

Strategies for enhancing the therapeutic targeting of phosphatidylserine in oncological disorders and viral infections

Iona Shu-Yi Easthope

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Corpus Christi College
Department of Biochemistry
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Abstract

Phosphatidylserine (PS) is exposed on a variety of tumour cell types, virus-infected cells and virions. Bavituximab, a PS-targeting antibody with a human immunoglobulin G (IgG) constant region, possesses anti-viral and anti-cancer activity that is mainly mediated by antibody-dependent cell-mediated cytotoxicity (ADCC). ADCC of therapeutic antibodies is naturally limited *in vivo* by the prevailing serum concentrations of IgG which compete for Fc γ receptor (Fc γ R) binding. The binding of bavituximab to Fc γ RIIIA is shown here to be reduced in the presence of either whole serum or polyclonal IgG. However, the use of IdeS, a highly specific IgG-cleaving enzyme derived from *Streptococcus pyogenes*, presents a potential solution to this problem. IdeS cleaves in the hinge region of IgG between amino acid residues Gly236 and Gly237 but is thought to possess an additional binding interface with its IgG substrate. Firstly, the location of the IdeS-IgG binding interfaces was investigated using truncated IgG1 variants and systematic amino acid mutagenesis. IdeS bound an IgG1 fragment crystallisable (Fc) domain lacking the hinge region, thereby confirming the presence of a secondary binding site. Secondly, residues involved in the IdeS-IgG interaction were mutated to create an IdeS-resistant form of bavituximab. Pre-treatment of competing IgG with IdeS fully restored binding of IdeS-resistant bavituximab to Fc γ RIIIA, while pre-treatment of serum produced a partial improvement in binding. Lastly, bavituximab, the PS receptor TIM-1, and the related protein TIM-4 were tested for binding to a model alphavirus, Semliki Forest Virus. Only TIM-1, the smallest of these constructs, was found to bind the virus; this implies that receptor size may be an important consideration in the design of PS-targeting anti-viral agents. Overall, these data suggest possible methods for improving the therapeutic targeting of PS, either by optimisation of antigen binding regions or by enhancement of Fc γ R binding.

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List of Abbreviations

ADCC	antibody-dependent cell-mediated cytotoxicity
β 2-GP1	β 2-glycoprotein 1
BCR	B cell receptor
BSA	bovine serum albumin
CD	cluster of differentiation molecule
CDC	complement-dependent cytotoxicity
CHKV	Chikungunya virus
CMV	cytomegalovirus
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
EEV	equine encephalosis virus
ELISA	enzyme-linked immunosorbance assay
Ep-CAM	epithelial cell adhesion molecule
F(ab)	fragment, antigen-binding
FBS	foetal bovine serum
Fc	fragment, crystallisable
Fc γ R	Fc γ receptor
FcRn	neonatal Fc receptor
GAS	Group A <i>Streptococcus</i>
GP	glycoprotein

GPI	glycosylphosphatidylinositol
h-	human
HAH	human anti-hinge
HDX	hydrogen-deuterium exchange
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	human immunodeficiency virus
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
HSV	herpes simplex virus
IdeS	Immunoglobulin-degrading enzyme of <i>Streptococcus pyogenes</i>
Ig	immunoglobulin
iIgG	intact immunoglobulin G
IL	interleukin
IMGT	The International Immunogenetics Information System
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibition motif
K_D	equilibrium dissociation constant
LB	lysogeny broth
m-	murine
mAb	monoclonal antibody
MARV	Marburg virus
mAU	milli absorbance units
MDM	monocyte-derived macrophage
MAC	membrane attack complex
MALS	multi-angle light scattering
MLV	Moloney murine leukemia virus
MW	molecular weight
MWCO	molecular weight cut-off

NEB	New England BioLabs
PBS	phosphate-buffered saline
PBST	PBS, 0.05% Tween-20
PC	phosphatidylcholine
PCR	polymerase chain reaction
PE	phosphatidylethanolamine
PEI	polyethyleneimine
PGN	Peregrine Pharmaceuticals
PI	phosphatidylinositol
PMN	polymorphonuclear leukocytes
PNGase F	peptide N-glycosidase F
PS	phosphatidylserine
P/S	penicillin-streptomycin
PSR	phosphatidylserine receptor
RF	relative fluorescence
ROS	reactive oxygen species
RRV	Ross River virus
SAXS	small-angle X-ray scattering
scIgG	singly-cleaved immunoglobulin G
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SFV	Semliki Forest virus
Sib35	Streptococcal immunoglobulin-binding protein 35
SINV	Sindbis virus
SpeB	streptococcal pyrogenic exotoxin B
SPR	surface plasmon resonance
T20N100	20 mM Tris-HCl, 100 mM NaCl, pH 7.5
T20N100C5	20mM Tris-HCL pH 7.2, 100 mM NaCl, 5 mM CaCl ₂
TAM	Tyro/Axl/Mer

TB	Terrific Broth
TCR	T cell receptor
TIM	T cell, immunoglobulin and mucin
TNF	tumour necrosis factor
UPLC	ultra performance liquid chromatography
VSV	vesicular stomatitis virus

Abstract

Phosphatidylserine (PS) is exposed on a variety of tumour cell types, virus-infected cells and virions. Bavituximab, a PS-targeting antibody with a human immunoglobulin G (IgG) constant region, possesses anti-viral and anti-cancer activity that is mainly mediated by antibody-dependent cell-mediated cytotoxicity (ADCC). ADCC of therapeutic antibodies is naturally limited *in vivo* by the prevailing serum concentrations of IgG which compete for Fc γ receptor (Fc γ R) binding. The binding of bavituximab to Fc γ RIIIA is shown here to be reduced in the presence of either whole serum or polyclonal IgG. However, the use of IdeS, a highly specific IgG-cleaving enzyme derived from *Streptococcus pyogenes*, presents a potential solution to this problem. IdeS cleaves in the hinge region of IgG between amino acid residues Gly236 and Gly237 but is thought to possess an additional binding interface with its IgG substrate. Firstly, the location of the IdeS–IgG binding interfaces was investigated using truncated IgG1 variants and systematic amino acid mutagenesis. IdeS bound an IgG1 fragment crystallisable (Fc) domain lacking the hinge region, thereby confirming the presence of a secondary binding site. Secondly, residues involved in the IdeS–IgG interaction were mutated to create an IdeS-resistant form of bavituximab. Pre-treatment of competing IgG with IdeS fully restored binding of IdeS-resistant bavituximab to Fc γ RIIIA, while pre-treatment of serum produced a partial improvement in binding. Lastly, bavituximab, the PS receptor TIM-1, and the related protein TIM-4 were tested for binding to a model alphavirus, Semliki Forest Virus. Only TIM-1, the smallest of these constructs, was found to bind the virus; this implies that receptor size may be an important consideration in the design of PS-targeting anti-viral agents. Overall, these data

suggest possible methods for improving the therapeutic targeting of PS, either by optimisation of antigen binding regions or by enhancement of Fc γ R binding.

Chapter 1

Introduction

1.1 Antibodies as therapeutic agents

Therapeutic antibodies were first developed in the 1980s and are now one of the fastest growing drug classes [151]. Although serum therapy had been used since the 1890s to treat infectious diseases, the use of antibodies with a defined specificity only became viable with the development of hybridoma technology in 1975 [35]. This breakthrough enabled the production of large quantities of homogeneous, monoclonal antibodies (mAbs). Currently, 49 mAbs are approved or under review for use in the European Union or United States [150] and there are over 200 more at various developmental stages [203]. Almost half have been licensed for use against cancer; the other major indications targeted are auto-immune disorders and transplant rejection [88]. Only two have so far been approved for infectious diseases: palivizumab (MEDIV-493/SYNAGIS®) for respiratory syncytial virus (RSV) infection and axibacumab (ABthrax®) which targets *Bacillus anthracis*.

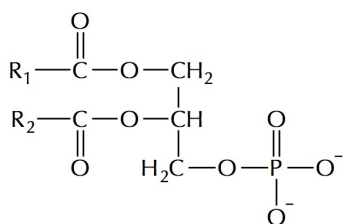
The advantages that have contributed to the success of mAbs include high specificity, relatively low toxicity, long half-life (due to engagement of the antibody receptor FcRn, which promotes antibody recycling) and ability to recruit immune effector functions. These effector functions

are expanded upon in Sections 1.8 and 1.9. Despite the potential of antibodies to bind to almost any antigen, the number of current targets is low compared to the number of mAbs under investigation. Of the 206 anti-cancer mAbs that entered clinical study between 1980 and 2005, 91 mAbs were specific for only 10 antigens, of which the top three were epithelial cell adhesion molecule (Ep-CAM), mucin-1, and epidermal growth factor receptor (EGFR) [151]. There is a continuing challenge to find novel, viable targets for anti-cancer and anti-pathogen mAbs, particularly in the case of cancer where there is a requirement to differentiate between self and non-self antigens. The ideal target is specific to the pathogen or the tumour cell, abundantly expressed, and not secreted (such there is no possibility of a secreted antigen competing with the target) [141]. Phosphatidylserine, a glycerophospholipid found in cell membranes, is one such candidate in the treatment of both oncological disorders and viral infections, and is the subject of discussion in Sections 1.2 to 1.7.

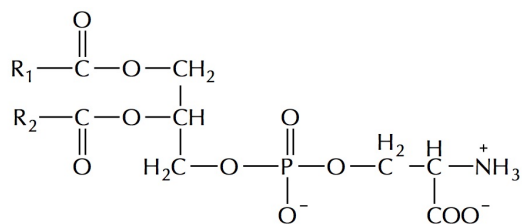
1.2 Phosphatidylserine has multiple biological functions

1.2.1 Phosphatidylserine in the plasma membrane

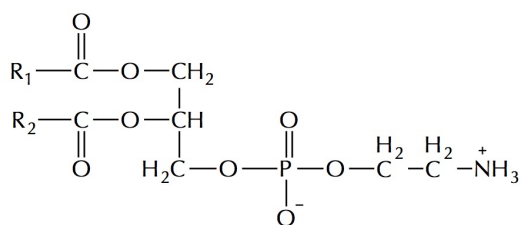
Phosphatidylserine (PS) is an anionic glycerophospholipid, a category of lipids with a major structural role in eukaryotic cells [185]. Glycerophospholipids are composed of a three-carbon glycerol backbone, a phosphorylated headgroup, and two hydrocarbon fatty acid chains [193]. The most basic glycerophospholipid is phosphatidate, for which the headgroup consists of a phosphate group only. Phosphatidate forms the structural basis for the glycerophospholipids found commonly in membranes: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidic acid (PA) (Figure 1.1). Diphosphatidylglycerol (cardiolipin) is found mainly in the inner mitochondrial membrane. PS comprises 5–15% of total phospholipids in mammalian cells, with the highest percentage in the plasma membrane [185, 205].



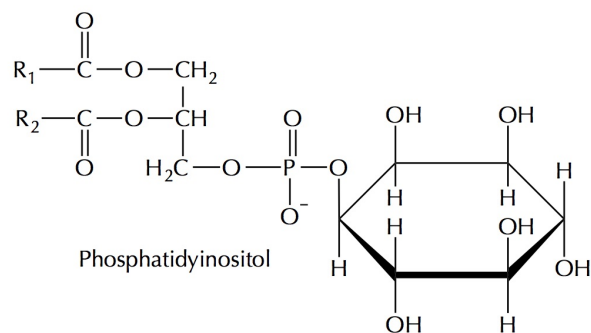
Phosphatidate



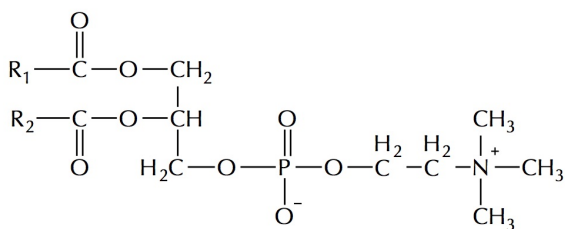
Phosphatidylserine



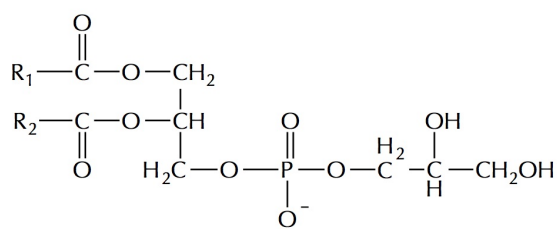
Phosphatidylethanolamine



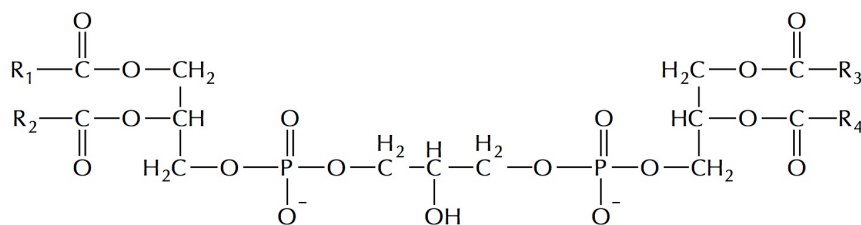
Phosphatidylinositol



Phosphatidylcholine



Phosphatidylglycerol

Diphosphatidylglycerol
(cardiolipin)**Figure 1.1:** Structures of common glycerophospholipids in eukaryotic cell membranes.

In healthy cells, PS is usually localised on the inner leaflet of the plasma membrane [38]. Maintenance of asymmetry is due mainly to the type IV subgroup of P-type ATPases (flippases), such as ATPB81, which rapidly shuttle PS from the exoplasmic to the cytoplasmic leaflet of the membrane [38, 205]. Once achieved, asymmetry is a relatively steady state and requires additional factors for disruption [161]. This is due to the slow rate of transbilayer lipid movement, on average 10^{-15} s^{-1} ; the high energy barrier for this movement is imposed by the resistance of the hydrophobic core to the passage of polar head groups plus additional resistance against the insertion of additional phospholipids in the destination bilayer [46, 185].

Disruption of asymmetry is thought to be mediated by two protein groups: ATP-binding cassette (ABC) transporters (floppases) and scramblases. ABC transporters, of which ABCA1 is the family member with PS, phosphatidylcholine (PC) and cholesterol selectivity, export lipids from the inner to the outer leaflet in an ATP-dependent manner. Scramblases are ATP-independent translocators that translocate phospholipids bidirectionally across the membrane regardless of headgroup. They are active in erythrocytes, activated platelets and apoptotic cells and are thought to be predominantly responsible for the collapse of asymmetry and the exposure of PS.

PS is exposed on the surface of activated platelets following blood vessel injury [212]. Contact between PS and plasma coagulation factors then increases the formation rate of thrombin and the conversion of soluble fibrinogen into an insoluble fibrin clot [212]. PS is also found on the surface of exosomes derived from platelets and dendritic cells, viable monocytes, mature macrophages and activated B cells [38].

1.2.2 Role of phosphatidylserine in apoptosis

Perhaps the most widely-known function of PS is as an 'eat-me' signal on apoptotic cells to stimulate engulfment by phagocytes ('efferocytosis') [148]. Fadok *et al* [63] found that phagocytosis of apoptotic HL60 cells by bone-marrow-derived macrophages (BMDM) was

correlated with exposure of PS on the cell membrane. Loading of PLB985 cells with phospho-L-serine or phospho-D-serine showed that PS recognition was stereospecific for the L isomer [63]. Induction of apoptosis in Jurkat cells produced an increase in PS of over 260-fold within 1–2 h of induction [19]. The pathways leading to PS exposure during apoptosis have not yet been clearly defined, although the process has been shown to be independent of cytochrome c release or DNA fragmentation, to be caspase-dependent, and to be stimulated by a sustained elevation of cytosolic calcium [9].

Efferocytosis can be carried out both by ‘professional’ phagocytes, such as macrophages and dendritic cells, and ‘non-professional’ phagocytes, including epithelial cells in close proximity to the apoptotic cell [81]. Numerous receptors have been identified that enable phagocytes to recognise PS and these are discussed in detail in the next section. However, PS exposure alone is thought to be insufficient for efferocytosis, given that many cell types as described above express PS without being engulfed. This may be due to a requirement for other pro-apoptotic signals in conjunction with PS: other known ‘eat-me’ signals include calreticulin and annexin I (intracellular proteins that colocalise with PS under apoptotic conditions), deposition of complement proteins, and changes in surface glycosylation [81]. Live cells may also express ‘don’t-eat-me’ signals that overrides PS exposure. Thirdly, oxidation of PS molecules in apoptotic cells may also have a role in signalling: for example, MFG-E8 (Section 1.4) has a preference for oxidised PS over unoxidised PS [148].

1.3 Phosphatidylserine binding leads to downregulation of immune responses

The hallmark of apoptosis, compared to necrosis or pathogen engulfment, is the non-inflammatory nature of the process. The immunosuppressive effects of PS are mediated by PS engagement by receptors expressed on T and B lymphocytes and dendritic cells—these receptors are described in Section 1.4. One main mechanism by which tolerance is achieved

is the increased production of anti-inflammatory cytokines, such as IL-10 and TGF- β , from monocytes and macrophages following efferocytosis. This is accompanied by the decreased production of various pro-inflammatory cytokines such as TNF- α , IL-1 and IL-12 [38, 195].

The role of PS in tolerance induction was confirmed using mice injected with PS-containing liposomes prior to antigen stimulation [82]. Mice that received PS liposomes had a reduced accumulation of leukocytes, as well as antigen-specific CD4⁺ T-cells, in the lymph nodes. When lymph node T cells were examined *ex vivo* for their response to antigen stimulation, levels of the pro-inflammatory cytokines IL-2 and IFN- γ were decreased while levels of TGF- β were elevated. PS treatment also caused a reduction in the size and number of germinal centres and decreased levels of antigen-specific antibodies in serum.

Evidence regarding the direct effect of PS on dendritic cells (DCs) is conflicting. Hoffmann *et al.* [82] found that DC maturation and antigen presentation to T cells were unaffected by exposure to liposomes containing 30% PS. On the other hand, a group led by Shilyansky found that 30% PS liposomes inhibited DC maturation and predisposed the DCs to apoptosis [40]. The PS-treated DCs showed a reduced capacity to stimulate T cell proliferation; furthermore, the affected CD4⁺ T cells produced lower levels of IFN- γ and IL-12. Surprisingly, IL-10 expression was also reduced, while TGF- β was unaffected. Downregulation of DC activity was due to inhibition of NF- κ B nuclear translocation, via an increase in I κ B levels in immature DCs and reduced I κ B phosphorylation in stimulated DCs [56]. Phosphorylation of p38 MAPK, which is required for production of IL-12, was also inhibited.

T cells appear to be affected both directly and indirectly. PS-containing extracellular vesicles from ovarian tumour fluids have been found to directly inhibit T-cell activation in a PS-dependent manner [99]. This has been attributed to the binding of PS by a PSR expressed by the T cell. Furthermore, a recent study showed that phagocytosis mediated by T-cell, immunoglobulin and mucin protein-1 (TIM-1), a protein that binds PS (Section 1.4), triggered antigen presentation via MHC class II and cross-presentation via MHC class I. This presentation pattern maintained a greater proportion of T cells in a naïve phenotype and increased proliferation of regulatory T cells (Tregs).

