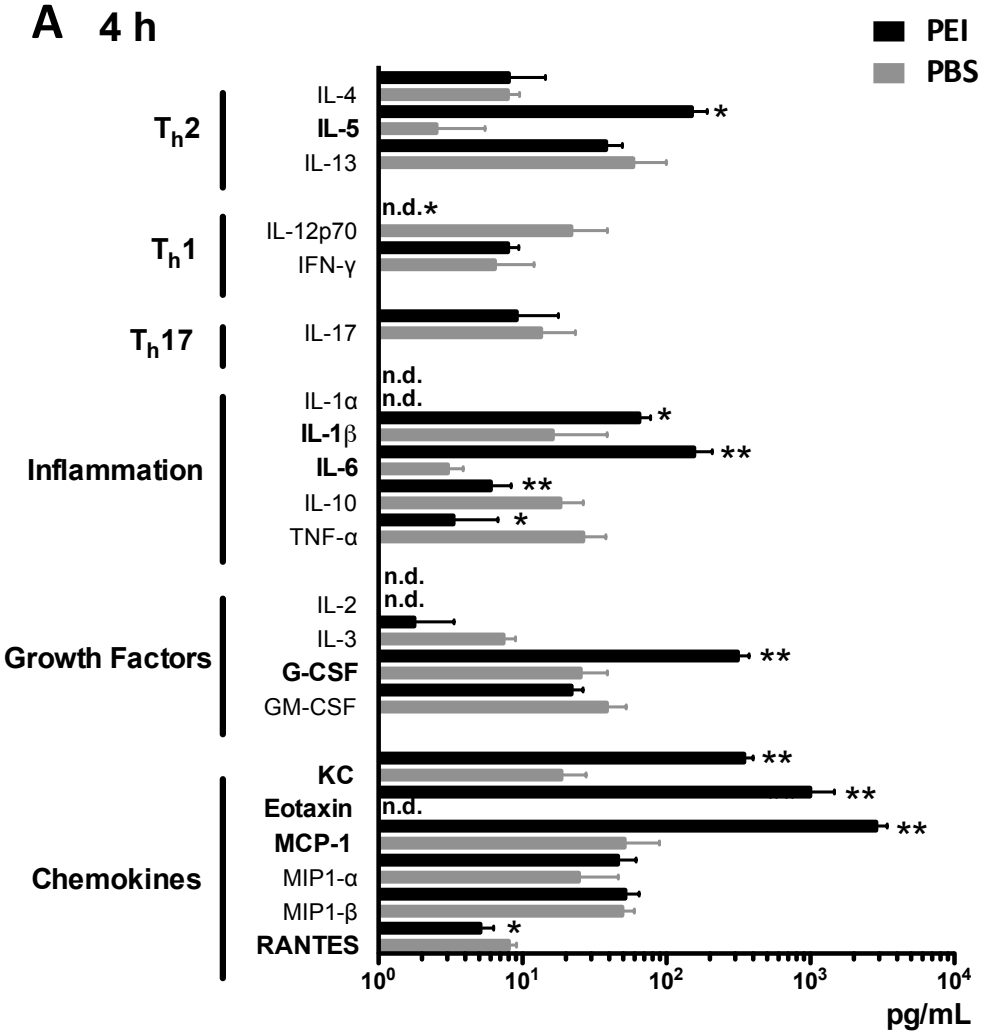
Taken together these findings suggest that PEI both directly stimulates the immune system and has effects on antigen and antigen delivery. PEI induces the recruitment of leukocytes, including robust numbers of inflammatory monocytes that have the capacity to develop into DCs and macrophages that are important for antigen presentation. PEI can enhance the interaction of glycoprotein antigen with these immune cells, possibly via induction of endocytosis and phagocytosis of PEI-antigen complexes that might potentiate antigen presentation. My findings suggest that factors contributing to PEI adjuvant activity likely include 1) complex formation with $gp140_{97CN54}$ antigen; 2) immune cell recruitment, including robust delayed recruitment of inflammatory monocytes and induction of cytokine release; 3) enhancement of antigen interaction with target immune cells; which potentially results in the observed potent adjuvant activity that is characterised by the induction of strong Ab responses in a mixed T_h1 and T_h2 context.