Taken together, the evidence suggests that the therapeutic targeting of PS would lead to a blockade of PSR binding by lymphocytes and a reversal of the immunosuppressive effects of PS. To achieve this, small molecules could be used to inhibit PSR binding; alternatively, soluble PSRs may be used to compete with cell surface PSRs on lymphocytes. In both cases, an understanding of the mechanisms of PSR binding is required, which is the subject of the following section.

1.4 Phosphatidylserine is recognised by multiple receptor families

Phosphatidylserine recognition by phagocytes occurs both directly and via bridging molecules. Of particular relevance to this thesis are the TIM protein family (Section 1.4.1) and β 2-glycoprotein 1 (Section 1.4.2), which are discussed below. Throughout this work, phosphatidylserine receptor (PSR) will be used to refer to any molecule that binds PS. This differs from some usage in the literature which refers to a protein originally thought to mediate apoptotic clearance of phagocytes and thus designated 'PSR' [62]. This protein was subsequently found to be predominantly localised in the nucleus and to be important for embryogenesis but not for apoptotic cell clearance [21, 126]. It is therefore no longer thought to be a functional PSR.

1.4.1 TIM family

The T-cell, immunoglobulin and mucin (TIM) protein family is a set of membrane-bound proteins that directly bind PS. They are composed of an immunoglobulin V-like (IgV) domain which forms the PS-binding pocket, a long mucin domain which is heavily O-glycosylated, a transmembrane domain and, for most members, a cytoplasmic tail (Figure 1.2) [69]. Eight family members (TIM-1 to TIM-8) are found in the mouse, whereas humans express only three (TIM-1, TIM-3, TIM-4) which are homologous to the mouse proteins of the same denomination. An alignment of the full-length human TIM-1, TIM-3 and TIM-4 proteins is given in Figure 1.3.

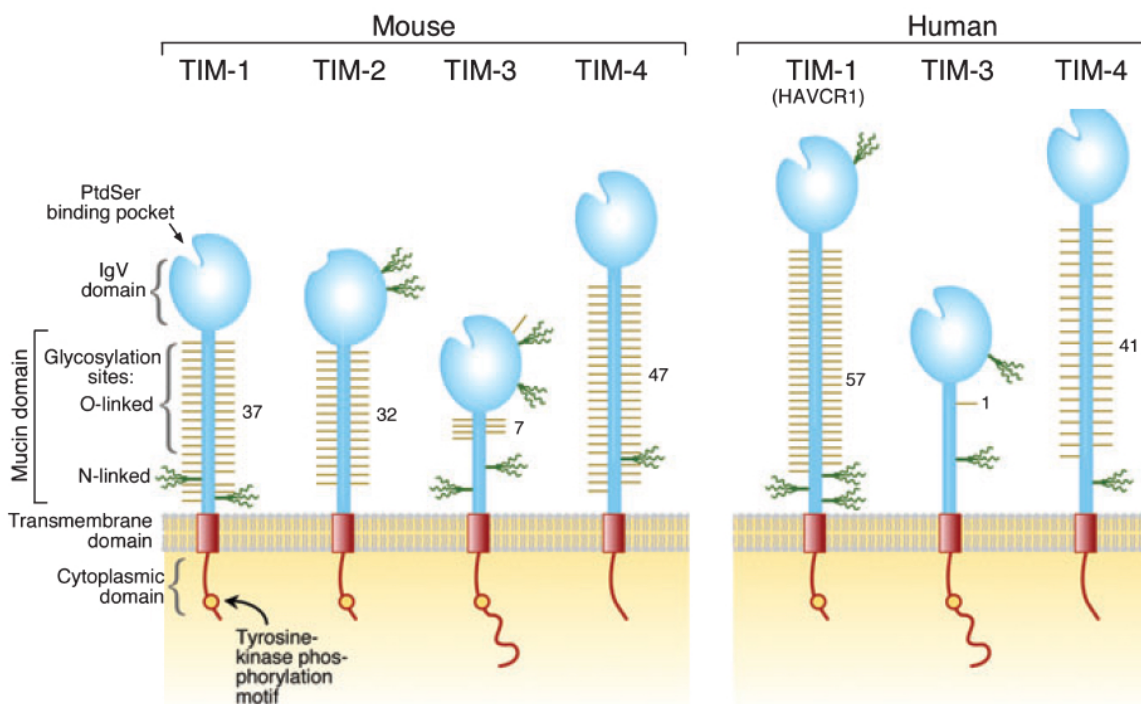


Figure 1.2: Domain structures of the murine and human TIM family members. While the length of the mucin domain is variable, all TIMs contain an IgV domain at the N-terminus. Both murine and human TIM-4 lack the tyrosine kinase phosphorylation motif found in other family members. Figure reproduced with publisher's permission from Freeman *et al.* (2010) [69] (license number 3700331106945).

Sequence conservation of the IgV domain is ~60% but much lower for the full-length protein due to variability in the mucin domains [158].

TIM-1 is expressed on T-cells, predominantly on activated CD4⁺ (T_H2) cells but also to a lesser extent on T_H1 cells, B cells and mast cells [105]. It acts as a co-stimulatory molecule and, upon antibody cross-linking, triggers the proliferation of CD4⁺ T-cells [53]. Additionally, TIM-1 binds PS and facilitates clearance of apoptotic and necrotic cells. It was separately cloned as Kidney Injury Molecule-1 (KIM-1) and found to be upregulated over one-thousand-fold on proximal tubule epithelial cells (PTC) in the post-ischaemic kidney [28, 87]. TIM-1 is also known as HAVCR-1 due to its initial discovery as a receptor for Hepatitis A virus [65, 97].

The other members of the TIM family found in humans, TIM-3 and TIM-4, are also involved

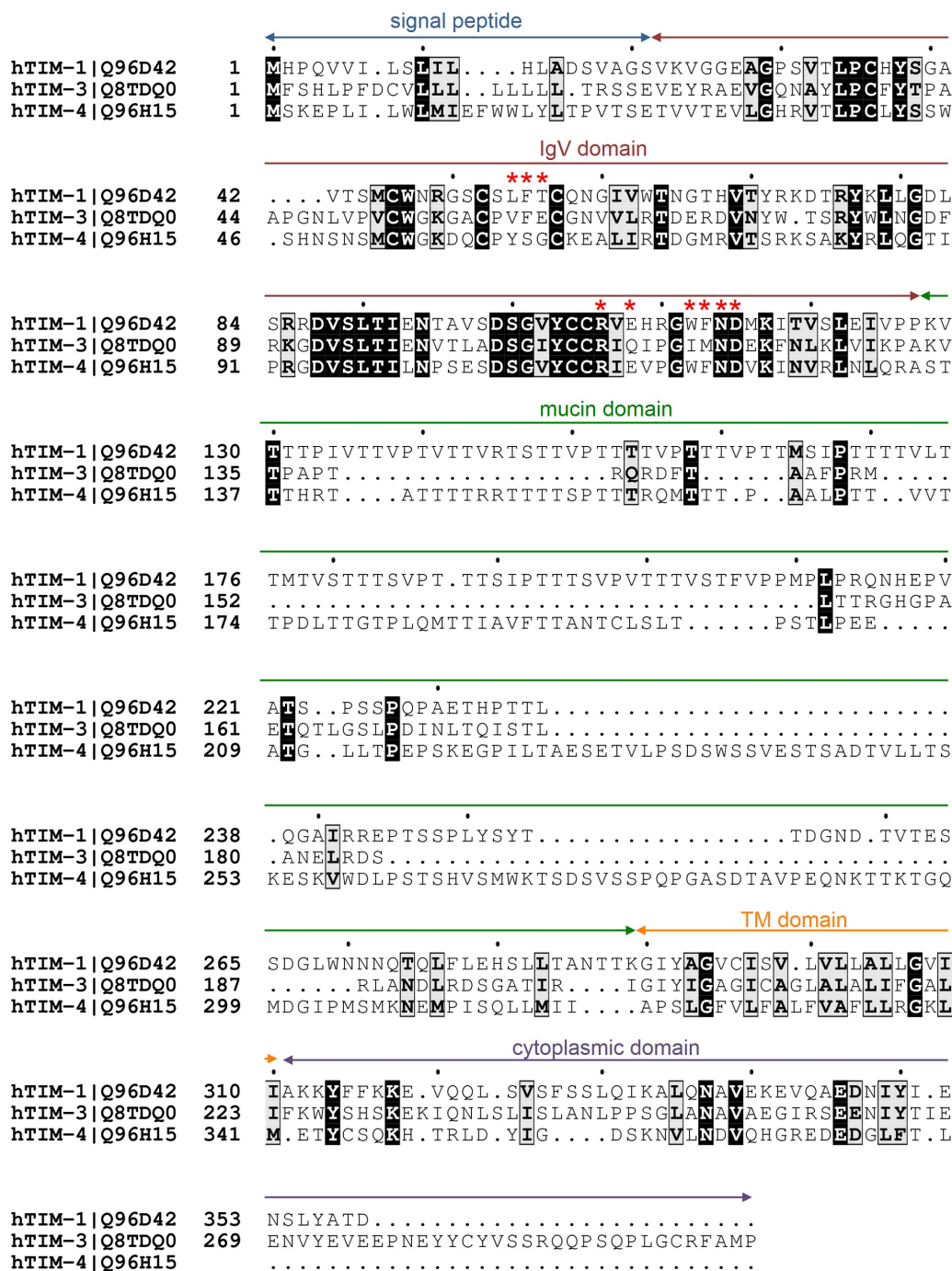


Figure 1.3: Sequence alignment of the human TIM-1, TIM-3 and TIM-4 proteins. UniProt identifiers are given after the name of each protein. Domain boundaries are marked above the alignment: signal peptide in blue, IgV domain in red, mucin domain in green, transmembrane (TM) domain in yellow, cytoplasmic domain in purple. Residues involved in interaction with phosphatidylserine, from alignment with the mouse TIM-4 sequence (Figure 1.5) and structure (Figure 1.4), are indicated with red asterisks. Alignment was done with T-Coffee [54] and shading according to physicochemical properties with ESPrnt 3.0 [153]. Strictly conserved residues are white with a black background, residues with a similarity score > 0.7 in bold black and residues with a similarity score < 0.7 in regular black.

in immune regulation. TIM-3 is expressed on differentiated T_H1 cells, a subset of activated CD4⁺ (T_H2) cells, some CD8⁺ cells and at lower levels on T_H17 cells. Engagement of TIM-3 by its ligands PS or galectin-9 produces an inhibitory signal that leads to apoptosis of T_H1 and T_c1 cells [69]. TIM-3 was also shown to be important for the clearance of apoptotic cells in vivo, as mice treated with anti-TIM-3 mAb had increased numbers of apoptotic cells in splenic follicles and increased levels of anti-dsDNA antibodies in serum. Co-expression of TIM-3 with Programmed Death 1 (PD-1), another inhibitory receptor, is associated with T-cell exhaustion in the context of chronic viral infection [93].

In contrast to the other members of the family, TIM-4 is not expressed on T-cells but instead is found on mature macrophages and dendritic cells [69]. There is also some evidence for expression in peripheral lymphoid tissues such as the spleen, lymph nodes and Peyer's Patches [53, 163], giving it the alternative name of SMUCKLER (Spleen, Mucin-Containing, Knockout of Lymphotoxin). TIM-4 also binds PS with high specificity and promotes phagocytosis of apoptotic cells by macrophages, while concomitantly decreasing production of nitric oxide [204]. However, the mechanism by which this occurs remains uncertain as TIM-4 lacks the longer cytoplasmic tail and tyrosine phosphorylation motif found in other family members (Figure 1.2). Studies have shown that the transmembrane and cytoplasmic regions are dispensable for engulfment, although an intact cytoskeleton is required [138]. It was therefore suggested that recruitment of other proteins was required for downstream signalling.

The structures of the murine TIM-1, TIM-2, TIM-3 and TIM-4 IgV domains were determined by X-ray crystallography [34, 158]. Figure 1.4A shows the structure of the murine TIM-4 IgV domain bound to phosphatidylserine. The ligand binding site is formed by the cavity between the CC' and FG loops and contains a metal ion identified as either sodium or calcium. Asn97 and Asp98 in the FG loop are involved in the metal ion coordination, as is the phosphate group of PS. The serine residue of PS inserts into the cavity and interacts with Ser38 and Lys39 on the CC' loop, and Glu89 on the FG loop. The outer lip of the FG loop is made up of the hydrophobic amino acids Trp95 and Phe96, which interact with the ligand's fatty acid tail. These contacts are shown in detail in Figure 1.4B. Mutagenesis of residues Trp95, Phe96, Asn97 and Asp98

resulted in 70–85% lower binding to PS liposomes relative to the wild type protein, confirming their role in ligand binding.

Although structural characterisation has been carried out only for the murine TIM (mTIM) proteins, the human orthologs (hTIM) appear to be functionally equivalent. A sequence alignment of the IgV domains from mTIM-4 and hTIM-4 is given in Figure 1.5. The sequence homology between these two proteins is 64% for this domain [149] and the WFND motif (residues 95–98) is fully conserved. Moreover, binding studies showed that mTIM-1, mTIM-4, hTIM-1 and hTIM-4 were highly selective for phosphatidylserine, with little to no binding observed for phosphatidylethanolamine, phosphatidylinositol and phosphatidylcholine [102, 127].

Interestingly, a recent study by Tietjen *et al.* identified differential sensitivity of the TIM proteins to PS density [182]. Binding of murine TIM-4 to vesicles containing 30% PS was 60% stronger than binding to 10% PS vesicles. Hill binding analysis produced a coefficient of 2.40 ± 0.08 , indicating a stoichiometry of at least 3 PS per protein. Furthermore, X-ray reflectivity, a technique that measures the reflected X-ray intensity from an interfacial surface, was used to determine the orientation of TIM-4 bound to a lipid membrane. The data showed TIM-4 bound to a lipid bilayer with residues Lys39 and Arg25 probing into the lipid headgroup region, and Arg46 and Lys100 sitting at the buffer interface (residue numbering to match Figure 1.5). Molecular dynamics simulations indicated that this was a stable interaction. These basic residues were therefore suggested to provide weaker interactions with additional PS molecules in the bilayer. In contrast, murine TIM-1 and TIM-3 proteins bound PS with significantly less apparent co-operativity, with TIM-3 showing a weaker affinity at all PS percentages tested. TIM-4 thus possesses the greatest sensitivity to PS exposure levels of all the TIM proteins examined.

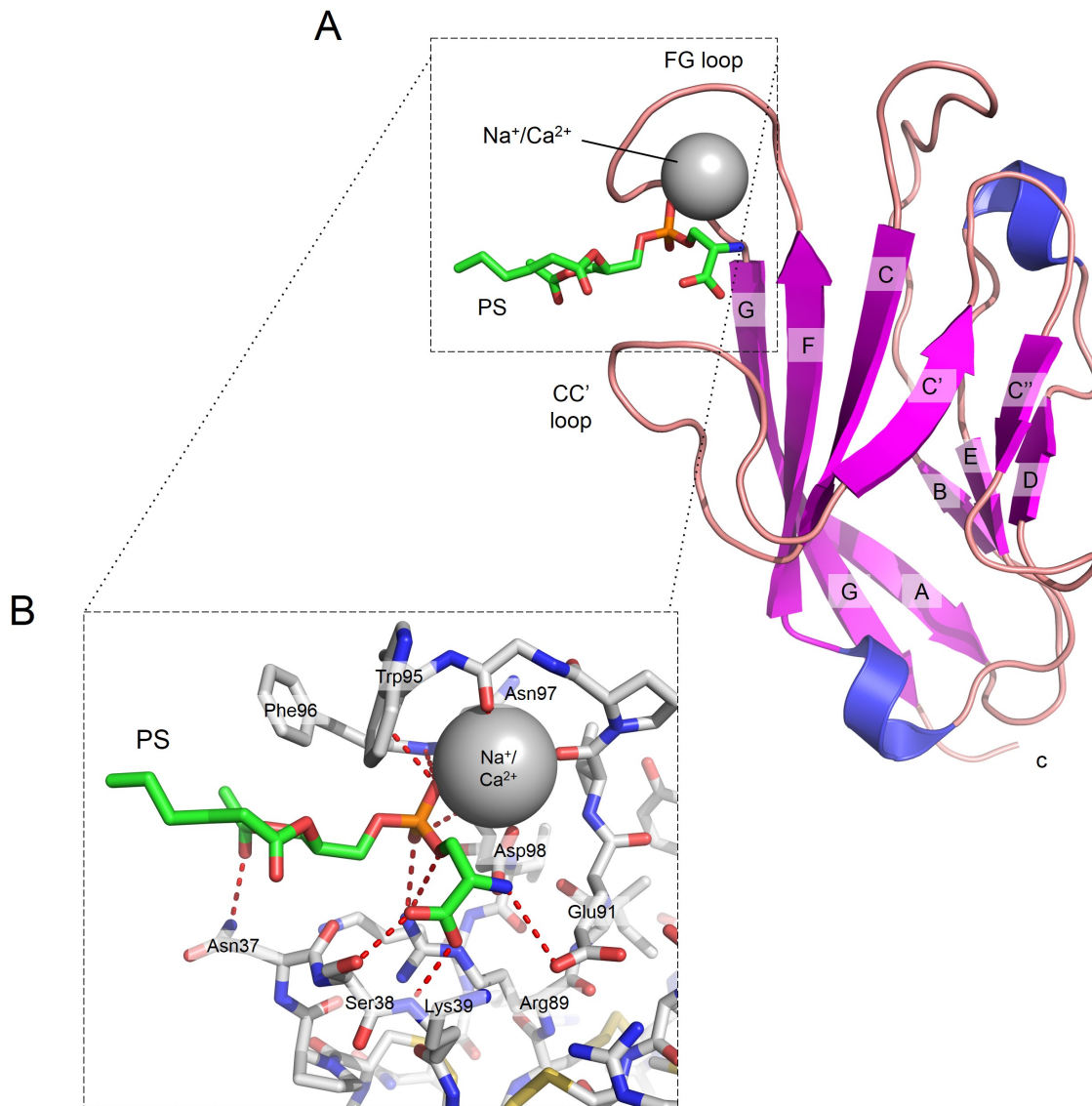


Figure 1.4: Structure of the murine TIM-4 IgV domain in complex with dicaproyl-phosphatidyl-L-serine (PS) (PDB 3BIB). **A**] X-ray crystal structure of the IgV domain as a cartoon representation, coloured by secondary structure. Helices are in blue, β -sheets in purple and loops in pink. Secondary structure elements are labelled in capital letters and the C-terminus is labelled in lower-case. The N-terminus is located behind β -strand G. The CC' and FG loops that flank the binding site are indicated. The grey sphere identifies the sodium/calcium ion. Phosphatidylserine is given in stick representation with carbon in yellow, oxygen in red, nitrogen in blue and phosphorus in orange. **B**] Magnification of the ligand binding site formed by the FG and the CC' loops. Both mTIM-4 and phosphatidylserine are shown in stick representation. Oxygen is coloured in red, nitrogen in blue, phosphorus in orange, and carbon in light grey for mTIM-4 and yellow for phosphatidylserine. Figure was produced using PyMol, Schrödinger LLC.

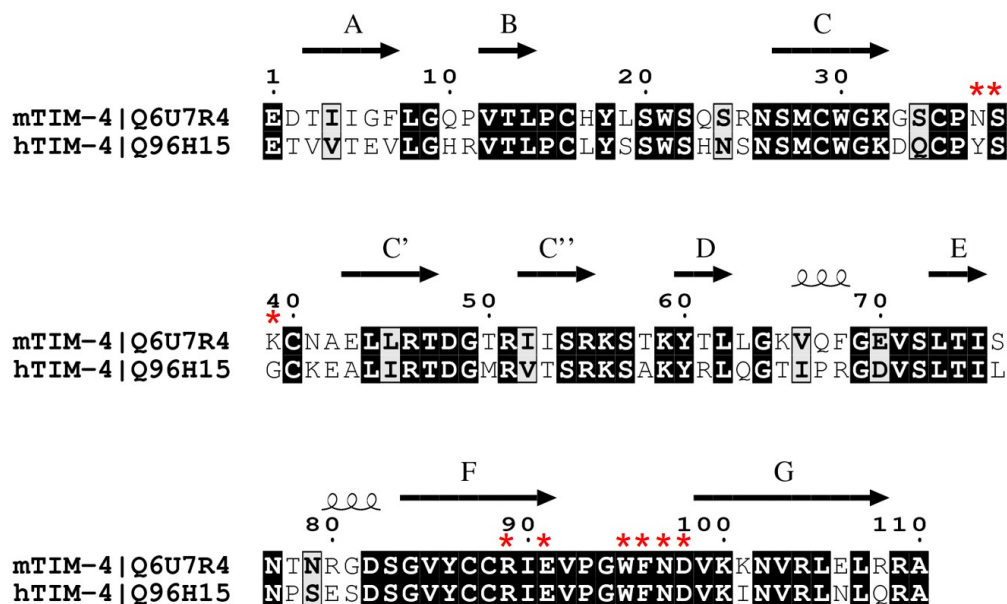


Figure 1.5: Sequence alignment of the IgV domains from murine TIM-4 and human TIM-4. Secondary structure elements are derived from the X-ray crystal structure of murine TIM-4 IgV (3BIB) and are given above the alignment: arrows indicate β -sheets and coils indicate α -helices. β -sheets are labelled according to the nomenclature used in Figure 1.4 and ref. [158]. Sequences are numbered from the start of the IgV domain. Residues involved in the interaction with phosphatidylserine, as shown in Figure 1.4, are indicated with red asterisks. Alignment was done with T-Coffee [54] and shading according to physicochemical properties with ESPrpt 3.0 [153]. Strictly conserved residues are white with a black background, residues with a similarity score > 0.7 in bold black and residues with a similarity score < 0.7 in regular black.

1.4.2 β 2-glycoprotein 1

The plasma protein β 2-glycoprotein 1 (β 2-GP1), also known as apolipoprotein H, is composed of a single chain of 326 amino acid residues, with a molecular weight of ~ 43 kDa. It binds cardiolipin, phosphatidylserine, phosphatidylglycerol, and phosphatidic acid all with a dissociation constant of $\sim 1 \times 10^{-8}$ M. It also binds phosphatidylinositol with a slightly higher dissociation constant of $\sim 1 \times 10^{-7}$ M [75]. No binding has been observed to phosphatidylethanolamine or phosphatidylcholine membranes [75, 199].

The amino acid residues involved in phospholipid binding have been established from site-directed mutagenesis [122, 157, 164], X-ray crystallography [22, 162] and nuclear magnetic resonance (NMR) [83]. The X-ray data showed that the protein consisted of four complement control protein (CCP) domains, also known as Sushi domains, plus a fifth distinct C-terminal domain (Figure 1.6). The fifth domain is distinguished from domains 1–4 by a CKNKEKKC motif (amino acids 281–288) and a flexible C-terminal hydrophobic loop [162]. The CKNKEKKC motif provides an area of positive charge and is predicted to interact with negatively charged phospholipid headgroups. This is consistent with the mutagenesis studies which found that Lys284, Lys286 and Lys287 were critical for binding to cardiolipin [164]. Additionally, a peptide matching the CKNKEKKC sequence was found to inhibit phospholipid binding, while deletion or replacement of the flanking cysteine residues in this peptide reduced the inhibitory effect [86]. This is because the corresponding cysteine residues are involved in two of the three disulphide bonds that help to maintain the domain conformation: Cys245–Cys296, Cys281–Cys306 and Cys288–Cys326. Cys281–Cys306 in particular is thought to be particularly important for clustering the additional positive charges on Lys305, Lys308, His310, Lys317 and Lys324 in close proximity to Lys284, Lys286 and Lys287 [157]. Furthermore, amino acids Leu313, Phe315 and Trp316 on the flexible hydrophobic loop also contribute to phospholipid binding [122]. It is hypothesised that this loop inserts into the lipid bilayer, with the tryptophan at the lipid-water interface, to anchor β 2-GP1 into the membrane.

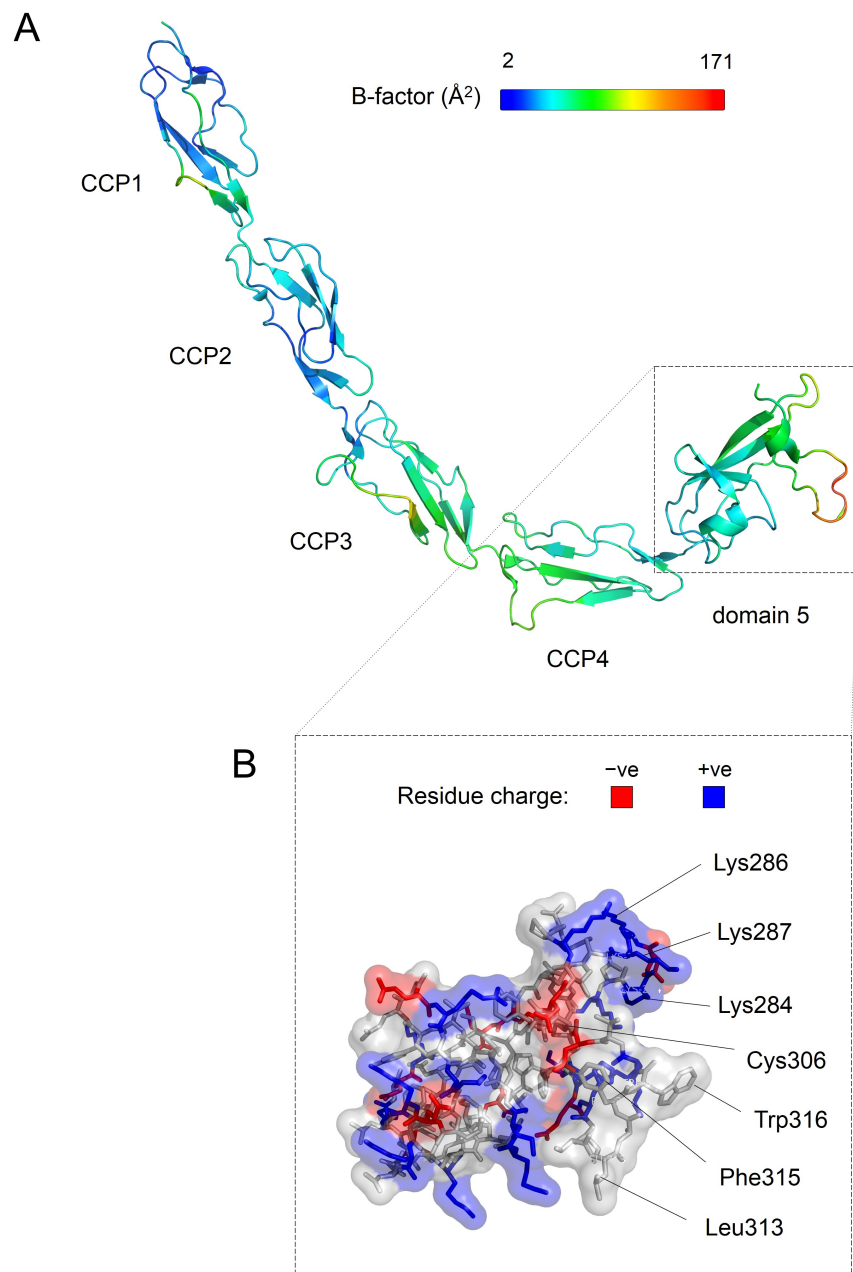


Figure 1.6: The structure of β 2-glycoprotein 1 (PDB 1C1Z) as determined by X-ray crystallography. **A**| The secondary structure of the full length protein as a cartoon representation. This structure is coloured by B-factor to highlight the flexible C-terminal loop thought to be involved in membrane insertion. **B**| Domain V of the protein as a stick and surface representation. The domain is coloured according to residue charge. Amino acid residues known to be important in phospholipid binding are annotated. Figure was produced using Pymol, Schrödinger LLC.

Table 1.1: Binding affinities of β 2-glycoprotein 1 to phospholipid membranes at various ionic strengths, as measured by ellipsometry. Data obtained from Willems *et al.* (1996) [199].

Membrane PS:PC ratio (mol%)	[NaCl] (mM)	[CaCl ₂] (mM)	K_D (μ M)
5:95	120	0	26.0
10:90	120	0	3.7
10:90	120	3	14.0
20:80	60	0	0.032
20:80	120	0	0.17
20:80	120	1	1.63
20:80	120	3	14.0

The broad selectivity for negatively charged phospholipids indicated that binding is governed by ionic interactions rather than the exact structure of the headgroup, and would be affected by the ionic strength of the solution. In accordance with this, Willems *et al* tested binding of β 2-GP1 to planar phospholipid bilayers using ellipsometry and showed that increasing the concentration of NaCl or CaCl₂ decreased the measured affinity (Table 1.1) [199]. Taking the 10:90 mol% PS:PC membrane with 120 mM NaCl and 3 mM CaCl₂ to be the most physiologically relevant of those tested, β 2-GP1 can therefore be considered to have a relatively low affinity (K_D 14.0 μ M) for cell membranes exposing PS. However, addition of anti- β 2-GP1 antibodies significantly increases the adsorption of β 2-GP1 to bilayers containing 20% PS [199].

The importance of β 2-GP1 function *in vivo* remains uncertain. It is thought to have a role in thrombotic regulation by competing with clotting factors for binding to exposed phospholipid on activated platelets [11]. Domain 1 of β 2-GP1 then recruits macrophages by interacting with members of the lipoprotein receptor-related protein (LRP) family [116]. It is involved in clearance of apoptotic cells by the same mechanism. β 2-GP1 has also been implicated in antiphospholipid syndrome (APS), as it is one of the plasma substrates recognised by antiphospholipid antibodies [52]. The clinical symptoms of the resulting autoimmune syndrome are varied but typically include thrombosis and recurrent pregnancy loss. Antibodies have been identified that bind to all five domains of β 2-GP1 and are generally

of low affinity to their antigen. However, antibody binding to $\beta 2$ -GP1–phospholipid complexes is greater than to $\beta 2$ -GP1 alone [120]. There is evidence that $\beta 2$ -GP1 binding to phospholipids induces substantial conformation changes [18], which may expose a neo-epitope on $\beta 2$ -GP1 for antibody binding. It may also be that clustering of $\beta 2$ -GP1 molecules on a phospholipid surface increases the avidity of antibody binding. One antibody that recognises $\beta 2$ -GP1 is 3G4, a mouse monoclonal antibody which cross-reacts with human and bovine $\beta 2$ -GP1 [146]. This antibody has been developed into bavituximab, a mouse–human chimera which is discussed further in Section 1.7.

1.4.3 Other PSRs

The majority of known phosphatidylserine receptors, like the TIM proteins and $\beta 2$ -GP1, act to promote clearance of apoptotic cells. Additional examples of those that bind directly to PS include the following:

- LRP1/CD91, which recognises calreticulin colocalised with PS [81].
- Brain-specific angiogenesis inhibitor 1 (BAI1). BAI1 has the dual function of binding PS and promoting engulfment by interaction with ELMO/Dock180; the latter protein is involved in rearrangement of the actin cytoskeleton for engulfment [38].
- Stabilin-2, a multifunctional scavenger receptor expressed by several macrophage cell lines and human monocyte-derived macrophages. Downregulation of stabilin-2 was found to inhibit phagocytosis of apoptotic cells [38].

Indirect receptors include the following:

- Milk fat globule-epidermal growth factor 8 (MFG-E8) is secreted by professional phagocytes and bridge PS to $\alpha_v/\beta_{3/5}$ integrins [81].

- Gas6 and Protein S, which bridge PS to the Tyro-3-Axl-Mer (TAM) receptor family [81]. This receptor family has been implicated in phosphatidylserine-dependent uptake of multiple virus species (Table 1.2).

1.4.4 Modulatory PSRs

Some proteins that bind PS have a repressive effect on phagocytosis. One such example is Annexin V (AnxA5), which modulates both coagulation and phagocytosis of apoptotic and necrotic cells [38]. It binds with high specificity to membranes with exposed PS in a calcium-dependent manner; this forms the basis for apoptosis detection assays using fluorescently labelled AnxA5. However, binding is influenced not only by the density of PS but also the presence of other phospholipids in the membrane such as PE. AnxA5 was found to block phagocytosis by macrophages but not dendritic cells; this may have a function in the clearance of necrotic cells by dendritic cells, thereby promoting an immune response [81].

A second set of receptors in this category is the CD300 family, for which the best described member is CD300a. CD300a is expressed on a broad range of cells, including T cells, B cells, neutrophils and eosinophils [170]. It binds both PS and PE, but shows greater affinity for PE [170]. Its inhibitory activity was initially indicated by the presence of immunoreceptor tyrosine-based inhibition motifs (ITIM) on its cytoplasmic tail and was then confirmed experimentally: blocking of CD300a increased the efficiency of phagocytosis by macrophages [170]. In addition, anti-CD300a and anti-CD300c antibodies increased the NK-cell-mediated killing of tumour cells [106], which are known to have exposed PS (Section 1.6).

Aside from the inhibition of engulfment, the other immunomodulatory effects of CD300a correlate well with those following PS recognition by other PSRs such as TIM proteins: inhibition of B-cell receptor and T-cell receptor signalling, reduced reactive oxygen species (ROS) production in neutrophils and suppression of the effects of cytokines on eosinophil proliferation [170]. It therefore indicates the complex nature of apoptotic cell clearance, as triggering of engulfment is likely to be dependent on the balance between expression of

multiple ligands, including PS and PE, and multiple phagocyte receptors which mediate downstream signalling.

1.5 Role of phosphatidylserine in the viral life cycle

Viruses from numerous families induce PS exposure on infected host cells and/or incorporate PS on the outer leaflet of the virion envelope. This can be a by-product of the host response to the virus; however, evidence is accumulating that viruses also harness the immunomodulating properties of PS to promote attachment, transduction and egress.

1.5.1 Phosphatidylserine exposure on virus-infected cells

Loss of PS asymmetry on infected cells may occur following induction of apoptosis, which is part of the host anti-viral defence mechanism. Cells express multiple sensors for the presence of viruses, such as the RNA-dependent protein kinase (PKR) which responds to viral double-stranded DNA (dsDNA) by dimerisation and phosphorylation. PKR subsequently phosphorylates the translation initiation factor eIF2 α which not only causes an arrest of protein translation but also activates the Fas-associated death domain (FADD) signalling cascade that leads to apoptosis [61]. Another pro-apoptotic transcription factor is p53: this protein is activated by unregulated entry into the S-phase of the cell cycle during viral replication.

In addition, viral infection frequently perturbs calcium homeostasis by altering membrane permeability, interacting with proteins involved in calcium signalling, or directly sequestering calcium. Changes to cytosolic calcium levels, within or without the context of apoptosis, can activate PS exporters and inhibit PS translocases; the net result is membrane PS asymmetry [10, 211].

Furthermore, viruses commonly manipulate the mechanisms of apoptosis. To allow replication to proceed, viruses can block apoptosis by inactivation of p53, expression of anti-apoptotic

protein mimics such as Bcl-2, inhibition of caspases and secretion of soluble TNF receptor (TNFR) decoys [14]. Conversely, viruses have also been found to promote apoptosis as a means of transmission. Small, membrane-bound apoptotic bodies produced by the dying cell can provide a convenient vehicle for dissemination, as they are consumed by phagocytes without initiating a host response [79]. The timing of apoptosis induction is crucial: if too early, an insufficient number of viral progeny will have been produced. For example, human immunodeficiency virus (HIV) produces both pro-apoptotic and anti-apoptotic proteins, illustrating the importance of modulating the host death response. The Nef protein increases cell surface expression of TNF while downregulating CD4 and MHC class I expression. The gp120/gp41 complex crosslinks to CD4 lymphocytes and induces apoptosis. The Tat and Vpr proteins appear to have dual functions: on the one hand, Tat causes increased expression of Fas and Vpr induces cell cycle arrest in the G2 phase, both of which are pro-apoptotic actions. On the other hand, these proteins have also been shown to elevate Bcl-2 levels and to interrupt TCR-mediated cell death signalling. It is possible that the exact role of these proteins reflects the chronology of viral replication [79].

Given the variety of possible apoptotic induction factors, viral infection therefore commonly causes PS exposure on the host cell surface; further details regarding specific virus families are given in Table 1.2.

1.5.2 Mechanisms of phosphatidylserine exposure on enveloped viruses

Phosphatidylserine is exposed on enveloped viruses from a broad range of families (Table 1.2). This may occur following budding from the plasma membrane or from intracellular organelles. The herpesviruses bud from Golgi-derived vesicles [156] which may not have lipid asymmetry or, like the plasma membrane, lose the asymmetry upon viral infection.

In the case of plasma membranes, PS may already be present across the outer leaflet for reasons given in Section 1.5.1. Viruses may also selectively bud from lipid rafts [27, 180] that are enriched in phosphatidylserine [140]. There is accumulating evidence for this phenomenon

across multiple virus families, including HIV, influenza, Ebola and Marburg viruses. The lipid composition of HIV-1 and HIV-2 viruses was found to differ from lipid composition of host cell membranes [6]: this is a key indicator of selectivity regarding point of egress from the plasma membrane. Although in this study, the percentage of PS in infected cell membranes was lower than in uninfected cell membranes, the percentage of PS in the virus was comparable to or higher than the infected cell. The HIV-1 Gag protein was later found to associate with rafts following its membrane localisation, while disruption of raft formation by cholesterol depletion impaired virus release and reduced infectivity [136].

The haemagglutinin (HA) and neuraminidase glycoproteins of the influenza virus was found to associate with rafts [27, 160]. Thirdly, studies of Ebola and Marburg viruses showed significant proportions of the Ebola glycoprotein (GP) and the Marburg NP were raft-associated [13]. The Ebola VP40 protein, which regulates budding from the host cell plasma membrane, has a preference for membranes containing at least 10% PS; this preference was found to be specific for PS over the other anionic phospholipids tested (phosphatidylinositol, phosphatidylinositol bisphosphate and phosphatidylinositol trisphosphate) [176]. Furthermore, virions from both species incorporated the raft glycolipid GM1 during budding [13].