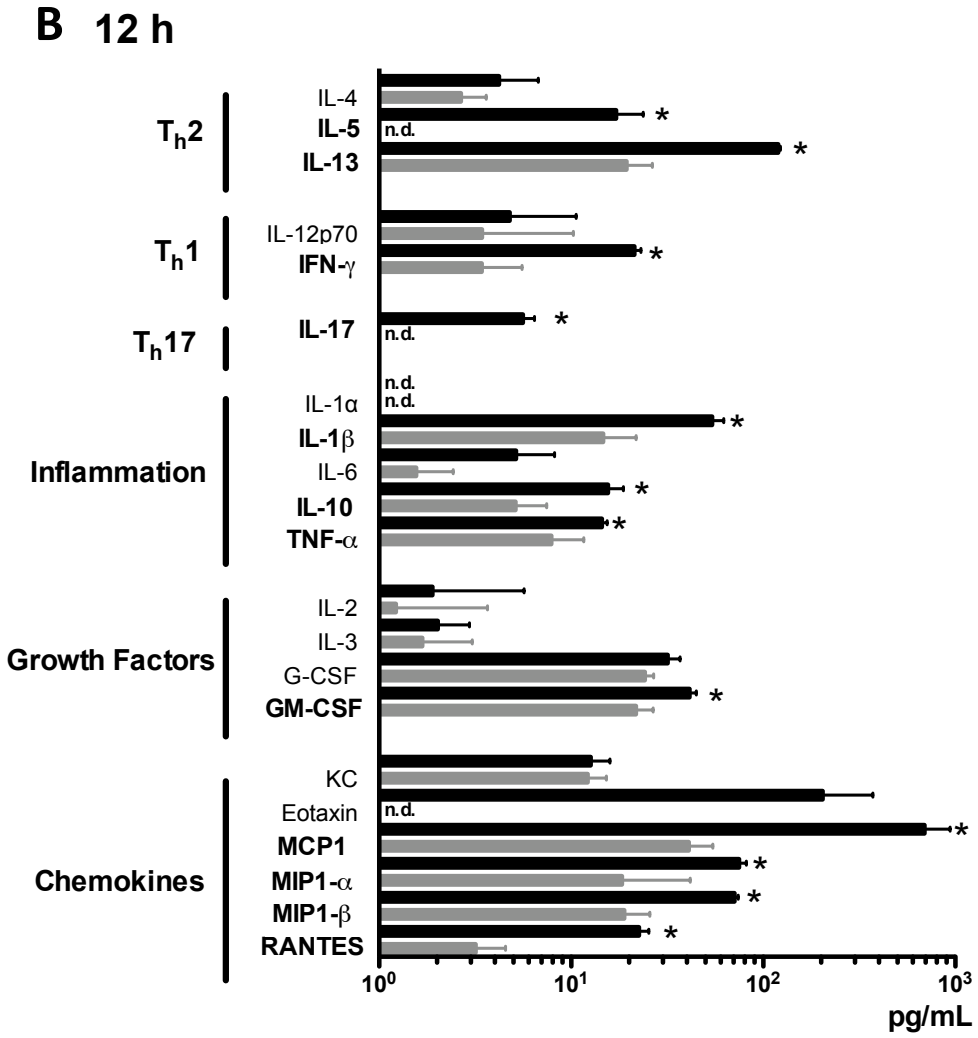
Key chapter findings

In this chapter, PEI was found to:

- **Induce an early innate increase in peritoneal leukocyte numbers, with particular elevation of neutrophils, eosinophils, and strong elevation of monocyte levels following i.p. PEI administration. Early innate increases in cytokines, including T_h1 and T_h2 cytokines, and chemokines were also observed.**
- **Form PEI-gp140$_{97CN54}$ particles in TEM**
- **Enhance OVA association with all studied peritoneal leukocyte populations, including APCs, particularly monocytes and DCs, and to associate itself with all studied peritoneal leukocyte populations following i.p. administration**
- **Be effectively taken up by peritoneally-derived macrophages and be partially targeted to endosomal or phagolysosomal compartments**
- **Enhance uptake of gp140$_{97CN54}$ by peritoneally-derived macrophages**
- **Lack a direct effect on BMDC expression of activation- and maturation- surface markers *in vitro***