1.5.3 Phosphatidylserine enhancement of virus entry

Apoptotic mimicry, defined by the exposure of PS on the virion surface, can be advantageous to the virus by promoting uptake into both professional and non-professional phagocytes while circumventing the host immune response. Furthermore, as most 'bystander' cells are capable of phagocytosis, using engulfment as an entry mechanism would enable a virus to broaden its host cell tropism without expression of additional receptor proteins [7].

There is a growing body of evidence that virus infectivity is enhanced by PS receptors on the cell surface. A comprehensive study by Morizono & Chen [130] evaluated most of the known phosphatidylserine-binding receptors for enhancement of viral entry. Using lentiviral vectors pseudotyped with the virus of interest, they determined that MFG-E8, Axl/Gas6, TIM-1 and

TIM-4 are viral receptors, albeit with varying degrees of efficacy. Of these, TIM-4 seemed the least effective: enhancement of transduction was seen with Ross River Virus (RRV), gp64 and VSV-G but not with Sindbis or 2.2 1L1L pseudotypes. Even where enhancement of transduction was seen, it was markedly lower than that produced by TIM-1 and Axl/Gas6.

Following binding of one or more of these receptors, viral entry into the cell can proceed by various endocytic mechanisms; some, such as Ebola, have been shown to use caveolae and clathrin-mediated endocytosis as well as actin- and microtubule-dependent pathways [85]. A study of Ebola virus entry found that Axl, an indirect PSR, promoted macropinocytosis of Ebola pseudovirions [85]. Inhibition of phospholipase C (PLC), which interacts with Axl and is required for macropinocytosis, led to inhibition of pseudovirus uptake. However, for most viruses, the pathways leading from PSR engagement to endocytosis are still unclear. Use of PS varies between viruses, between cell lines and between PSRs, and the process may require additional cofactors. In some cases, PS may be one of multiple entry factors, not all of which are required *in vivo*.

Taking HIV-1 as an example, it remains uncertain whether PS on the virion or on the host cell, or both, has a role in viral infection. Callahan *et al.* [33] found that PS was present on the surface of HIV-1 infected Jurkat cells and monocytes. PS was also present on virions produced from 293T cells and H9 cells. Annexin V and PS-containing lipid vesicles inhibited infection of monocytes and monocyte-derived macrophages but not of Jurkat cells or primary CD4⁺ T cells. This was attributed to the presence of a PSR specifically expressed on macrophages. The role of cellular PS was not considered to be a factor as monocytes, unlike their descendent macrophages, do not constitutively expose PS and did not stain with Annexin V in this study. Additionally, host cell PS receptors were found to have a role post-entry [17] (Section 1.5.4).

In contrast, a later investigation by Moody *et al.* [129] used a panel of PS-targeting antibodies and found that none of the antibodies bound HIV-1 virions using a capture assay. Only one, PGN632, bound the virus using a SPR assay, with a fast off-rate. However, the control antibody used here, which is specific to a conformational epitope of gp120 [67], also did not bind; it

is possible that conditions of virus immobilisation to the chip caused distortion of the virus structure and altered the exposure of the PS ligand. Despite these observations, a selection of the antibodies were found to inhibit HIV-1 infection, but only with the use of peripheral blood mononuclear cells and primary viral isolates. Use of epithelial cell targets or pseudoviruses showed no inhibition, and inhibition was specific to CCR5-tropic HIV-1. Notably, both the F(ab')₂ and the Fc portions of the antibodies were required for inhibition of infectivity. The design of the infectivity assays also did not eliminate the possibility of the antibodies binding to host cell PS. These findings suggests that the inhibitory effect is mediated by binding cell-associated, rather than virion-associated, PS. Finally, the antibodies that bound PS directly, instead of via the bridging molecule β 2-GP1, were the more effective inhibitors.

Table 1.2 lists the virus species for which PS has been found to be present on the membrane; evidence for active involvement of PS in the viral life cycle (as opposed to simply passive incorporation of PS into the viral membrane) is highlighted in bold. Some of the variation identified between results so far may be a result of experimental factors: use of pseudoviruses in place of live viruses, gain-of-function studies involving single receptors, and limitations of method sensitivity. For this reason, the methods supporting the researchers' conclusions have also been given.

Table 1.2: Evidence supporting the role of PS in viral binding, transduction and infection. See footnotes on page 31 for explanation and abbreviations.

Genus ¹	Species	Details	Experimental evidence	Refs
<i>Arenaviridae</i>²				
Arenavirus	Junin	Binding of infected cells with bavituximab ³ (PGN401) and PGN635 antibodies	Immunofluorescence	[47]

continued on next page

Table 1.2 continued

Genus	Species	Details	Experimental evidence	Refs
		Binding of virions with PGN antibodies.	None provided	[47]
Mamm- arenavirus	Pichinde	Binding of virions and infected cells with an anti-PS antibody	Immunofluorescence	[171]
		Therapeutic effect of anti-PS antibody in virus-infected guinea pigs⁴.	Overall survival. Reduction in viral load. ADCC quantification.	[171]
<i>Bunyaviridae</i>				
Phlebovirus	Punta Toro	Improvement in survival after treatment of infected hamsters with bavituximab (PGN401) and ribavirin	Survival curves Reduction in virus titres	[47]
		Binding of virions and infected cells with PGN antibodies	None provided	[47]
<i>Filoviridae</i>				
Ebolavirus	Ebola (various)	Binding of virions and infected cells with PGN antibodies	None provided	[47]
		Binding of infected cells with bavituximab	Flow cytometry; immunofluorescence	[57]

continued on next page

Table 1.2 continued

Genus	Species	Details	Experimental evidence	Refs
		Binding of virions with bavituximab	ELISA	[57]
		~15-fold enhancement of Ebola pseudovirus entry by hTIM-1	Entry assay using hTIM-1-expressing 293T cells and MLV pseudovirus carrying Ebola GP.	[91]
		TIM-1 and TIM-4 enhancement of pseudovirus transduction, in a manner dependent on PS–TIM interaction.	Flow cytometry to determine uptake of Ebola pseudoviruses into HEK-293T cells expressing TIM-1 (some TIM-1 constructs with mutations in the PS-binding domain).	[128]
		TIM-1 binding to pseudovirions and enhancement of viral transduction.	Quantification of transduced, TIM-1-expressing cells 24 h after transduction.	[103]
		Annexin V binding to pseudovirions	ELISA	[128]
		Selective binding and preferential budding of VP40 from membranes containing > 5–10 mol% PS.	Binding: SPR using lipid vesicles	[2]
			Budding: confocal imaging of budding into lipid vesicles.	[176]
		VP40 oligomerisation and plasma membrane localisation dependent on PS content of membrane.	Confocal microscopy using PS-deficient CHO-K1 cell line PSA-3.	[2]

continued on next page

Table 1.2 continued

Genus	Species	Details	Experimental evidence	Refs
Marburgvirus	Marburg (MARV)	PS exposure on exoplasmic leaflet dependent on plasma membrane localisation of VP40.	Flow cytometry using Annexin V of cells expressing a localisation-deficient VP40 mutant.	[2]
		Tyro-3 (Dtk) and Axl expression increased infection of Ebola and Marburg viruses.	Infectivity of both live virus and pseudovirus tested with stable transductions of Vero E6 and Jurkat cells.	[167]
		Axl increases macropinocytosis of Ebola pseudovirions.	Quantification of infected cells 24 h after virus transduction.	[85]
		~3-fold enhancement of MARV pseudovirus entry by hTIM-1.	Entry assay using hTIM-1-expressing 293T cells and MLV pseudovirus carrying MARV GP.	[91]
		Tyro-3 (Dtk) and Axl expression increased infection of Ebola and Marburg viruses.	Infectivity of both live virus and pseudovirus tested with stable transductions of Vero E6 and Jurkat cells.	[167]
<i>Flaviviridae</i>				
Flavivirus	Dengue	Binding of infected cells with a PS-targeting antibody	None provided	[47]
		Direct binding of virions by TIM-1 and TIM-4.	Pull-down assay; ELISA	[121]

continued on next page

Table 1.2 continued

Genus	Species	Details	Experimental evidence	Refs
		Enhancement of HEK-293T and CHO-745 viral uptake and productive infection by TIM-1 & TIM-4 (up to 500-fold) and TIM-3 (a much lesser extent). This was dependent on binding of virion-associated PS and independent of the cytoplasmic tail of TIM-1. Similar results were observed with TAM receptors and their ligands Gas6 and ProS; however, the tailless and kinase-dead AXL variants were found to enhance endocytosis but not a productive infection.	Uptake: qPCR of viral RNA, fluorescence microscopy. Infectivity: measurement of newly synthesised NS1 viral proteins.	[121]
	Yellow fever	Binding of infected cells and virions by PGN antibodies.	None provided	[47]
<i>Herpesviridae</i>				
Cytomegalovirus	CMV	Exposure of PS on hCMV virions.	Flow cytometry using Annexin V-FITC; clotting assays as evidence for the presence of pro-coagulant phospholipids.	[179]
		Binding of mCMV virions and infected cells with an anti-PS antibody.	None provided.	[47]
		Binding of mCMV-infected cells with bavituximab.	Immunofluorescence.	[171]

continued on next page

Table 1.2 continued

Genus	Species	Details	Experimental evidence	Refs
Simplexvirus	HSV	PGN632, a human PS-targeting antibody, as effective as the standard treatment Zirgan at reducing corneal lesions in a rabbit model of ocular herpes.	Scoring of disease parameters in HSV-1-infected rabbits.	[42]
		Exposure of PS on HSV-1-infected ND7 (neuron-like) cells	Immunofluorescence staining with Annexin V	[71]
		Exposure of PS on HSV-1 and HSV-2 virions.	Flow cytometry using Annexin V-FITC; clotting assays as evidence for the presence of pro-coagulant phospholipids.	[179]
<i>Orthomyxoviridae</i>				
Influenza virus A	Influenza A	Binding of influenza-infected cells with bavituximab	Flow cytometry	[171]
		Engulfment of influenza-infected cells promoted by PS	Phagocytosis of infected, labelled HeLa cells by macrophages	[168]
<i>Poxviridae</i>				
Orthopoxvirus	Vaccinia	Binding of infected cells by bavituximab	Flow cytometry.	[171]
		PS detected on virions.	Confocal microscopy using Annexin V with fixed virus.	[123]

continued on next page

Table 1.2 continued

Genus	Species	Details	Experimental evidence	Refs
		Exposure of PS on VSV-infected cells	Immunofluorescence with Annexin V.	[123]
		PS found to be vital for a productive infection: delipidated viruses bound cells but did not enter or replicate. Pre-treatment with Annexin V reduced the infectivity, but not the binding, of virions. Annexin V pre-treatment of cells had no effect on either binding or infectivity.	Flow cytometry of cells following 1 h binding / 1 h binding + 2 h infection, as required.	[123]
<i>Retroviridae</i>				
Lentivirus	HIV-1	Binding of virions and infected cells with bavituximab	None provided.	[47]
		Detection of PS on HIV-1-infected Jurkat T and U937 & THP-1 monocytic cells.	Flow cytometry with Annexin V.	[33]
		Detection of PS on HIV-1 virions.	Pull-down assay using Annexin V.	[33]
		PS enhancement of HIV-1 infection, but not binding, of macrophages (U937 and THP-1 cell lines and MDM). This effect was not seen with Jurkat T-cells or primary CD4 ⁺ T-cells and was dependent on the HIV envelope proteins.	Binding: measurement of cpm following incubation of cells with radiolabelled virus for 3 h, in presence/absence of Annexin V or PS vesicles. Infection: RT assay following infection in presence/absence of Annexin V.	[33]

continued on next page

Table 1.2 continued

Genus	Species	Details	Experimental evidence	Refs
		Anti-PS antibodies inhibit infectivity of PBMCs with CCR5-tropic primary HIV-1 isolates (not of epithelial cells or with pseudovirus or with CXCR4-tropic virus). Only PGN632 was found to bind virions and with a fast off-rate.	Binding studies: capture assays & SPR. Infectivity: luciferase reporter assay using TZM-b1 cells & p24 HIV antigen ELISA using cell culture supernatant.	[129]
<i>Rhabdoviridae</i>				
Vesiculovirus	VSV	Binding of virions and infected cells with an anti-PS antibody	None provided	[47]
		Binding of infected cells with bavituximab.	Flow cytometry	[171]
<i>Togaviridae</i>				
Alphavirus	CHKV	8-fold enhancement of CHKV pseudovirus entry by hTIM-1	Entry assay using hTIM-1-expressing 293T cells and MLV pseudovirus carrying CHKV GP.	[91]
		3–5-fold enhancement of CHKV pseudovirus entry by hTIM-1	Entry assay using hTIM-1-expressing H3 cells and VSV pseudovirus carrying CHKV env	[128]

continued on next page

Table 1.2 continued

Genus	Species	Details	Experimental evidence	Refs
	EEEV	~15-fold enhancement of EEV pseudovirus entry by hTIM-1	Entry assay using hTIM-1-expressing 293T cells and MLV pseudovirus carrying EEV GP.	[91]
	RRV	Enhancement of entry by hTIM-1	Immunofluorescence using immune ascitic fluids to detect virus levels 2 days after infection of hTIM-1-expressing cells	[91]
	SFV	Increase in exposure of PS from 6–30% on ‘aged’ (post-20 h-incubation) virions	Phospholipase A ₂ degradation.	[5]
	SINV	Enhancement of entry by hTIM-1	Immunofluorescence using immune ascitic fluids to detect virus levels 2 days after infection of hTIM-1-expressing cells	[91]

¹ Taxonomy according to the International Committee on Taxonomy of Viruses, 2014 classification. ² Family names are given in bold italics preceding those of genus and species. ³ Bavituximab, also known as PGN401, is an antibody that binds PS via β 2-glycoprotein 1. ⁴ Entries in bold indicate direct evidence for the involvement of phosphatidylserine in the viral life cycle, as opposed to membrane exposure or virion incorporation without further activity.

CHKV, Chikungunya virus; CMV, cytomegalovirus; EEEV, Eastern Equine Encephalitis virus; HIV, human immunodeficiency virus; HSV, herpes simplex virus; MARV, Marburg virus; MLV, Moloney murine leukemia virus; RRV, Ross River virus; SFV, Semliki Forest virus; SINV, Sindbis virus; VSV, vesicular stomatitis virus. ELISA, enzyme-linked immunosorbant assay; GP, glycoprotein; MDM, monocyte-derived macrophage; PGN, Peregrine Pharmaceuticals; PS, phosphatidylserine; RT, reverse transcriptase; SPR, surface plasmon resonance.

1.5.4 Post-entry and egress

PS and PSRs are also involved in post-entry steps of infection. The presence of TAM receptors on dendritic cells were found to potentiate a post-entry step of HIV-1 pseudovirus infection, rather than affecting binding [17]. Receptor engagement also led to downregulation of type I interferon production.

More surprisingly, expression of TIM-1 in 293T cells enhanced HIV-1 virus entry but also inhibited the release of HIV-1 virions from the cell surface [113]. The net effect was a reduction in total virus production from the TIM-1-expressing cell line, which was dependent on the phosphatidylserine-binding capacity of TIM-1. TIM-1 expression was also observed to induce PS exposure in the absence of any viral infection. Furthermore, the virions produced had incorporated TIM-1 into their envelopes. The other human TIM family members, TIM-3 and TIM-4, had similar effects both with HIV-1 and with Ebola and murine leukemia pseudoviruses. Two additional PSRs, Axl and RAGE, were tested for inhibitory effects: Axl inhibited the production of Ebola only, while RAGE inhibited HIV-1 and Ebola but not MLV production. The authors proposed a mechanism whereby the TIM-1 and other PSRs crosslink to PS; as both the PS and the PSR can be found on the virus and the host cell, the resulting network of interactions would inhibit viral release.

1.5.5 Engagement of PSRs by non-enveloped viruses

Despite the absence of PS on their surface, some non-enveloped viruses also demonstrate a form of ‘non-classical’ apoptotic mimicry [7]. The SV40 major capsid protein VP1 binds Axl and Tyro3 by mimicking the PS-bridging molecule Gas6 [59]. siRNA knockdown of Axl indicated that this receptor had a role both during and after virus entry. Another example is the poliovirus, an enterovirus and thus traditionally classified as non-enveloped. However, it was recently observed to exit cells non-lytically in PS-enriched vesicles, containing multiple virus particles [41]. These vesicles enabled en bloc infection of neighbouring cells and were

therefore more efficient at propagating infection than free virus particles. Thirdly, infection of hepatitis A virus, another non-enveloped virus, is promoted by TIM-1 (also known as HAVCR-1, hepatitis A virus receptor-1) [97]. It is not yet known whether this virus engages TIM-1 using a capsid protein or if it is transferred between cells using a similar vesicular mechanism.

1.6 PS is exposed under conditions of tumourigenesis

PS has been found to be a specific marker of tumourigenesis. Tumourigenic cell lines derived from human melanomas, squamous cell carcinomas, rhabdomyosarcoma, prostate cancer, renal cell cancer and glioblastoma exposed significantly more PS than did cell lines from non-tumourigenic counterparts [152, 184]. The extent of PS exposure was correlated with the level of malignancy: in comparison to non-tumourigenic melanocytes, PS exposure on the SBcl-2 non-malignant cell line was elevated four-fold and exposure on the metastatic lines WM9 and WM164 were elevated up to eight-fold and eleven-fold, respectively [152]. Primary cell cultures from rhabdomyosarcoma, malignant melanoma and glioblastoma also showed increased PS exposure, albeit to a slightly lesser extent than cell lines [152]. This was attributed to the fact that tumours are heterogeneous and contain differentiated, pre-malignant and malignant cells. In none of these cases were the tumour cells found to be apoptotic, as propidium iodide staining verified that the plasma membranes were intact. Furthermore, activated monocytes bound and lysed tumourigenic cell lines in preference to a non-tumourigenic cell line [184].

PS was detected *in vivo* on the vasculature of various solid tumour types in mice, including lymphoma, non-small-cell lung carcinoma, colorectal carcinoma and melanoma, using an anti-PS murine IgM antibody [147]. The percentage of PS-positive vessels was 10–40% in tumours, while no staining was seen of vessels in non-neoplastic organs. Whole organism imaging of rats using radiolabelled bavituximab, another PS-targeting antibody (Section 1.7), showed specific localisation of bavituximab to prostate tumours [92].

PS exposure on the outer membrane leaflet appears to be one mechanism of immune evasion by tumours, via apoptotic mimicry. PS binding by macrophages and dendritic cells triggers

the immunosuppressive response detailed in Section 1.3 [100]. In addition to membrane-localised PS, there is evidence for soluble PS [100] and PS-containing microvesicles [99] in the tumour environment. The latter were found to inhibit T cell activation by interrupting the TCR signalling cascade [99] (Section 1.3).

Much of the evidence for the role of PS in downregulating tumour recognition and removal has been provided by PS blockade. Antibody targeting of CD300a and CD300c, related proteins that bind PS and PE (Section 1.4.4), increased NK-cell-mediated killing of tumour cells *in vitro* [106]. Use of MFG-E8 to compete for PS indicated that this inhibitory effect was at least partly due to PS binding; however, an additional ligand, probably PE, seemed to be involved in this process, as competing for PS alone provided incomplete inhibition. When MFG-E8 itself was used in conjunction with chemotherapy drugs, a greater effect on tumour cell killing was observed with the combination therapy compared to either agent alone. Specifically, the effects included increased levels of tumour apoptosis, as measured by caspase 3 activity, decreased numbers of Treg cells, increased IFN- γ production from T cells, and increased production of IL-12, IL-23 and TNF- α from dendritic cells [94]. The same study also showed that addition of the anti-MFG-E8 antibody switched the MFG-E8 receptor from $\alpha_v\beta_{3/5}$ integrins to Fc receptors, thereby promoting Fc receptor-mediated uptake of treated tumour cells and stimulating an immune response. Finally, anti-PS antibodies such as bavituximab have successfully been used to target tumours, a strategy discussed in Section 1.7.

1.7 Targeting PS in a clinical context

1.7.1 Development of bavituximab

Phosphatidylserine is an attractive target for anti-cancer and anti-viral agents due to its specific exposure on pathological cells i.e. virus-infected, tumourigenic or apoptotic cells. This concept is already being exploited by Peregrine Pharmaceuticals, a California-based company developing monoclonal antibodies against cancer and viral infections. Their lead product is

bavituximab, also referred to as PGN401, which is a chimeric monoclonal antibody (mAb) directed against $\beta 2$ -GP1. Phase III clinical trials are currently underway for bavituximab in combination with docetaxel in the treatment of non-small cell lung cancer (NSCLC) [183]. Additional clinical trials of bavituximab in combination with other chemotherapy drugs for treatment of HER2-negative metastatic breast cancer, melanoma, rectal cancer and liver cancer are in Phase I and Phase II stages [139].

Bavituximab was derived from the mouse monoclonal 3G4, a mouse IgG3 mAb which binds domain II of $\beta 2$ -GP1 [115, 181]. A related antibody, 2aG4, was produced by fusing the 3G4 variable regions to a murine IgG2a heavy chain constant region, which increased its affinity for murine Fc γ receptors (Fc γ R). Bavituximab was developed for use in humans and contains the 3G4 mouse variable regions fused to human IgG1 heavy chain and Ig κ light chain constant regions (Figure 1.7A). Domain V of $\beta 2$ -GP1 interacts with PS; this family of antibodies thus indirectly coordinate 2 $\beta 2$ -GP1 molecules clustered on a PS-presenting bilayer as shown in Figure 1.7B. The K_D for bavituximab binding to $\beta 2$ -GP1 seems to be < 1 nM [115, approximation from graphical data]; this is a much higher affinity than reported for the binding of $\beta 2$ -GP1 alone to PS (Section 1.4.2). Bavituximab recognises rat and human $\beta 2$ -GP1 with equal affinity, but does not bind to murine $\beta 2$ -GP1; experiments in mice therefore involved coadministration of human $\beta 2$ -GP1 with the relevant PS-targeting mAb [206].

1.7.2 Oncological applications

Bavituximab and its antibody relatives have been demonstrated both *in vitro* and *in vivo* to have anti-tumour and anti-viral activity, although the leading pre-clinical and clinical evaluations are in treatment of solid tumours. In mice, administration of 3G4 or 2aG4 reduced tumour growth, both when administered alone [146] or in combination with docetaxel [84, 206]. The combination therapy was significantly more effective than either agent alone and, in both cases, the mice receiving combination therapy had increased survival rates. Tumour shrinkage seemed mainly to be due to destruction of tumour vasculature: histologic examination of

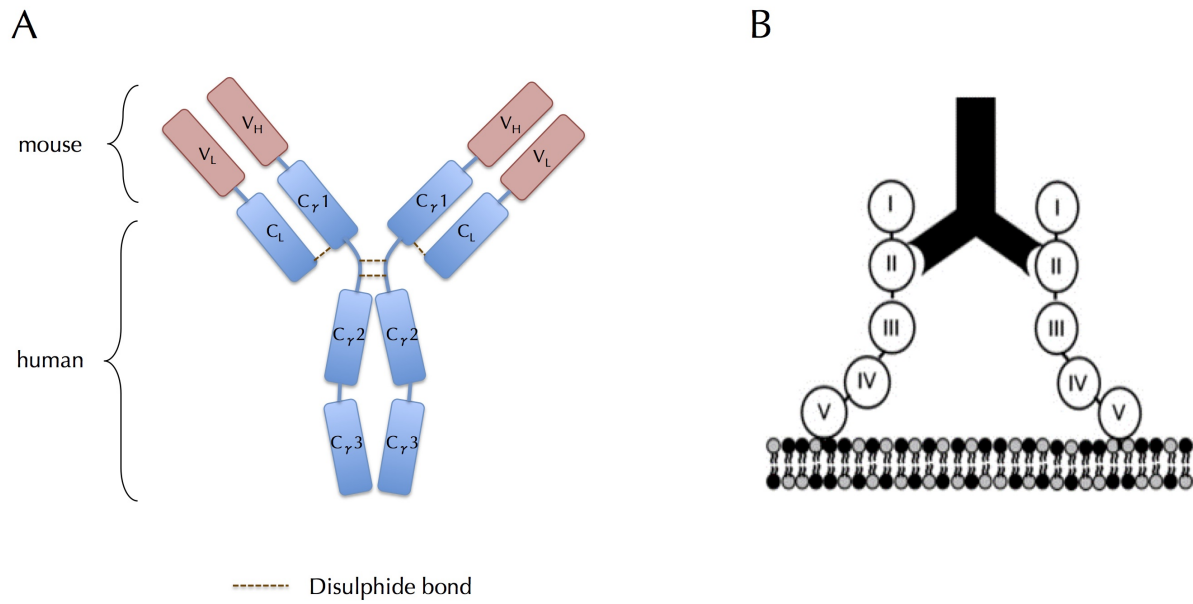


Figure 1.7: Mode of bavituximab–phosphatidylserine interaction, via β 2-glycoprotein 1. **A|** Bavituximab is a chimeric antibody with mouse variable and human constant regions. **B|** Bavituximab coordinates two molecules of β 2-glycoprotein 1, which in turn bind anionic phospholipids (grey). Subfigure B reproduced with publisher’s permission from Thorpe (2010) [181] (license number 3701541000459).

tumours from treated mice showed vascular collapse between 2 and 6 days, followed by necrosis of the solid tumour [80, 181].

The immunological mechanisms behind the activity of these anti- β 2-GP1 antibodies are varied, but seem to be predominantly antibody-dependent cellular cytotoxicity (ADCC) [171] (Section 1.8). The localisation of effects to tumour vasculature, when used in combination with radiotherapy or chemotherapy, is partly due to the increased propensity of endothelial cells to expose PS compared to other cell types. For example, irradiation of human umbilical endothelial cells (HUVECs) triggered PS exposure, while irradiation of A549 lung cancer cells did not [80]. Subsequent incubation of the treated cells with 2aG4 and macrophages led to specific lysis of the HUVECs and not of the A549 cells.

Furthermore, tumour infiltrates from 2aG4 or 3G4 treated mice showed decreased numbers of myeloid-derived suppressor cells (MDSCs) and M2 (anti-inflammatory, pro-tumour) macrophages, along with increased numbers of M1 macrophages (pro-inflammatory, anti-tumour) [206]. One likely reason for this change is 2aG4/3G4 stimulation of MSDC differentiation into M1 macrophages and dendritic cells (DCs), as discovered from *in vitro* assays. The antibodies were also found to repolarise M2 macrophages to an M1 phenotype, mediated by antibody binding to vesicles associated with the macrophage surface. Macrophages incubated with these antibodies produced more NO, higher amounts of pro-inflammatory cytokines IL-12 and TNF- α , and increased levels of T cell stimulatory molecules. Both cell lysis [80] and macrophage repolarisation [206] were dependent on the Fc portion of the 2aG4 antibody used; this indicates that the antibody is binding to PS, preventing its recognition by PSRs and blocking its anti-inflammatory signals. Instead, the antibody Fc is binding FcRs, thereby stimulating an immune response. Finally, anti-PS antibodies stimulate dendritic cell maturation and antigen presentation: treatment of F98 glioma cells prior to incubation with dendritic cells and T cells improved rates of F49 cell lysis [181].

1.7.3 Anti-viral applications

The anti-viral effects of 2aG4, 3G4 and bavituximab have mainly been demonstrated *in vivo* using animal models. Treatment of Pichinde virus-infected guinea pigs with bavituximab significantly improved survival compared to a control group [171]. The effect of bavituximab was comparable to ribavirin, the current standard treatment for Lassa fever virus, and a combination treatment of ribavirin plus bavituximab was more effective than either agent alone. Bavituximab treatment also markedly improved survival from 25% to 100% in mice given an 80% lethal infectious dose (LD₈₀) of mouse cytomegalovirus (mCMV). In Punta Toro virus-infected hamsters, similar improvements in survival were seen following treatment with bavituximab and with a bavituximab/ribavirin combination treatment [47].

Analysis of the plasma from the treated guinea pigs showed that bavituximab did not affect levels of anti-Pichinde antibodies or the proliferative ability of splenocytes; however, it did reduce plasma viral titres. One mechanism of action of these antibodies therefore seems to be opsonisation and clearance of virus from the bloodstream. The second mechanism is antibody-mediated cellular cytotoxicity of infected cells: evidence for this was derived *in vitro* by comparing cytotoxicity of Pichinde virus-infected fibroblasts with and without bavituximab treatment. Bavituximab significantly increased the levels of ADCC by the peritoneal effector cells [171]. Further specific examples for the anti-viral activity of PS-targeting antibodies are provided in Table 1.2.

1.8 Importance of receptor binding for antibody effector functions

1.8.1 Antibody structure

Knowledge of the antibody structure-function relationship is important for the development of therapeutic mAbs, such as bavituximab, with maximal desired efficacy and minimal off-target effects. All antibodies, or immunoglobulins (Ig), share the same structural basis, being

composed of two identical heavy polypeptide chains and two identical light polypeptide chains [1]. These polypeptide chains fold into repeating units of 110 amino acids, termed 'Ig domains': two layers of β -pleated sheets held together by disulphide bonds. The chains can be divided into a variable region, which confers antigen specificity, and a constant region that dictates the antibody isotype. Four heavy chain types are found in humans: α (alpha), δ (delta), ϵ (epsilon), γ (gamma) and μ (mu), which give rise to the corresponding isotypes IgA, IgD, IgE, IgG and IgM [188]. Only two light chains, λ (lambda) and κ (kappa), are expressed; these can combine with any one heavy chain type. The Ig isotypes differ in form and function, as detailed in Table 1.3.

The focus of this discussion will be human IgG, due to its potency in activating an immune response, its substantially greater serum concentration compared to the other isotypes, and the fact that the majority of licensed therapeutic antibodies, plus some at the pre-clinical stage such as bavituximab, use the IgG format [88]. There are four human IgG subtypes, designed 1–4, which differ primarily in the hinge region (Figure 1.8). The structure of a human IgG1, the anti-HIV antibody B12, is shown in Figure 1.9. This structure illustrates the characteristic 'Y' shape of antibodies: the ends of the two F(ab) domains form the antigen-binding regions while the Fc region mediates effector functions through interactions with Fc receptors (FcRs) or complement.

1.8.2 Effector functions of IgG antibodies

Antibodies have multiple effector functions *in vivo*, which are described in further detail in this section. A discussion of mAb effector functions in a therapeutic context can be found in Section 1.9.

Neutralisation

Neutralisation involves specific binding to the pathogen and subsequent reduction of its infectivity [1]. This process is best exemplified by anti-viral antibodies, which bind either to

Table 1.3: Human antibody isotypes.

Isotype	Subtypes	Serum conc. (mg/mL) ¹	Secreted form ²	Functions ²
IgA	IgA1, 2	0.54–4.04 Mean 1.88	Monomer, dimer, trimer	Mucosal immunity Complement activation by lectin or alternative pathways
IgD	Single	<0.01–0.11 Mean 0.02	Monomer	Naïve B cell antigen receptor
IgE	Single	0.02–5.49 Mean 0.55	Monomer	Defence against helminths Immediate hypersensitivity
IgG	IgG1–4	5.81–16.30 Mean 10.20	Monomer	Opsonisation of antigens Complement activation via classical pathway Antibody-dependent cell-mediated cytotoxicity Feedback inhibition of B-cells Neonatal immunity
IgM	Single	0.37–1.95 Mean 1.01	Pentamer	Naïve B cell antigen receptor Complement activation

¹ Serum concentrations for IgM, IgG and IgA are the means for adults of both genders derived from values in ref.[177], Table 1. Serum concentrations for IgD and IgE from ref. [31].

² Information for form and function sourced from refs. [1] and [39].

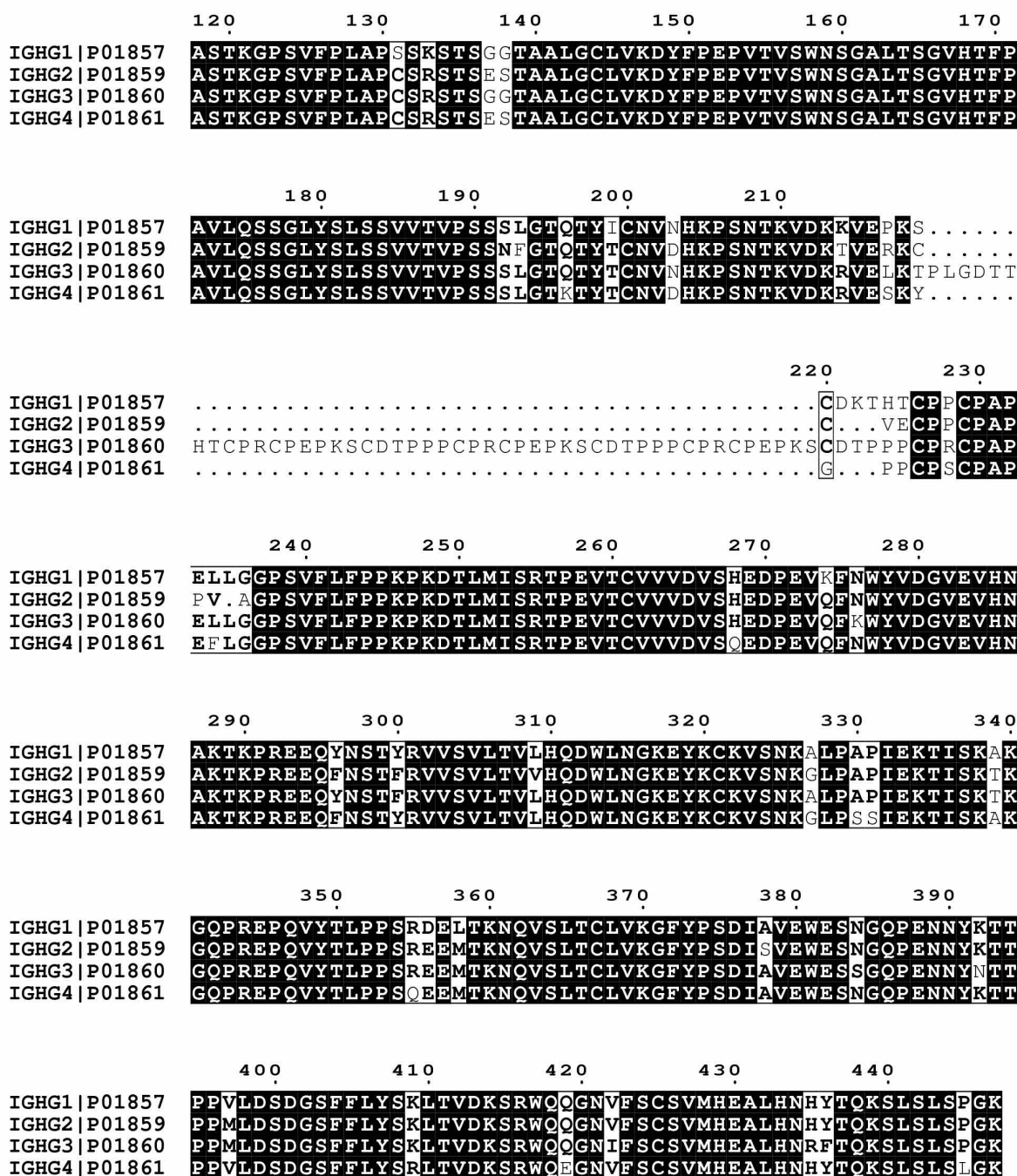


Figure 1.8: Sequence alignment of the human IgG heavy chain constant regions (CH1, hinge, CH2 and CH3). The relevant UniProt identifiers are given after each antibody subclass. Sequence numbering is given for IgG1 following the Eu convention [60]. Alignment was done with T-Coffee [54] and shading according to physicochemical properties with ESPrpt 3.0 [153]. Strictly conserved residues are white with a black background, residues with a similarity score > 0.7 in bold black and residues with a similarity score < 0.7 in regular black.