5.4. Chapter appendix




B 12 h
Th2
Th1
Th17
Inflammation
Growth Factors
Chemokines
IL-4
IL-5
IL-13
IL-12p70
IFN-γ
IL-17
IL-1α
IL-1β
IL-6
IL-10
TNF-α
IL-2
IL-3
G-CSF
GM-CSF
KC
Eotaxin
MCP1
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RANTES
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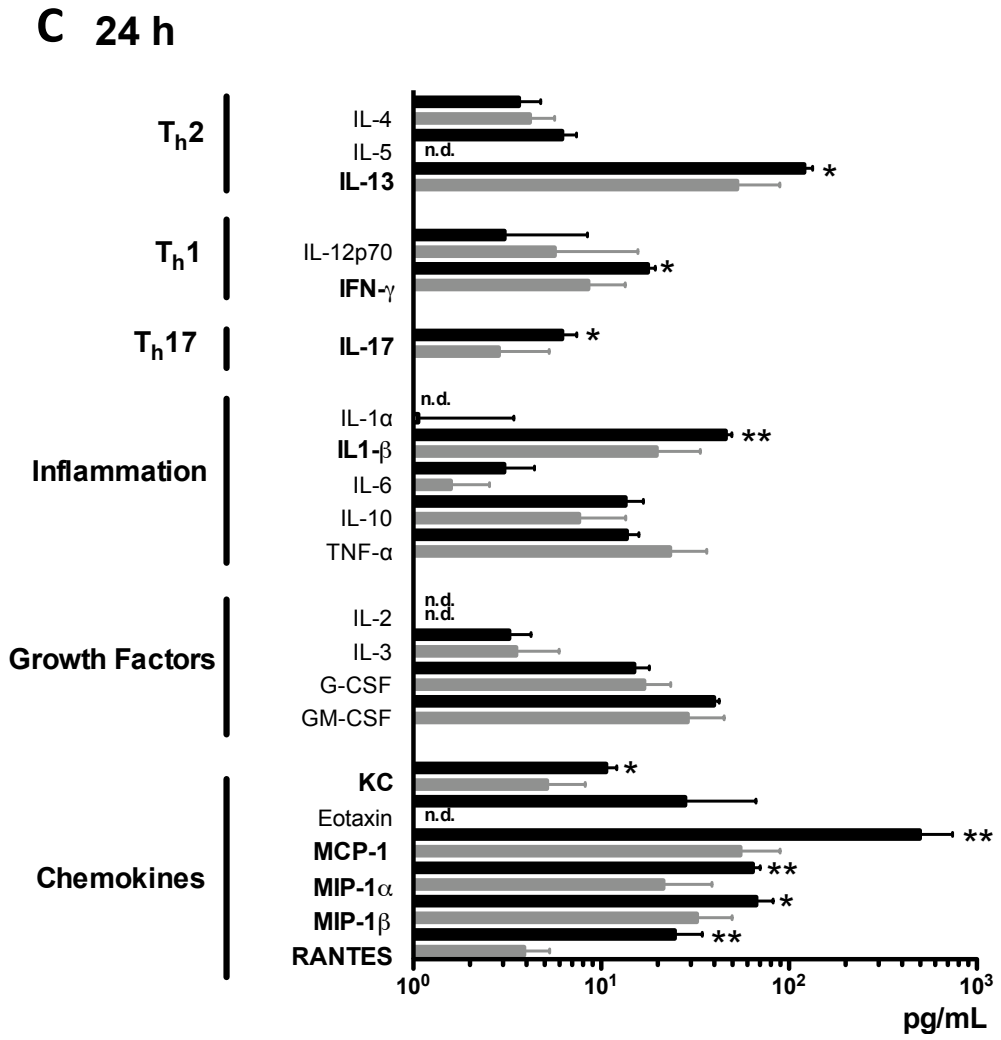

Figure I. Early innate cytokine induction in the peritoneum 4, 12 or 24 h after i.p. PEI administration. 12 week female BALB/c mice (*n*=5) were injected i.p. with 80 µg PEI, PBS only, or 1 mg Alhydrogel in a total volume of 500 µl. At 4 (**A**), 12 (**B**), or 24 h (**C**) mice were sacrificed and peritoneal lavages collected for cytokine profiling using Luminex™ bead-based analysis. Data are represented as means +/- SD of biological replicates (mice). The increased soluble factors in the presence of PEI are depicted in bold. The average detection threshold was approx. 20 pg/mL (exceptions: RANTES 2.78 pg/mL, Eotaxin 257 pg/mL). Mann-Whitney test (**P*<0.05, ***P*<0.01).