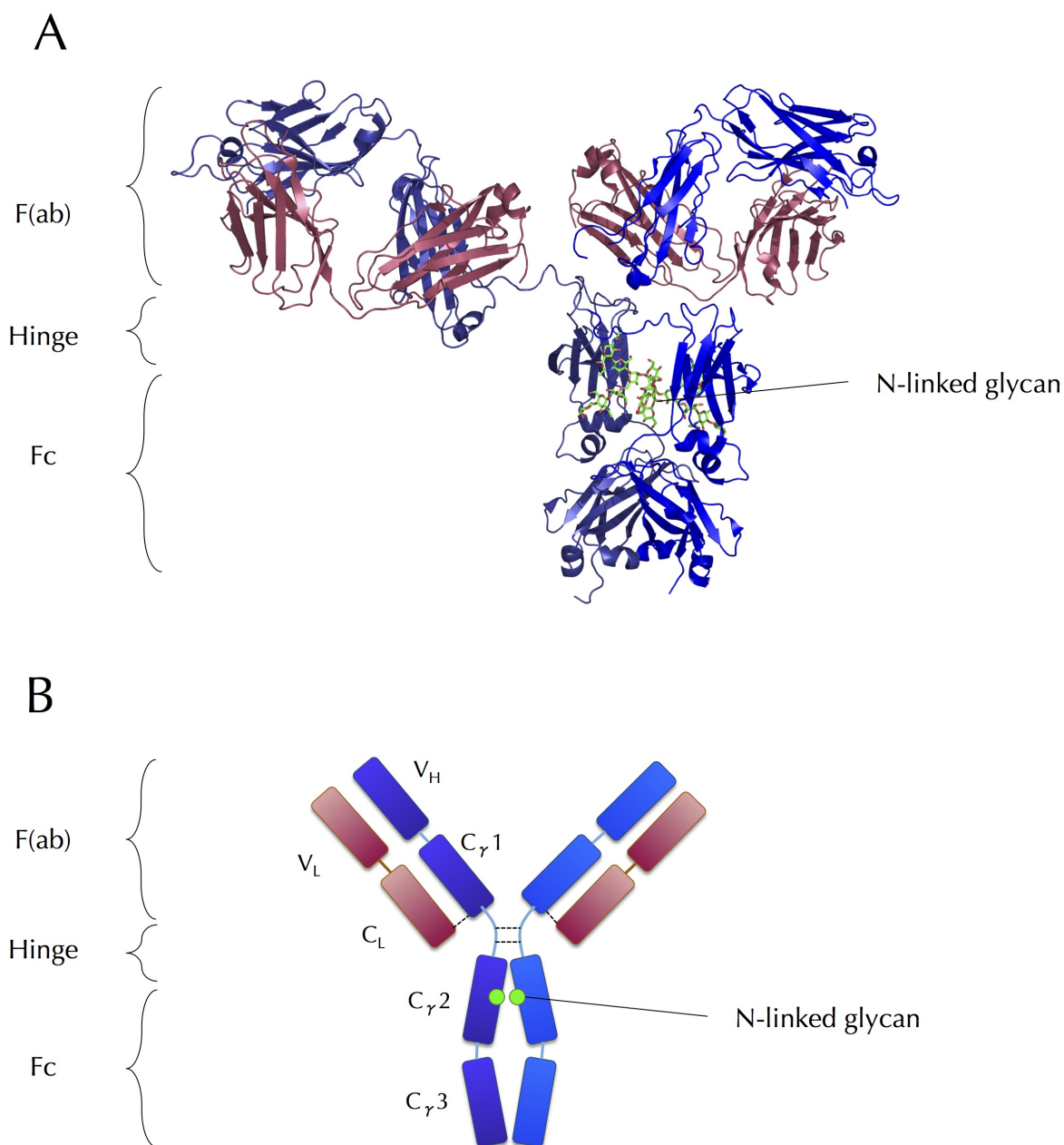


Figure 1.9: Structure of human IgG1 antibody B12. **A**| Cartoon representation of the human IgG1 antibody B12 crystal structure (PDB 1HZH). Heavy chains are coloured dark blue and light blue, light chains are coloured dark pink. The N-linked glycans on the $C_{\gamma 2}$ domain are in stick representation with carbons in green, nitrogens in blue and oxygens in red. The F(ab), hinge and Fc domains are indicated with curly braces. **B**| Schematic of a human IgG antibody using the same colouring as panel A. Each box represents an Ig domain fold, with the variable and constant domains of one heterodimer labelled accordingly. The positions of the N-linked glycans are shown with green circles. Disulphide bonds are indicated using black dotted lines.

viral coat proteins involved in cell binding or entry, or their cellular receptors. The antibodies thereby block membrane fusion, in the case of enveloped viruses, or cell penetration, in the case of non-enveloped viruses [48]. Antibodies can also neutralise bacterial toxins such as tetanus toxin, which binds to neuromuscular junctions and inhibits neurotransmission [1, 118]. Neutralisation is dependent only on the F(ab) regions of the antibody and can be carried out by antibodies of any subclass.

Opsonisation

Opsonisation is the process of coating pathogens with antibodies to promote phagocytosis [1]. Mononuclear phagocytes and neutrophils express FcRs that bind immunoglobulins, stimulating pathogen uptake and degradation. The most efficient receptors involved in phagocytosis are Fc γ RI, the high affinity receptor expressed on phagocytes and neutrophils, and Fc γ RIIA and Fc γ RIIC, lower affinity receptors also expressed on these cell types (Section 1.8.3). Opsonisation may also occur with the complement protein C3, which is also recognised by phagocytes.

Antibody-dependent cell-mediated cytotoxicity

Antibody-dependent cell-mediated cytotoxicity (ADCC) was first described as a response primarily executed by natural killer (NK) cells via Fc γ RIIIA. Receptor binding stimulates NK cells to secrete pro-inflammatory cytokines such as IFN- γ and to discharge the cytotoxic contents of their granules [1]. Recently, further studies in mouse models indicate that monocytes and macrophages may instead be the main effector cells of ADCC, as removal of the inhibitory Fc γ RIIB, which is not expressed on most NK cells, had a positive effect on cytotoxic antibody activity [133].

Activation of the classical complement pathway

The complement system is a set of proteases that become stably activated when attached to microbial surfaces or to antibodies [1]. The classical pathway is antibody-dependent and is initiated by the binding of protein C1q to the Fc domains of IgG–antigen complexes. This leads to the cleavage and activation of C4 and C2 to form the C3 convertase complex. Subsequent cleavage of C3 into C3a and C3b triggers a variety of immunological functions. For example, C3b is a ligand for phagocyte receptors; additionally, the peptides derived from proteolysis of C3 and other complement proteins are pro-inflammatory. Components C5–C9 form the membrane attack complex (MAC) which, once deposited, enables lysis of the target cell. This is termed complement-dependent cytotoxicity (CDC).

The complement cascade requires clustering of Fc domains and is therefore efficiently triggered by one surface-bound IgM or several IgG molecules [124]. Out of the IgG subtypes, IgG1 and IgG3 are more efficient initiators than IgG2 or IgG4 [188]. This is mainly due to reduced binding of the latter subtypes. Binding of C1q is dependent on residues Leu235, Asp270, Lys322, Pro239 and Pro331; in IgG2, Leu235 is mutated to alanine while in IgG4, Pro331 is replaced by serine (Figure 1.8).

1.8.3 Fc receptors

The Fc gamma receptors (Fc γ Rs) have different expression patterns on leukocytes and varying affinities for IgG. A schematic diagram of human Fc γ Rs and the neonatal receptor FcRn is given in Figure 1.10. The α chain of each receptor contains either two or three immunoglobulin-like extracellular domains and is required for ligand binding; in Fc γ RII it is also responsible for signal transduction. In other receptors the α chain is associated with additional γ chains that mediate signal transduction, or glycosylphosphatidylinositol (GPI) in the case of Fc γ RIIIB [1]. FcRn is the exception in that its α chain resembles that of MHC class I molecules and is associated with β 2-microglobulin [154]. Binding of immune complexes causes receptor

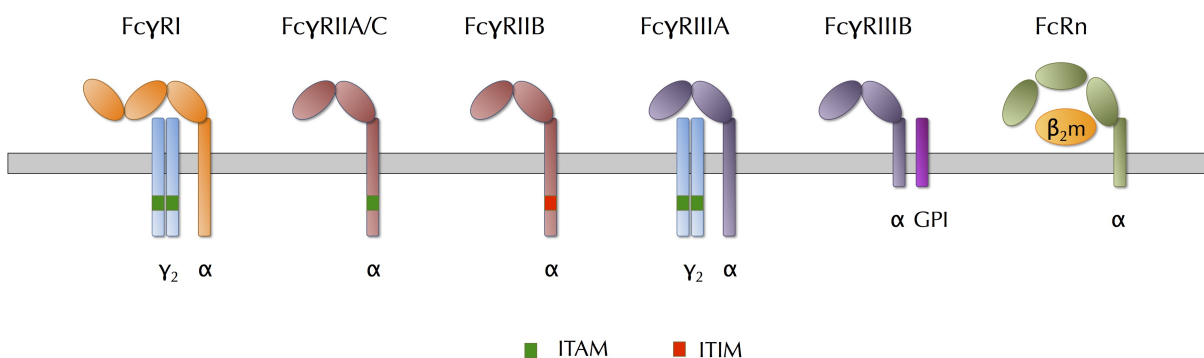


Figure 1.10: Schematic diagram of the domain structure of human FcγRs [133, 135]. Individual receptors are shown inserted into a plasma membrane (grey). The intracellular signalling motifs ITAM (immunoreceptor tyrosin-based activation motif) and ITIM (immunoreceptor tyrosine-based inhibition motif) are given as green and red squares. FcRn is associated with a β_2 -microglobulin subunit.

clustering in the plane of the membrane and triggers phosphorylation of the immunoreceptor tyrosine-based activation or inhibition motifs (ITAM or ITIMs). These motifs then recruit protein tyrosine kinases which mediate downstream signalling. These receptors' affinities for IgG, cell distributions and functions are given in Table 1.4.

The interaction site for classical FcγRs is in the lower hinge region of IgG1. This is exemplified by the crystal structures for the IgG1–FcγRIII complexes [66, 145, 175], for which an illustration is given in Figure 1.11. The receptor binds IgG asymmetrically in a 1:1 stoichiometry, causing an opening of both the FcγRIII D1–D2 interdomain angle and of the IgG Cγ2 domains [201]. The D2 domain of FcγRIIIA makes extensive contacts with the lower hinge and upper Cγ2 regions of IgG, particularly the IgG residues Leu234 to Pro238, Pro329 and D265. Additionally, the FcγRIIIA glycan attached to Asn162 interacts with the Asn297 glycan on Cγ2 chain B [66].

The IgG residues involved in FcγR and FcRn binding were confirmed by site-directed mutagenesis of IgG1 followed by analysis of binding to Fc receptors [166]. Importantly, mutations E233P, L234V, L235A and G236X (where X indicates a deletion) were chosen to create a lower hinge resembling that of IgG2; these mutations caused significant reduction in binding to all FcγR, in agreement with crystal structure data. Furthermore, some mutations were found

Table 1.4: Characteristics of the human FcγRs and their binding affinities for IgG.

FcγR	Isoforms	Classification	Subclass preference	Allele	K _D (μM)	Cell distribution	Function
FcγRI (CD64)	-	Activatory	1 = 3 > 4	-	0.015	Macrophages, neutrophils, dendritic cells, eosinophils	Phagocytosis, phagocyte activation
FcγRII (CD32)	A	Activatory	3 > 1 > 4 = 2	H131	0.19	Macrophages, neutrophils, dendritic cells, eosinophils, platelets	Phagocytosis, phagocyte activation (inefficient)
				R131	0.29		
				-	8.3	B cells, dendritic cells	Feedback inhibition
FcγRIII (CD16)	A	Activatory	3 > 1 >> 4 > 2	V158 F158	0.50 0.85	NK cells	ADCC
				NA1 NA2	5.0 5.0	Neutrophils	Phagocytosis (inefficient)
FcRn	-	-	N/A	-	0.13	Macrophages, neutrophils, dendritic cells, endothelium	Recycling Neonatal immunity

Subclass preferences from refs. [30] and [188]. K_D values are calculated for IgG1 using ref. [29]. All other information from [1] and [29].

to selectively improve binding to particular receptors: for example, S298A was found to reduce binding to Fc γ RII and increase binding to Fc γ RIIIA, while E333A, K334A and A339T all had no effect on binding to Fc γ RII but increased binding to Fc γ RIIIA.

1.8.4 Fc glycosylation

The Asn297-linked glycan on IgG is integral to the structure and effector functions of the antibody. IgG Fc glycans are typically of the complex and biantennary type, containing a high proportion of core fucose, partial galactosylation and bisecting *N*-acetyl-D-glucosamine (GlcNAc); up to 20% are sialylated [50]. Removal of the Asn297 glycan, either enzymatically (deglycosylation) or by expression in bacterial systems (aglycosylation), results in a more ‘closed’ conformation due to inward movement of the C γ 2 regions compared to the natively-glycosylated structure [20, 104]. However, in-solution structural analysis using small-angle X-ray scattering (SAXS) found the aglycosylated form to take a more open conformation [20]. The authors suggest that, rather than the closed conformation being predominant, loss of the oligosaccharide instead results in increased flexibility. This reasoning is consistent with evidence showing a decreased thermal stability of deglycosylated C γ 2 domains [125, 210]. Concomitant with the structural alterations, deglycosylated IgG has reduced affinity for all classical Fc γ R and also for FcRn [90, 166]. The decrease in receptor binding translates into a loss of effector function, such as ADCC and CDC.

With regard to specific glycan structures, absence of the core fucose has been found to increase binding to Fc γ RIIIA up to fifty-fold; as a results, the afucosylated antibodies were more potent inducers of ADCC *in vitro* [165]. The ‘fucose effect’ can be rationalised by comparison of the structures of afucosylated and fucosylated IgG1 Fc bound to Fc γ RIIIA: a steric clash between the Fc fucose and the Fc γ RIIIA Asn162 oligosaccharide reduces the overall stabilising carbohydrate-carbohydrate interactions between antibody and receptor [66]. Finally, the role of sialic acid may be immunomodulatory, as hypersialylation decreases the cytotoxic effect of antibodies *in vivo* [96]. Moreover, the anti-inflammatory effect of IVIg is due specifically to

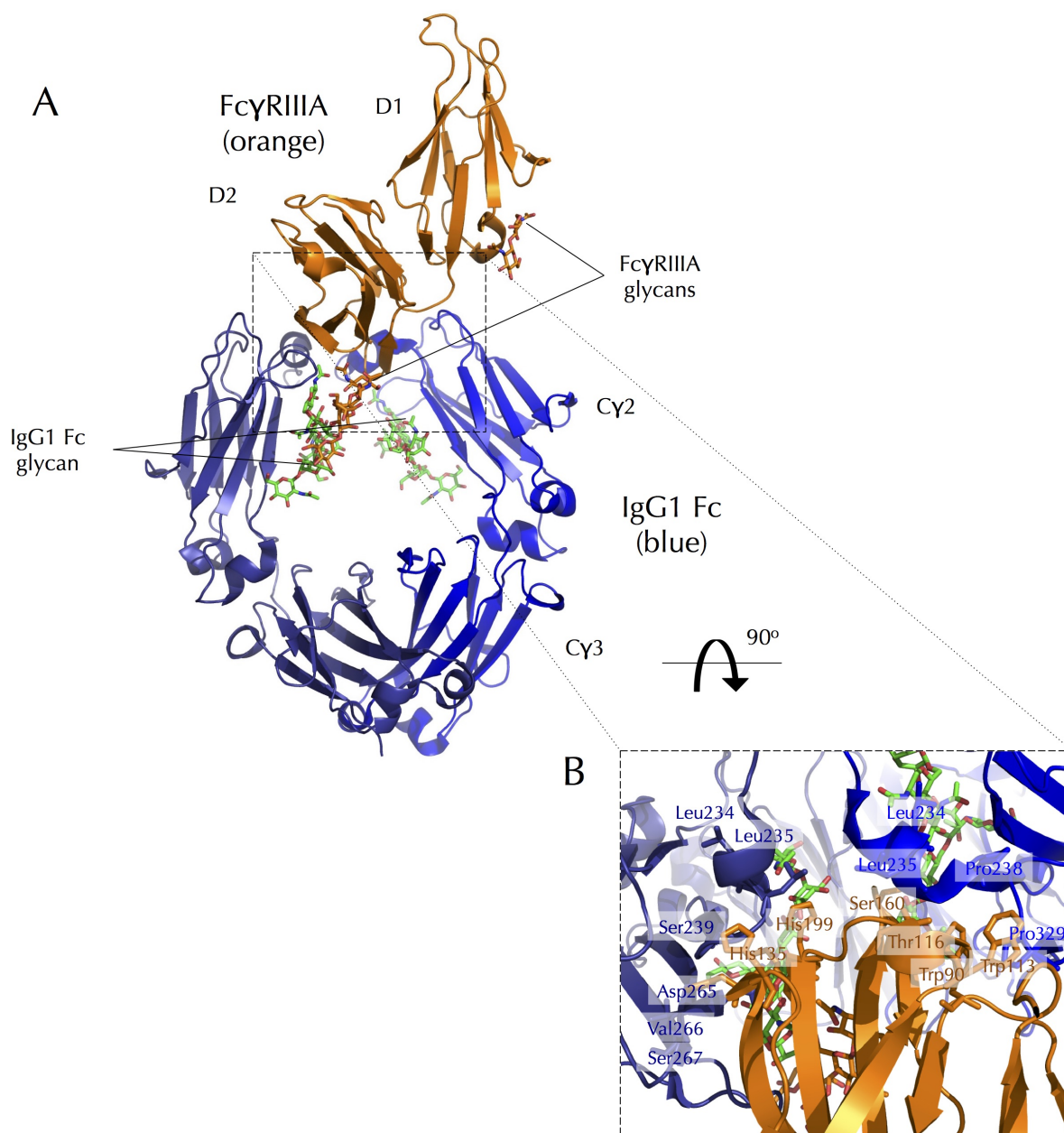


Figure 1.11: Crystal structure of the human IgG1 Fc–FcγRIIIA complex (PDB 3SGJ). **A** | Both Fc and FcγRIIIA are shown in cartoon representation. Fc heavy chains are coloured dark blue and light blue with the Cγ2 and Cγ3 domains labelled. FcγRIIIA is coloured orange with the D1 and D2 Ig-like domains labelled. Glycans are given in stick representation with nitrogens in blue, oxygens in red, and carbons in green or orange for Fc and FcγRIIIA, respectively. **B** | Close-up view from above of the binding interface between IgG1 and FcγRIIIA. Chains and glycans are coloured as in panel A. Residues involved in the interaction are given in stick representation and labelled in text colours to match that of the relevant chain.

α 2,6-linked sialic acid. This appears to be due not to a reduction in Fc γ R binding but possibly mediated by interaction with other receptors such as siglecs (sialic acid-binding Ig-like lectins) [207].

1.9 Optimising the efficacy of therapeutic antibodies

1.9.1 Mechanisms of action of therapeutic antibodies

The majority of monoclonal antibodies (mAbs) licensed for therapeutic use are chimeric, humanised or fully human antibodies of the IgG isotype [88]. Many of these mAbs are thought to have multiple mechanisms of action, not all of which are mediated by the immune system. One well-established function in the latter category is the perturbation of signalling within tumour cells. For example, trastuzumab (Herceptin[®]) binds to the growth factor HER2 on the plasma membrane and inhibits its dimerisation and thereby its signalling [73]. Binding also decreases HER2 expression by triggering internalisation and/or shedding. Other antibodies promote apoptosis by interacting with the TRAIL receptors on the cell surface, stimulating the extrinsic apoptotic pathway.

As with endogenous IgG, therapeutic mAbs can promote ADCC and CDC through interactions with Fc γ Rs and C1q. These are thought to be the primary mechanisms of action for anti-cancer mAbs. Moreover, ADCC may be the predominant mechanism *in vivo* as tumour cells are known to express higher levels of complement inhibitors [187]. For example, trastuzumab and rituximab (Rituxan[®]) have been shown in mouse models to be dependent on Fc γ Rs for their anti-tumour activity [43]. Moreover, patients homozygous for the Val158 polymorphism on Fc γ RIIIA, which increases affinity for IgG1 (Table 1.4), had greater response rates to rituximab than patients who were carriers for Phe158 [197]. Rituximab treatment also promotes CDC, as complement proteins colocalise with rituximab on the cell surface, and the blocking of complement inhibitors CD55 and CD59 enhance the activity of rituximab *in vitro* [189]. Interestingly, ADCC and CDC do not appear to be synergistic: the deposition of complement

proteins, specifically C3b, on rituximab-coated cells inhibited NK cell-mediated ADCC. This indicates that findings from *in vitro* ADCC assays should be interpreted with caution, as they are often performed in media depleted of complement and with higher effector:target cell ratios than *in vivo* [189].