CHAPTER 6

General Discussion

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6.1. Summary of main findings

Despite the development of multiple prevention and therapeutic strategies to fight HIV-1 infection over the past years, an effective prophylactic vaccine will be needed to halt the pandemic. The development of such vaccine has encountered many challenges, one of which is the lack of an (mucosal) adjuvant that elicits the required type of immune response. The work presented in this thesis was intended to explore a new adjuvant strategy that might contribute to the design of an effective HIV-1 Env-based immunisation method. The main findings of my research have been summarised in the previous chapters, and so here I will only very briefly reiterate the major findings.

- The work presented herein is the first demonstration of PEI as a mucosal adjuvant as well as the first demonstration of PEI as an adjuvant in *in vivo* (glyco-)protein immunisation
- As such, PEIs comprise a new group of glycoprotein adjuvants that can enhance the immunogenicity of HIV-1 Env in mice and rabbits via systemic and mucosal administration routes, and the adjuvant activity is a shared attribute of PEI of different sizes, structures and biodegradability.
- The adaptive immune response elicited by PEI adjuvant included robust IgG, and after mucosal administration, IgA. The T cell response was mixed T_h1 + T_h2 type as determined by the production of balanced IgG1/IgG2 responses and release of both T_h1 and T_h2 cytokines.
- PEI interacted directly with antigen, modestly altering its antigenicity, and resulting in formation of particles that associated with and were taken up by APCs *in vitro* and *in vivo*. PEI also induced the recruitment of leukocytes to the region of administration.

6.2. Implications for HIV-1 vaccine development

6.2.1. General concepts

Recent developments in HIV-1 vaccine research have provided the insight that sterile protection against HIV-1 infection requires strong cellular and humoral responses, in a mixed T_h1 + T_h2 context, particularly at the mucosa. As the sole target of nAbs, HIV-1 Env is the principal immunogen for development of a HIV-1 subunit vaccine, but adequate enhancement of Env glycoprotein immunogenicity has proven a daunting challenge, particularly in the context of mucosal immunisation and the requirement for strong mucosal responses. A safe and effective adjuvant will be needed to meet this challenge. Since heterologous prime-boost regimens have shown particular promise recently, in particular the combination of vector-based and subunit vaccination to employ both arms of the immune system (Girard et al., 2011), the ideal adjuvant would be formulated in the prime and/or boost immunisation and should allow and encourage both T cell and B cell responses. Here, PEIs were shown to represent a new class of glycoprotein adjuvants that are particularly potent inducers of mucosal immune responses and create a mixed T_h1 + T_h2 setting for immune activation. Taken together, my observations that PEI induces broad, robust antibody responses to glycoprotein and that PEI has robust mucosal activity in a mixed T_h1 + T_h2 immune context, generally superior to CTB, combined with successful use of PEI as delivery agent in DNA vaccines, as well as the very recent observation that PEI is capable of glycoprotein antigen cross-presentation *in vitro*, which can potentiate NK-cell and glycoprotein-specific and CTL responses, gives this new class of glycoprotein adjuvants a very unique all-round functionality that meets the general requirements for (HIV-1) subunit vaccination.

Few of the existing and newer adjuvants have so far proven to present such a broad range of desirable characteristics. Some adjuvants, such as MF59, ISCOM, and ADVAX allow immune induction in a mixed T_h1 + T_h2 context and can act mucosally, thus meeting some of the above-mentioned requirements, but the

first two have reactogenicity problems and none of the studied formulations has yet allowed the induction of sterile protection against HIV-1 infection, therefore suggesting that optimisation of existing adjuvanted formulations and design of new adjuvants is needed (Coffman et al., 2010; Girard et al., 2011).

6.2.2. PEI cytotoxicity

In the development of new adjuvants safety is always a major concern, since adjuvant activity is often achieved through local tissue damage or local inflammation that might also result in side effects and toxicity. As such, most of the newer adjuvants are more reactogenic than Alum (Petrovsky et al., 2007). PEI has repeatedly been shown to have some cytotoxic behaviour (Moghimi et al., 2005), indications of which were also found in my research. This cytotoxic behaviour might partially underlie its adjuvant activity, through stimulation of DAMP- and PAMP-sensing pathways via the release of intracellular DAMPs, as was recently demonstrated for Alum adjuvants (Marichal et al., 2011). The combination of PEI's cytoxicity, endosomal/lysosomal rupture effects, and its ability to target DNA into cells, suggest that it is possible that PEI might mediate adjuvant activity similar to Alum, by induction of cell death and resulting release of host cell DNA that can act as a PAMP to stimulate innate immune responses. This would imply that PEI similar to Alum might induce local tissue damage. But, in the case of Alum this possible local necrotic effect has not posed a problem on the adjuvant's safety profile, as it has been successfully incorporated in a wide range of safe, worldwide licensed human vaccines. Furthermore, PEI was demonstrated to induce only limited release of pro-inflammatory cytokines TNF-**α** and IL-6 in line with observed safety of PEI in humans in Phase I and II trials on PEI-based DNA vaccines and cancer treatment (Lisziewicz et al., 2005a; Lisziewicz et al., 2005b; Ohana et al., 2004). Finally, in the context of DNA transfection, a reverse correlation was found between PEI cytotoxicity and the amount of DNA complexed with it (Boeckle et al., 2004; Fahrmeir et al., 2007; Yue et

al., 2011), similarly it is possible that the binding of antigen to PEI and the formation of complexes has a reducing effect on PEI cytotoxicity that is observed with PEI alone.

6.2.3. PEI mode of action

Many questions remain regarding the precise mode of action of PEI. It was demonstrated in my research that PEI has both a) direct effects on the immune system, including immune cell recruitment and cytokine release, although a direct effect on DC activation and maturation was not found, and b) effects on glycoprotein antigen, such as enhanced targeting to immune cells and the formation of nanoparticles. Either of these of the combination of the two might underlie the observed glycoprotein adjuvant activity and the induction of T_h1 + T_h2 antibody and cellular responses. It is unclear whether PEIs capacity to induce strong glycoprotein-specific antibody responses is related to its uniquely strong ability to deliver DNA into cells or the properties that are thought to underlie this capacity: strong cationic charge, buffering capacity, and membrane-disrupting effects. Interestingly, previously, PEI's ability to cross-present glycoprotein antigens was found to result from its endosome-rupture effect (Chen et al., 2011). Older studies have shown that the uptake of BSA by APCs could be enhanced by the replacement of its side chain carboxyl groups for polycationic aminoethylamide groups, suggesting that the cationic charge and presence of amide groups in itself might indeed play a role (Apple et al., 1988; Muckerheide et al., 1987). A recent study has shown the role of PEI branch length on transfection efficiency, which was explained by the increased capacity of longer PEI branches to disrupt and pass cellular membranes and therefore implying that the well known PEI-induced 'proton sponge effect' only partially underlies endosome-rupture and PEI's robust transfection efficiency (Yue et al., 2011; Akinc et al., 2005). Despite these findings that transfection-related mechanisms were suggested to underlie PEI's ability to cross-present protein antigens and is likely to explain the enhanced interaction of antigen with leukocytes and other immune enhancing effects seen in the presence of PEI, my own findings

that similar adjuvant activity could be observed with various structural as well as biodegradable PEI forms that are known to quickly degrade into short branched PEI forms following administration, suggest that PEI adjuvant activity is not directly related to transfection efficiency, and that the potential membrane-disrupting role of long PEI branches can only partially explain PEI adjuvant activity. Furtheremore, despite recent publications on the activation of TLR4 and TLR5 by PEI, the observed lack of direct PEI-mediated activation of BMDC *in vitro* in my studies, and other findings in the Sattentau lab in TLR-reporter cell lines, MYD88- and TRIF-deficient mice, suggest that PEI does not enhance immune responses via activation of TLR-pathways. As such, the mechanism of PEI adjuvant activity remains undefined at the molecular level, and it is unclear by what means PEI triggers the observed leukocyte recruitment. PEI was shown by other members in our lab to induce the release of IL-1β, IL-18 and caspase-1 by BMMs, whereas PEI-induced antibody responses appeared not dependent on activation of the inflammasome pathway in NLRP3-deficient mice, which when taken together, further suggests that PEI adjuvant mechanism might have a common ground with the adjuvant mechanism of Alum (personal communication Amin Moghaddam and William Hilson). Furthermore, a recent study demonstrated that PEI activates the complement system in a dose-dependent manner, but it is unclear to what extent complement activation is needed for its adjuvant activity (Merkel et al., 2011).