Both ADCC and CDC are affected by antigen density and antibody affinity. Alemtuzumab (Campath-1H) is an anti-CD52 mAb used for the treatment of T and B cell leukemia; CDC, ADCC and apoptosis have all been proposed as mechanisms of alemtuzumab-mediated cell lysis. Flow cytometric analysis of lymphoid cells from patients treated with alemtuzumab found that CD52 expression levels were higher in patients who responded to antibody therapy than in non-responders [72]. Additionally, a comparison of two anti-Ep-CAM antibodies of differing affinities discovered that levels of ADCC were higher with the use of the higher-affinity antibody and also with the upregulation of Ep-CAM on target cells [187]. Levels of rituximab-mediated CDC were also observed to correlate with expression of the CD20 antigen on B cells [189]. The abundant expression of a target antigen is therefore an important factor in the success of therapeutic antibodies, and provides some explanation for the multiplicity of clinically approved mAbs targeting the same antigen (Section 1.1).

Finally, the timing of therapeutic antibody administration may be an important determinant of clinical outcome. The majority of evidence for this seems to be in the treatment of infectious disease, particularly passive antibody therapy. The efficacy of serum therapy was found to diminish rapidly after the onset of symptoms [35]—for example, in patients with pneumococcal pneumonia, serum therapy was most effective if provided within 3 days of disease onset [36].

1.9.2 Receptor binding of mAbs *in vivo*

The antibody concentration required for clinical efficacy is frequently higher than that predicted from *in vitro* work. To use trastuzumab as an illustration, *in vitro* studies found that the use of 0.1 µg/mL anti-HER2 antibody effectively stimulated ADCC, with the efficacy

proportional to the level of HER2 expression on the cell line [112]. In contrast, at therapeutic doses the serum concentrations of trastuzumab are approximately one-hundred-fold higher, reaching a peak of 123 $\mu\text{g/mL}$ [74]. Preithner *et al* discovered that this was due to the presence of competing IgG in human serum, which significantly reduced the induction of ADCC by trastuzumab [143]. The inhibitory effect was especially pronounced at low antigen surface densities—for instance, the cell line KATO III, which expresses high levels of Ep-CAM and low levels of HER-2, was effectively lysed by both the anti-Ep-CAM antibody MT21 and trastuzumab. However, the addition of 50% human serum resulted in a greater reduction in trastuzumab-mediated lysis than MT21-mediated lysis.

Mechanisms to overcome this are being explored *in vitro*. A study using an antibody targeting Lewis-Y, a tumour-associated carbohydrate antigen, demonstrated a reduced susceptibility to serum inhibition of ADCC with an afucosylated variant of the antibody [132]. This effect was attributed to the higher affinity for Fc γ RIIIA seen with IgG lacking fucose, compared to natively glycosylated IgG (Table 1.4). Another option is to reduce the levels of serum IgG, allowing therapeutic mAbs to bind the receptors. Baruah *et al* demonstrated this concept with EndoS, a bacterial enzyme from *Streptococcus pyogenes* that cleaves complex glycans from IgG but has reduced activity with oligomannose glycans [12]. As discussed in Section 1.8.4, deglycosylation reduces IgG binding to Fc γ Rs. Accordingly, pre-treatment of serum with EndoS restored binding of an EndoS-resistant mAb almost to levels in the absence of serum. This approach could similarly be applied using other methods to abolish the binding of endogenous IgG, such as IdeS, an IgG-specific protease which will be discussed in the next section.

1.10 IdeS is a potential tool for modulating antibody efficacy

1.10.1 Enzymatic activity of IdeS

IdeS is a cysteine protease produced by *Streptococcus pyogenes*. First discovered in 2000 from a screen of Group A *Streptococcus* (GAS) culture supernatant proteins [111], it was found to

Table 1.5: Dissociation constants and enzyme kinetic parameters for activity of IdeS with human immunoglobulin G subclasses.

Immunoglobulin		K_D (μM)	K_m (μM)	$V_{\max}$ (nmol min ⁻¹)	k_{cat} (s ⁻¹)
IgG1	full-length	2.6	6.8	0.13	0.12
	Fc	5.6	NA	NA	NA
IgG2		32	18.9	0.09	0.02
IgG3		1.5	13.9	0.09	0.08
IgG4		3.0	11.9	0.12	0.10
IgM		35	NA	NA	NA
IgA		ND	NA	NA	NA

NA, not available; ND, not detectable. Data from ref. [3].

have sequence homology to the α -subunit of the human Mac-1 protein (CD11b). It was also identified independently as an IgG-cleaving protease and denoted IdeS (Immunoglobulin G-degrading enzyme of *Streptococcus pyogenes*) [196] and as Streptococcal immunoglobulin-binding protein 35 (Sib35) [98].

IdeS proteolytic activity is strictly restricted to IgG [196]. It binds to and cleaves all subclasses of human IgG, although it exhibits approximately ten-fold lower affinity for IgG2 [3]. The lower affinity for IgG2 is due to substantial differences in the cleavage site which is located between Gly236 and Gly237 (Eu numbering) in the lower hinge of the immunoglobulin (Figure 1.12). Binding affinities and kinetic parameters for human immunoglobulins are given in Table 1.5. The affinities of IdeS C94A, a catalytically inactive mutant, for IgG1 full length and IgG Fc were almost identical to wild type IdeS at 5.6 μM and 2.6 μM respectively [3]. IdeS is also active against monkey and rabbit IgG1, which share the consensus amino acid sequence ²³²PELLGG²³⁷ which spans that cleavage site and serves as a recognition sequence for the enzyme (Figure 2). Mouse and pig IgG1, which lack this sequence, were not cleaved under the assay conditions used.

Products of IdeS proteolysis are a F(ab')₂ of ~112 kDa and two ½Fc of ~25 kDa each. Interestingly, the initial cleavage of the first heavy chain proceeds approximately 100-fold

P01857|IGHG1_HUMAN EPKS.....
P01859|IGHG2_HUMAN ERKC.....
P01860|IGHG3_HUMAN ELKLTPLGDTTHTCPRCPEPKSCDTPPPCPRCPEPK
P01861|IGHG4_HUMAN ESKY.....

220
230
▼

P01857 IGHG1_HUMAN		C	DKTHT	CP	CP	CP	CP	AP	ELL	LG	GP
P01859 IGHG2_HUMAN		C	...VE	CP	CP	CP	CP	AP	VPV	AG	GP
P01860 IGHG3_HUMAN	SCDTPPPCPRCPEPKS	C	DTPPP	CP	R	CP	AP	ELL	LG	GP	GP
P01861 IGHG4_HUMAN		G	...PP	CP	S	CP	AP	EFL	LG	GP	GP

▲

Figure 1.12: Sequence alignment of the hinge regions from human IgG. The names of the antibody subclasses follow their respective UniProt identifiers. Alignment was done with T-Coffee [54] and shading according to physicochemical properties with ESPript 3.0 [153]. Strictly conserved residues are white with a black background, residues with a similarity score >0.7 in bold black and residues with a similarity score <0.7 in regular black. Numbering refers to IgG1 sequence (Eu numbering). The cleavage site between Gly236 and Gly237 is marked with red triangles.

		220		230	
HUMAN_IGHG1 P01857	EP	KSC	D...KTH	CP	P
MONKEY_IGHG AAA18103.1	EI	KTC	GGGSKPPR	CP	P
RABBIT_IGHG P01870	AP	STC	S...K...	P	T
MOUSE_IGHG1 P01868	VP	RDC	G...C.KP	C	I
PIG_IGHG K7ZLA7	GT	KTK	PP	CP	I

Figure 1.13: Comparison of the IgG1 hinge region from various mammalian species. Alignment was done with T-Coffee [54] and shading according to physicochemical properties with ESPript 3.0 [153]. Strictly conserved residues are white with a black background, residues with a similarity score > 0.7 in bold black and residues with a similarity score < 0.7 in regular black. UniProt identifiers are given after the vertical stroke, except for monkey IgG for which the GenBank ID is given. Numbering refers to the human IgG1 sequence (Eu numbering).

faster than that of the second chain (kcat values 10.1 s^{-1} and 0.10 s^{-1} respectively) [155, 192]. The resulting singly-cleaved IgG (scIgG) runs at approximately 138 kDa on denaturing non-reducing SDS-PAGE gels [26]. scIgG is indistinguishable from intact IgG (iIgG) using some non-denaturing biophysical techniques such as HPLC and gel filtration but can be separated from iIgG, F(ab')₂ and Fc monomers on SDS-PAGE [155].

IdeS is inhibited by some, but not all, of the well-known protease inhibitors such as iodoacetate and Z-LVG-CHN2 [196, 3]. Several 3-aminopiperidine-based peptide analogues were recently developed as the first selective non-covalent inhibitors of IdeS [15, 16]. Surprisingly, the human protein cystatin C, an inhibitor of many of the papain superfamily cysteine proteases, acts to enhance rather than inhibit IdeS function [190].

1.10.2 IdeS effects on the human host response

Following single cleavage, the Fc monomer that has been produced remains associated with the other heavy chain due to non-covalent interactions with its corresponding C γ 3 domain [49]. Additionally, cleavage below the hinge disulphide bonds maintains the structure of the F(ab')₂ antigen-binding domain. Antigen recognition is therefore largely unaffected, as proven experimentally in cellular assays [26, 64].

However, given that the lower hinge region is also the binding site for many Fc receptors (FcRs) (Section 1.8.3), IdeS activity thus obliterates most responses which depend on FcR binding. It is also striking that the single cleavage is sufficient to achieve almost complete knockdown of Ig effector function. Competition binding assays showed that scIgG exhibited approximately 20-fold loss in binding affinity to Fc γ RIIA, Fc γ RIIB and Fc γ RIIIA, relative to the intact mAb [26]. Binding of scIgG was thus comparable with that of F(ab')₂. Binding to Fc γ RI was less affected although notably reduced. In accordance with reduced Fc γ R binding, scIgG were almost entirely ineffective at mediating ADCC or CDC in cellular assays. However, a later study found some residual ADCC activity after 120 h [64]. The authors' explanation for this

was an increased proportion of myeloid lineage cells after 12 h, which mediate ADCC via the higher-affinity receptor Fc γ RI.

The exception to this trend was FcRn, which binds at a different site to Fc γ Rs, and bound to scIgG almost as strongly as to the intact antibody. This maintains the serum half-life of scIgG: studies of mice injected intraperitoneally with intact IgG, scIgG or F(ab')₂ showed that the F(ab')₂ disappeared from circulation within 2–8 days while iIgG and scIgG remained detectable at 35 days [26].

The anti-tumour efficacy of singly-cleaved trastuzumab (scIgG-T) was subsequently studied *in vivo*, using a mouse breast cancer model with high HER2 expressing BT474 cancer cells [64]. Intact trastuzumab produced almost complete inhibition of tumour growth compared to the isotype control. scIgG-T, in contrast, resulting in only 30% growth inhibition. Additionally, macrophage infiltration into the tumour was significantly reduced compared to intact trastuzumab, almost to the level of the isotype control in the case of CD11b-positive cells.

Furthermore, the biological activity of IdeS is not restricted to IgG proteolysis. The initial finding that IdeS bears some homology to the human CD11b/Mac protein prompted an investigation into a possible interaction of Streptococcal Mac-1/IdeS with polymorphonuclear leukocytes (PMNs) [108]. CD11b regulates several leukocyte functions including phagocytosis and oxidative burst [109]. Using flow cytometry, IdeS was found to bind to the surface of PMNs; this binding was partially inhibited by an anti-CD11b antibody and completely inhibited by an anti-Fc γ RIIIB (CD16) antibody. IdeS was also found to inhibit production of reactive oxygen species (ROS) by PMNs subjected to a variety of stimuli including serum-opsonised GAS. IdeS also significantly decreased phagocytosis and killing of GAS.

These effects could be due to a combination of Fc receptor binding activity and IgG proteolysis. To differentiate between these activities, IdeS from strain 5005 (IdeS5005) representing serotype M1 was produced with a C94A mutation. This mutant does not cleave IgG; however, it showed no difference in levels of phagocytosis inhibition, compared to the wild-type (WT) enzyme, at two out of the three concentrations tested [109].

1.10.3 Synergy of IdeS activities with other *S. pyogenes* proteins

Besides IdeS, *S. pyogenes* produces a variety of proteins to enable it to establish infection in the human host. These include streptococcal pyrogenic exotoxin B (SpeB) and M proteins. The M protein family consists of three subtypes: Mrp, Emm and Enn. M proteins are expressed on the bacterial surface and bind to various proteins found in human plasma, among them IgG and IgA [111, 178]. Binding of IgG via the Fc region has the advantage of sequestering anti-streptococcal antibodies and preventing a humoral immune response against the pathogen. Additionally, the bound IgG molecules form a shield that prevents recognition of bacterial proteins and deposition of complement.

The Emm1 protein was found to compete with C94S mutant IdeS for IgG binding, suggesting that these proteins recognise the same or overlapping sites on IgG [?]. Binding of human IgG to Emm1 reduced IdeS activity approximately four-fold. The binding affinity of human IgG to Emm1, as measured by ELISA, was ~14 nM, which was greater than to C94S-IdeS. A figure for the latter was not quoted in the same article but an earlier study quoted a K_D of 2.5 μ M for IdeS (strain MGAS5005, serotype M1) [3].

Additionally, IdeS more readily cleaves Fab-bound IgG than Fc-bound IgG, as shown by comparing cleavage of IgG bound to either Protein A-Sepharose (Fc-binding) or Protein L-Sepharose (Fab-binding). Similarly to the findings with Emm1-bound IgG, rate of IdeS activity with protein A-bound IgG was decreased ~4-fold compared to IgG in solution. Protein L-bound IgG, on the other hand, was cleaved equally effectively [?]. This is of benefit to the bacterium because Fc-bound IgG will remain on the bacterial surface and continue to form a protective shield to enable *S. pyogenes* to evade immune detection. In contrast, Fab-bound IgG is subject to faster IdeS-mediated cleavage, which prevents immune responses to bacterial antigens via exposed Fc.

A second *S. pyogenes*-derived protein found to interact with IdeS is SpeB. SpeB is another endopeptidase that also cleaves IgG in the hinge region between Gly236 and Gly237 i.e. the same site as IdeS [45]. It also degrades other host proteins and various bacterial proteins

including M protein and IdeS. It was questioned how biologically relevant the IgG-cleaving activity is, as under laboratory conditions SpeB is not expressed until the stationary phase of growth [196] whereas IdeS is expressed from log phase until late stationary phase. However, IdeS and SpeB could have complementary activity; additionally, SpeB is a much less selective enzyme than IdeS.

SpeB was discovered to degrade the N-terminal region of IdeS ([172]; unpublished data). The truncated IdeS is less immunogenic than the full-length IdeS while still retaining enzymatic activity, albeit lower than the uncleaved enzyme. Interestingly, neutrophil proteases also have the same effect on IdeS: the N-terminal region is cleaved producing a less immunogenic but still functional enzyme. This can be seen as another example of subversion of the host immune system by *S. pyogenes*.

1.10.4 Structural characterisation of IdeS

Most, but not all, strains of GAS produce IdeS [108]. Sequence analysis showed that IdeS proteins can be divided into 2 groups: complexes I and II [109]. Much greater sequence variation exists between complexes (~50%) than within complexes (3–6%); most of this variation occurs in the middle third of the protein sequence. All alleles tested appear to be enzymatically active apart from that derived from the strain M28, a complex II enzyme. This allele did inhibit ROS production; however, ROS inhibition alone was found to be insufficient for bacterial survival [173].

Amino acid sequence alignment between IdeS5005, IdeS8345 and an IdeS homologue from *S. equii* identified 6 histidine and 16 aspartate residues that were conserved. These residues in IdeS5005 were each replaced by alanine, the mutant proteins expressed in *E. coli* and the bacterial lysate supernatants screened for IgG endopeptidase activity [110]. This experiment identified residues His262, Asp284 and Asp286 as critical for enzymatic activity. Additionally, IdeS5005 D284A mutant was found to a greater extent in inclusion bodies compared to the

wild type and to the other mutants expressed, indicating that this mutation caused a degree of protein misfolding.

These findings were later supported by the solution of IdeS crystal structures by two groups, covering the wild type enzyme from strain MGAS5005 and its C94A catalytically inactive mutant [4] at 2.4 Å and 2.0 Å respectively, plus a C94S mutant at 1.9 Å [198]. The data indicates close structural homology between IdeS and members of the papain superfamily of proteases. The N-terminal domain forms mainly β -sheets whereas the C-terminal domain consists mainly of α -helices (Figure 1.14). One difference between IdeS and the papain superfamily proteases is the much narrower active site presented by IdeS, which correlates with its strict specificity for IgG.

Besides the active site cysteine, other key residues identified from the structures were Asp284 and His262, which together with Cys94 make up the catalytic triad. Mutant proteins D284A and H262A showed 20-fold lower binding affinity for IgG1 compared to the wild type protein in solution binding studies [4]. Additionally, SDS-PAGE analysis of the products from a range of mutant enzymes showed that K84A, D286A and Y255A mutant forms had considerably lower enzymatic activity. IgG binding affinities and kinetic constants for cleavage were determined using surface plasmon resonance (SPR), performed using IdeS containing mutations in and around the active site. Enzymatic constants were calculated using immobilised C94A mutant as a reporter to detect residual intact IgG. These experiments confirmed the importance of Lys84, Tyr255, His262, Asp284 and Asp286 for IdeS enzymatic activity [4].

There is conflicting evidence as to whether IdeS is active as a monomer or as a dimer. Crystal structures of IdeS produced both monomeric [198] and dimeric [4] structures. Support for dimerisation also came from kinetic analysis [191] which produced a sigmoidal velocity curve up to ~175 μ M, after which the rate declined. The authors speculated that this was due either to cooperativity, product inhibition or the presence or absence of an effector molecule. Gel filtration analysis produced two peaks, thought to represent monomeric and dimeric forms.