6.2.4. PEI in a mucosal setting

The work presented herein is the first demonstration of PEI as a mucosal adjuvant for protein immunisation. The complexities of mucosal immune regulation and the unique organisation of mucosal inductive sites require adjuvants to meet specific criteria to maintain activity via this route (Cripps et al., 2001; Neutra and Kozlowski, 2006; Ryan et al., 2001). The strong mucosal adjuvant activity of PEI via the i.n. route suggests that PEI allows successful penetration of antigen through mucosal surfaces and may effectively target antigen

to M cells (Neutra and Kozlowski, 2006; Ryan et al., 2001). It is possible that the formation of PEI-gp140$_{97CN54}$ particles facilitates mucosal immunisation, as particulate vaccines are generally more potent via the mucosal route (Ryan et al., 2001). Also, it is likely that mucosal adjuvant activity involves the protection of antigen from degradation by mucosal proteases, analogous to previously shown PEI-induced protection of RNA/DNA from degradation (Grzelinski et al., 2006; Urban-Klein et al., 2005).

Adjuvant name:	**Main constituents:**	**Immune bias*:**	**(selected examples of) actions found in experiments**	**Note:**	**Reactogenicity**:**	**References:**
Alum	Aluminium salts	T_h2	• Induced cell death resulting in release of host cell DNA that acts as DAMP <u>Other potential factors:</u> • NLRP3 inflammasome activation? (UA release, ATP release, and lysosomal damage, ROS, release PGE2 • Immune cell recruitment (eg. Monocytes, neutrophils, eosinophils) • Cytokine and chemokine release • Particulate vaccine • Depot?	Generallly not effective in HIV-1 immunisation	++	(Azizi et al., 2010; Dostert et al., 2008; Hornung et al., 2008; Kool et al., 2008a; Kool et al., 2008b; Kool et al., 2011; Kuroda et al., 2011; Lambrecht et al., 2009; Li et al., 2007; Marichal et al., 2011; Mbow et al., 2011; Petrovsky, 2007; Ryan et al., 2001; Serre et al., 2008)
MF59 and ASO3	Squalene-in-water emulsions	T_h1 + T_h2	<u>Undefined, probably:</u> • Depot formation • Immune cell recruitment (eg.DCs,eosinophils macrophages) • Apoptosis induction • Cytokine and chemokine release • Particulate vaccine • Tissue inflammation	**Mucosal** adjuvant activity Used with ΔV2 **HIV-1** $gp140_{SF162}$ trimer in NHP model, showed protection but not against heterologous virus challenge Considered too reactogenic for licensing in US	++++ (not licensed in the US, because considered too unsafe for use in human)	(Barnett et al., 2010; Carter and Reed, 2010; Dupuis et al., 2001; Dupuis et al., 1998; Frey et al., 2003; Morel et al., 2011; Ott et al., 1995; Petrovsky, 2007; Seubert et al., 2008)
AS04 **(MPL and formulations)**	MPL-A and aluminium salt	T_h1	• TLR4 ligand		++++	(Carter and Reed, 2010; Moore et al., 1999; Ryan et al., 2001; Salkowski et al., 1997)

liposomes	Combination of lipids and cholesterol	T_h1		Delivery vehicle: Macrophage membrane fusion and delivery of antigens into macrophages	Ability to **cross-present** protein antigens Potent activity requires co-formulation with other adjuvant, eg. MPL	++	(Ichikawa et al., 2002) (Anders et al., 2004; Baldridge et al., 2000; Prinz et al., 2006) (Hartmann et al., 2000; Heath et al., 1976; Owais and Gupta, 2000; Petrovsky, 2007; Tyrrell et al., 1976; Zheng et al., 1999)
CFA/IFA	Mineral or paraffin oil and surfactant peptidoglycan	T_h1 T_h17	+	Undefined, possibly: Inflammasome NLR, TLR ?	Gold standard experimental adjuvant Considered too reactogenic for use in man	++++	(Coffman et al., 2010; Gavin et al., 2006; Mayer-Barber et al., 2010; Moreira et al., 2008; Su et al., 2005; Vogel and Powell, 1995)
CT, LT, and Pertussis Toxin	Cholera toxin from *Vibrio Cholera*, heat labile toxin from *E.Coli* and Pertussis toxin from *Bordetella Pertussis*	T_h1 T_h2	+	Undefined, partially: • ADP-robosyltransferace activity and intracellular cAMP by subunit A, binding of PRR GM1 ganglioside by subunit B • DC maturation *in vitro*	Gold standard **mucosal** adjuvant Considered too reactogenic for use in man	++++	(Braun et al., 1999; Cong et al., 2001; Elson, 2005; Gagliardi et al., 2000; Imaoka et al., 1998; Lee et al., 2009; Lycke and Holmgren, 1986; Mutsch et al., 2004; Sack et al., 1980; van Ginkel et al., 2000)

ISCOMs and ISCOMATRIX	saponins	T_h1 + T_h2	Undefined, possibly: • Ag delivery: interaction with cholesterol en pore formation in cell membrane • Recruitment neutrophils, mast cells, macrophages, DCs and lymphocytes	Can **cross-present** protein antigens Works as **mucosal** adjuvant in mice Induced nAb responses to **HIV-1** gp120 in rabbits	++++	(Abusugra and Morein, 1999; Browning et al., 1992; Claassen and Osterhaus, 1992; Coffman et al., 2010; Davis et al., 2004; Hu et al., 1998; Maraskovsky et al., 2009; Morein et al., 1984; Ronnberg et al., 1995; Schnurr et al., 2009)
Flagellin	Flagellin from *S.typhimurium*	T_h1 + T_h2	• TLR5 ligand	**Mucosal** adjuvant activity in mice	+++	(Bates et al., 2008; Lee et al., 2006; Petrovsky, 2007)
CpG oligodeoxynucleotides	CpG motifs	T_h1	• TLR9 ligand		+++	(Ichikawa et al., 2002; Petrovsky, 2007; Prinz et al., 2006; Sparwasser et al., 1999)
Poly-IC	dsRNA derivative	T_h1	• TLR3 ligand and MDA5 ligand		+++	(Petrovsky, 2007)
ADVAX	Inulin	T_h1 + T_h2	Undefined	ADVAX-M (consisting of **α**-galactosyl ceramide) can work **mucosally**	Lack of reactogenicity	(Cristillo et al., 2011; Petrovsky, 2007)

PEI	**Polythyleneimine**	**T_h1 / T_h2**	**<u>Undefined, possibly:</u>** • **Recruitment early innate leukocytes in peritoneum: neutrophils, DCs and particularly monocytes** • **Early innate cytokine release** • **No direct stimulation DCs** **<u>Ag delivery:</u>** • **particle formation** • **enhanced targeting to APCs** • **receptor-mediated endocytosis?** • **Endosome/lysosome rupture ?**	**Robust mucosal adjuvant activity & can cross-present protein antigens**	**?**	**N/A** (Chen et al., 2011)

Table 6.1. Overview of licensed and widespread experimentally used adjuvants. * Experimental evidence of immune bias. ** Based on the literature, particularly (Petrovsky et al., 2007). Blue: licensed adjuvants. Red: PEI. Adapted from: (Coffman et al., 2010; Petrovsky, 2007; Ryan et al., 2001).

6.3. Future perspectives

6.3.1. Functional outcomes of Env adjuvantation by PEI

Having demonstrated the robust glycoprotein adjuvant activity of PEIs *in vivo*, the logical next step would be to investigate whether the PEI-induced immune responses can help in providing protection against HIV-1 infection. *In vitro* neutralisation assays comprising viruses pseudotyped with divergent heterologous glycoproteins will be used to further explore the breadth and efficacy of the PEI-induced antibody responses. Initial results of a small study that involved serum of rabbits immunised with $gp140_{97CN54}$ and PEI indicate that PEI-induced antibodies are able to neutralise heterologous pseudoviruses, but only of the neutralisation-sensitive Tier 1 group (data not shown). Further studies should provide more insight into the neutralisation capacity of PEI-induced antibodies. The role of non-neutralising antibody functions, such as ADCC, will be further investigated in intracellular cytokine staining ADCC (ICS-ADCC) assay, antibody-dependent cell mediated virus inhibition (ADCVI) or rapid and fluorometric antibody-dependent cellular cytotoxicity (RFADCC) assays (Forthal and Landucci, 1998; Gomez-Roman et al., 2006; Stratov et al., 2008). Since the NHP models are the closest available predictors of HIV-1 protection in man, a complete and rigorous investigation of PEI-mediated adaptive immune responses would involve testing in such challenge model. Also, my initial studies suggested the presence of T_h1 and T_h2 responses following PEI adjuvanted $gp140_{97CN54}$ immunisation, but PEI-induced T_h polarisation could be further investigated and more accurately determined as it is possible that other T_h components, such as T_h9 and T_{FH} are also involved.

6.3.2. Other potential vaccine uses for PEI

Development of effective mucosal immunisation strategies is not only needed for HIV-1 virus, but for a multitude of mucosally-transmitted pathogens including Herpes Simplex Virus 2 (HSV-2), *Chlamydia trachomatis,* Gonorrhoea, and constantly new arising strains of Influenza A, (WHO, www.who.int). It would therefore be valuable to study PEI adjuvant activity in the context of such distinct antigens in addition to a wider variation of HIV-1 specific antigens, as some of the results presented herein might have been $gp140_{97CN54}$ -specific. In fact, we have performed initial studies on PEI in combination with Influenza A haemagglutinin glycoprotein (HA PR8) and Herpes Simplex Virus 2 (HSV-2) glycoprotein D in the respective mice challenge models (data not shown). In the Influenza A model, a single immunisation of antigen in PEI resulted in protection against disease measured as body weight loss, and a 100% survival from >30% weight loss leading to sacrifice. Other adjuvants, including reference adjuvants Cholera Toxin B subunit (CTB) and CpG ODN (a TLR 9 ligand) failed to induce a similar level of protection. In the HSV-2 model, the presence of PEI adjuvant decreased the number of necessary humane endpoint sacrifices from 100% of mice in the HSV-2 glycoprotein D alone group to 20% in the PEI-adjuvanted group. These results support PEI's capacity to act as a general-purpose adjuvant. In addition to this, it would be informative to compare PEI's adjuvant activity to a broader set of licensed and experimental adjuvants. Furthermore, it would be interesting to include more biodegradable forms of PEI in the current analysis and to further investigate their individual potential and toxicity as adjuvants, particularly when used via the mucosal route. Finally, a recent paper demonstrated antiviral activity for PEI *in vitro*, suggesting that the formulation of PEI in microbicide agents could also be further investigated (Spoden et al., 2012).

6.3.3. Mechanism of action

In terms of its mode of action, further studies should aim at elucidating PEI-induced mucosal adjuvant activity by looking at leukocyte recruitment and cytokine effects at the mucosa, including the NALT as well as PEI-induced trafficking of antigen-associated leukocytes to the draining LNs following i.n. immunisation. Also, possible PEI-induced permeabilisation of mucosal surfaces would be worth an exploration especially in light of PEI's general membrane damaging potential. More in general, the possible role of cytotoxicity and release of DNA from damaged cells will be assessed through the use of DNAse treatment to potentially block adjuvant effects during PEI-adjuvanted immunisation. Further investigation of the contribution of PEI's exceptional cationic charge will be done through immunisation studies with charge-neutralised PEI. Finally, since confirmation of PEI's capacity to cross-present glycoprotein antigens *in vivo* would enable the induction of HIV-1 $gp140_{97CN54-}$ specific CTL responses that are likely to be required in addition to strong Ab responses for effective protection against HIV-1 infection, it would be interesting to investigate cross-presentation and the induction of CTL responses in the context of HIV-1 gp140.

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Begin at the beginning and go on till you come to the end; then stop.

Lewis Carrol, Alice in Wonderland, 1832-1898.