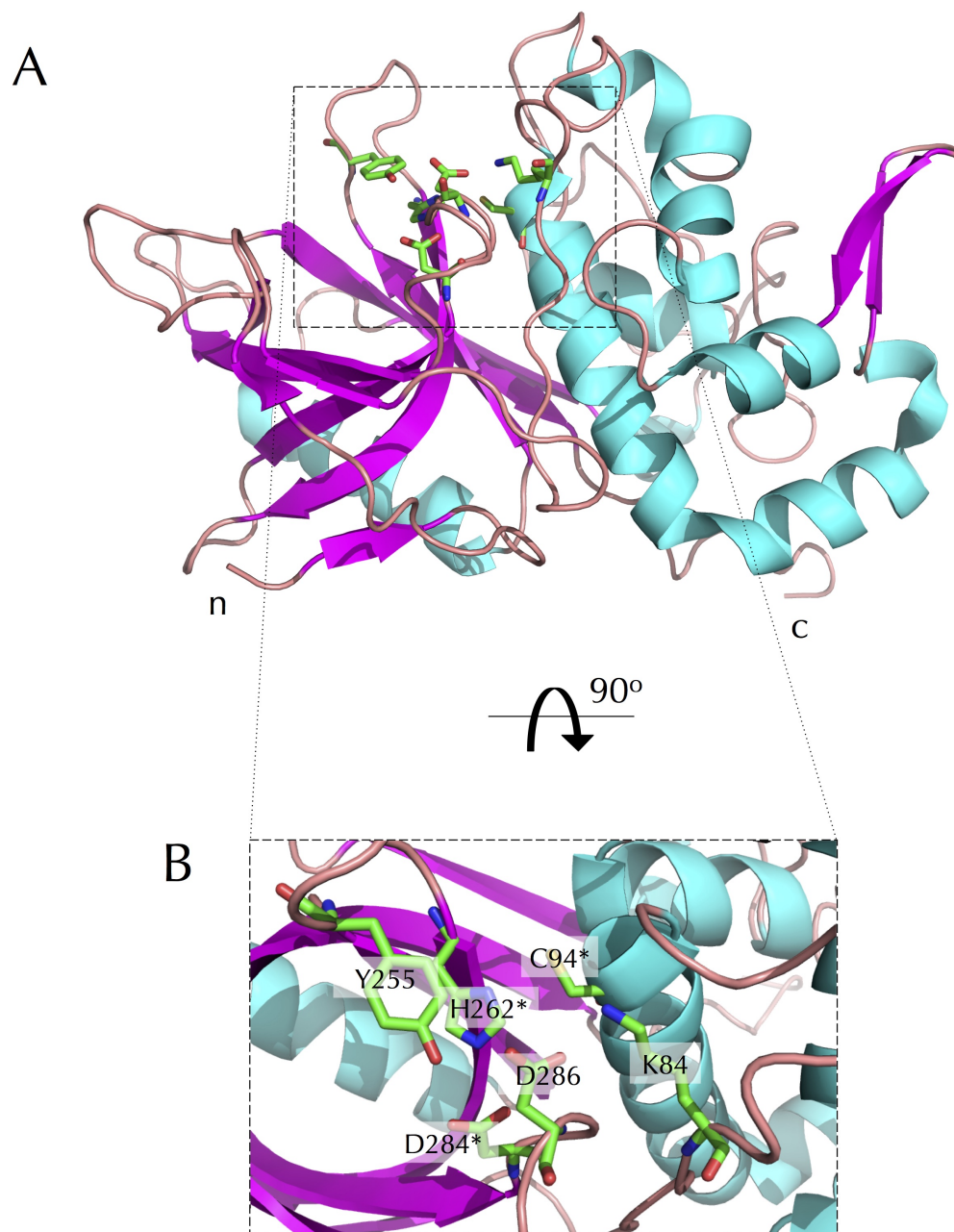


Figure 1.14: Crystal structure of wild type IdeS (PDB 2AU1). **A**| Cartoon representation of a single IdeS monomer, coloured by secondary structure (helices in blue, β -strands in dark pink and loops in light pink). The catalytic residues in shown in stick form with carbon in green, oxygen in red, nitrogen in blue. The N- and C-termini are indicated. **B**| This inset provides a top-down view of the active site, with a colour scheme matching that of the panel A. Residues identified from mutagenesis studies to be important for enzymatic activity are shown in stick representation and labelled with the single-letter amino acid code; an asterisk indicates the residues that constitute the catalytic triad.

When an isolated monomer peak was re-injected into the column, two peaks were observed, lending support to the idea of a monomer-dimer population in equilibrium.

However, more recent data has suggested that IdeS is active as a monomer [192]. Monitoring enzyme activity as a function of concentration showed no relationship between the two, indicating that dimer formation is not essential for IdeS activity. Gel filtration chromatography produced no equilibrium between potential dimer and monomer populations; instead two stable peaks were observed. Analysis of fractions from the first peak (the potential dimer) with SDS-PAGE and MALDI-TOF identified these proteins as truncated forms of IdeS which had formed aggregates. Finally, analysis of the cleavage rate for both the first and second heavy chains showed classical Michaelis-Menten kinetics and not a sigmoidal curve as expected from previous work. Finally, small-angle X-ray scattering (SAXS) analysis of an IdeS-Fc complex [55] showed 1:1 binding of IdeS to Fc, with the IdeS bound at the top of the hinge region.

Exploration of the specificity of IdeS showed that it did not cleave short peptides resembling the hinge region of IgG. The smallest known substrate of IdeS is the product of IgG proteolysis by papain (Fc_{papain}), which cleaves in the upper hinge region above the disulphide bonds. It was therefore hypothesised that IdeS may also contain an exosite for binding the C γ 2 or C γ 3 domains [191]. This correlates with the findings of Kinder *et al* [101], who created mutant IgG1 variants with varying degrees of resistance to proteolytic enzymes. The replacement of the IgG1 hinge region with that of IgG2, involving mutations E233P, L234V, L235A, G236X (where X indicates a deletion), conferred resistance to a number of proteases but not to IdeS. The additional incorporation of S239A, K326A, I332E and/or E333A mutations was required to provide resistance to IdeS, the I332E mutation being particularly significant. Furthermore, while the IgG1-to-IgG2 switch of the hinge region almost entirely ablated CDC and ADCC activity, the additional incorporation of the S239D and I332E mutations increased ADCC activity approximately eleven-fold relative to the IgG1 wild-type. However, no concurrent improvement in CDC stimulation was observed.

1.10.5 Biological activity of IdeS products

Products of IgG hinge cleavage are two $\frac{1}{2}$ Fc monomers and a F(ab')₂ domain. The $\frac{1}{2}$ Fc monomers are ineffective competitors for binding to bacterial surface proteins such as the M-proteins and are thus free to circulate. Unexpectedly, these fragments were found to decrease bacterial survival, as measured in whole blood assays, but only when pre-incubated with the blood [174]. This was attributed to the ability of $\frac{1}{2}$ Fc fragments to prime PMNs for ROS release: $\frac{1}{2}$ Fc-primed PMNs reached a greater maximum of ROS production at an earlier timepoint than a non-primed control. The priming effect observed may not translate into a bactericidal effect *in vivo* because $\frac{1}{2}$ Fc are diffusible and may therefore stimulate PMNs to discharge at some distance from the active site of infection.

Hinge cleavage exposes cryptic self-epitopes which can form a target for human anti-hinge (HAH) autoantibodies. The existence of these antibodies in healthy individuals has been known for some time [24]; however, their origins are not entirely clear. Studies of pooled serum showed that autoantibodies were biased towards the IgG3 isotype [25]. Binding studies using IgG3 autoantibodies and peptide fragments resembling specific hinge regions indicated greatest reactivity against peptides corresponding to the C-terminal of Fab and F(ab')₂ fragments. Reactivity against peptides resembling the N-terminal Fc was much lower while full-length equivalent antibodies were also not targeted. In the same study, HAH autoantibodies were found to reconstitute ADCC and CDC activity of F(ab')₂ domains targeted against MDA-MB-231 cells.

1.10.6 Therapeutic potential of IdeS

The rapid and specific action of IdeS against IgG holds promise for the treatment of various autoimmune diseases and for prevention of transplant rejection, such as kidney transplants for sensitised patients [77]. Rabbits injected intravenously with 5 mg IdeS showed complete clearance of IgG within 6 h [95]. IgG levels began to recover after 4 days. IdeS was also found to completely cure lethal rabbit IgG-induced thrombocytopenia in a mouse model.

Although IdeS is active against all subclasses of rabbit IgG, it cleaves only IgG2a/c and IgG3 mouse antibodies. In a mouse model of arthritis where the disease was induced by anti-CII IgG2a antibody, IdeS, IdeS treatment almost completely prevented the development or progression of arthritis, but only if administered within 1–2 days of disease onset. Moreover, the therapeutic effect was dominant: IdeS suppressed arthritis even when the disease was induced with a mixture of cleavable and non-cleavable antibodies. Finally, IdeS blocked antibody-induced relapse in a model of chronic (relapsing) arthritis. Importantly, IdeS was stated to have activity for only 3 h *in vivo*, although data for this is not shown [131].

IdeS has just passed Phase I clinical trials incorporating 24 healthy volunteers [200]. The maximal IdeS concentration used in this trial was 0.24 mg/kg with a peak serum concentration of ~9.5 mg/L. The entire IgG pool was cleaved to F(ab')₂ and Fc fragments within 6 hours after administration, and was mostly restored within three weeks. IdeS itself was rapidly cleared: the half life was calculated to be between 4.1 h and 4.9 h, depending on the dose received, and the majority had left the bloodstream within 24 h. This is due to the relatively small size of IdeS (~37 kDa), which is below the kidney glomerular filtration cut-off size of ~70 kDa [114]. No serious adverse effects were reported, and the positive results from this study have led to a Phase II trial in sensitised dialysis patients, which is currently underway.

1.11 Aims of this thesis

Given the utility of antibody-based therapeutics and the potential of phosphatidylserine (PS) as an anti-viral and anti-cancer target, the purpose of this thesis was to enhance the function of phosphatidylserine-targeting antibodies and to explore the therapeutic use of alternative phosphatidylserine receptors. Firstly, Chapter 3 investigates the binding of the endopeptidase IdeS to its IgG substrate using systematic site-directed mutagenesis of IgG and truncated IgG variants. The aim was to establish whether there is a secondary binding site for IdeS on IgG, in addition to the hinge cleavage region, and to map the binding interface(s) between these two proteins.

Secondly, Chapter 4 uses the specific IgG-cleaving activity of IdeS to improve Fc γ R binding of a therapeutic monoclonal antibody (mAb) in the presence of competing serum IgG. The effect of IdeS on levels of mAb binding was assessed *in vitro* using an IdeS-resistant form of bavituximab and the receptor Fc γ RIIIA.

Finally, Chapter 5 has a dual focus. The first section of this chapter reports on the attempted creation of alternative phosphatidylserine-targeting agents, in the form of IgG fused to the PS receptor TIM-4. The second section of this chapter investigates the binding of bavituximab, TIM-1 and TIM-4 to Semliki Forest Virus, in the hope that data from binding and structural studies would begin to clarify the levels of PS exposure on the virion and its accessibility to receptors.

Chapter 2

Materials and Methods

All solutions and media were prepared using 18.2 M Ω water from a Milli-Q Plus purification system (Millipore, UK). All chemicals were sourced from Sigma (UK) unless otherwise specified. Solutions of phosphate-buffered saline (PBS) were made from solid tablets (Sigma, P4417) according to manufacturer's instructions, to yield 10 mM phosphate buffer, 2.7 mM potassium chloride and 137 mM sodium chloride, pH 7.4.

2.1 Molecular biology and site-directed mutagenesis

General cloning protocols are given in Section 2.1.1 and methods used for cloning each protein are given in the following Sections 2.1.3 to 2.1.6. Commercial sources of deoxyribonucleic acid (DNA) encoding recombinant proteins are detailed in Table 2.3 and Table 2.4. Constructs that were generously provided by colleagues are given in Table 2.5. All primers were purchased from Thermo Scientific (Germany).

2.1.1 Cloning procedure

The cloning procedure outlined here is applicable to the creation of all constructs, with additional details and/or exceptions given in Sections 2.1.5 and 2.1.6.

Polymerase chain reaction

All polymerase chain reactions (PCR) used AmpliTaq Gold[®] or AmpliTaq Gold[®] 360 Master Mixes (both from Life Technologies, UK) as specified by the manufacturer. Each reaction mixture contained ~500 ng plasmid DNA and primers at a final concentration of 0.3 μ M. Thermal cycling was carried out according to Table 2.1. PCR products were then analysed and separated by agarose gel electrophoresis.

Table 2.1: PCR cycling specifications for the amplification of both non-fusion and fusion proteins.

Cycle	Function	Temperature (°C)	Time (mm:ss)
1	Initial denaturation	95	02:00
2 (x 30)	Denaturation	95	00:30
	Annealing	60	00:30
	Extension	72	02:00
3	Final extension	72	10:00

DNA electrophoresis

Agarose gels for DNA analysis and separation were made with 0.7–1.0% w/v agarose in 40 mM Tris, 20 mM acetic acid and 1 mM ethylenediaminetetraacetic acid (EDTA). GelRed nucleic acid stain (Biotium, Inc., USA) was added to enable DNA visualisation under UV light. Gels were run at 110 V for ~1 h, bands excised and DNA extracted using NucleoSpin[®] Gel and PCR kit (Macherey-Nagel, UK) or QIAquick Gel Extraction kit (Qiagen, UK).

Restriction digestion and ligation

PCR products (inserts) were digested with high-fidelity restriction enzymes (New England BioLabs (NEB), UK) for 1–16 h at 37°C. Destination plasmids were digested in parallel and fully digested plasmid was isolated by agarose gel electrophoresis. Inserts and plasmids were purified from reaction components or agarose using the NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel) or the QIAquick PCR purification kit (Qiagen). Prior to insert ligation, plasmids were treated with Antarctic Phosphatase (NEB) using T4 DNA Ligase for 20 mins at room temperature or overnight at 16°C. DNA was then transformed into XL-1 Blue competent cells (Agilent Technologies, USA) according to the manufacturer's protocol. The resulting bacterial cultures were streaked onto agar plates containing the appropriate antibiotic: 100 µg/mL carbenicillin for selection of ampicillin-resistant plasmids such as pHLSec, or 25 µg/mL Zeocin™ for selection of Zeocin™-resistant plasmids such as pFUSE.

Following overnight incubation of plates at 37°C, bacterial colonies were picked and cultured in Lysogeny Broth (LB) containing antibiotic at the previously-stated concentrations; as an alternative to LB containing Zeocin™, Fast-Media® Zeo TB (Invivogen) was also used. After 14–16 h growth at 37°C, bacterial cells were harvested and DNA extracted using NucleoSpin® Plasmid (Macherey-Nagel) or QIAprep Spin Miniprep Kit (Qiagen). Successful insertion of the desired DNA sequence was confirmed by sequencing (Source Bioscience, UK).

2.1.2 Expression vectors

pHLSec

The mammalian pHLSec expression vector was a gift from Dr Thomas Bowden (University of Oxford). pHLSec contains a secretion signal sequence, AgeI and KpnI restriction sites and a C-terminal hexahistidine tag [supplementary information in reference 8] (Appendix A). Proteins expressed from this vector therefore contain an ETG amino acid sequence at their N-terminus and a GTKHHHHHH sequence at their C-terminus, derived from these restriction sites.

pFUSE

The pFUSEss-CHlg-hG1 and the pFUSE-hIgG1-Fc2 vectors from Invitrogen (Appendix A) were used for the production of IdeS-resistant bavituximab and Fc fragments respectively. Both of these vectors contain a 20-amino acid Il-2 secretion signal sequence at their N-terminus, followed by a multiple cloning site (MCS). pFUSEss-CHlg-hG1 then incorporates the IgG1 constant region (Ala118–Lys447) while pFUSE-hIgG1-Fc2 contains the Fc region only (Asp221–Lys447) (Eu numbering [60]).

2.1.3 Bavituximab

Wild type bavituximab

The bavituximab protein sequence was obtained from the International IMmunoGeneTics Information System[®] (IMGT[®]) [107], chain ID 8734. This was back-translated into a DNA sequence, codon-optimised for expression in human cells, and synthesised by the GeneArt[™] gene synthesis service (Invitrogen). AgeI and KpnI restriction sites were added to the 5' and 3' ends, respectively, of both heavy and light chains, which were then PCR-amplified and ligated into pHLSec using these restriction sites. The IMGT[®] source codes and primers are given in Table 2.3. The full length amino acid and DNA sequences are given in Appendix B.

IdeS-resistant Bavituximab

Mutations chosen to confer IdeS-resistance were E233P, L234V, L235A, G236X (deletion), S239D and I332E. As mutagenesis is more successful in pFUSE than pHLSec vectors, it was decided to use pFUSEss-CHlg-hG1, containing the IgG1 heavy chain constant region, as a mutagenesis template and to subclone the bavituximab variable regions into this vector. These were initially incorporated into the pFUSEss-CHlg-hG1 vector (Invivogen), firstly with the use of Quikchange Lightning Site-Directed Mutagenesis kit (Agilent, UK) to delete Gly236 and

secondly with the use of Quikchange Multi Site-Directed Mutagenesis kit (Agilent) to carry out the amino acid substitutions. Table 2.2 lists the primers used in both steps. All primers were designed using the QuikChange® Primer Design online program (Agilent) and purchased from Thermo Scientific. The mutated vector was designated pFUSEss-CH1g-hG1_IdeS-R.

For creation of an IdeS-resistant bavituximab, the bavituximab heavy chain variable region was amplified from the source template using the primers 5'-CGTGAATTCGGAAGTGCAGCTGCAGCAGTCTGGC-3' (forward) and 5'-CGTGCTAGCGCTAGACACTGTACGGTGGTGCC-3' (reverse). The resulting inserts therefore incorporated the pFUSEss-CH1g-hG1 vector restriction sites EcoRI and NheI, respectively (underlined in primer sequences). Inserts were then restriction digested and ligated into pFUSEss-CH1g-hG1_IdeS-R at the 5' end of the IgG1 constant region, in frame with the IL-2 signal sequence. The resulting heavy chain was designated 'Bavi-H_IdeS-R'.

Table 2.2: Primers for mutagenesis to confer IdeS resistance in IgG1.

Mutation	Primers (5'-3')
G236X	Fwd: GCACCTGAACTCCTGGGACCGTCAGTCTTC Rev: GAAGACTGACGGTCCCAGGAGTTCAGGTGC
I332E	Fwd: GCCCTCCCAGCCCCCGAGGAGAAAACCATCTCCA Rev: TGGAGATGGTTTTCTCCTCGGGGGCTGGGAGGGC
E233P, L234V, L235A, S239D	Fwd: CACCGTGCCCAGCACCTCCAGTCGCGGGACCGGATGTCTTCTTCCCCC Rev: GGGGGAAGAGGAAGACATCCGGTCCCGCGACTGGAGGTGCTGGGCACGGTG

2.1.4 TIM-4

The human TIM-4 whole cDNA clone was obtained from Sino Biological, Inc. (China). The region corresponding to amino acids 24 to 153, the IgV domain plus some of the mucin domain (described in Section 1.4.1) was PCR-amplified and ligated into pHLSec for mammalian expression. Specific details and primers are listed in Table 2.3.

2.1.5 TIM-4-IgG1 fusion proteins

A list of TIM-4-IgG1 fusion proteins created is given in Table 2.4. All constructs cloned contain the human orthologs of both TIM-4 and IgG1. Fusion proteins are designated 'Protein A—Protein B', where the C-terminus of 'Protein A' is fused to the N-terminus of 'Protein B'. Each construct was created by overlap extension PCR. Specifically, a first round of PCR amplification was carried out separately for proteins A and B using the primers given in Table 2.4. The overhanging DNA products were used in a second round of DNA amplification with the forward primer from protein A and reverse primer from protein B. Final PCR products were restriction digested and ligated into pHLSec using the AgeI and KpnI restriction sites.

2.1.6 IgG1 Fc and C γ 2 domains

Fc single mutants

Single amino acid mutagenesis of the human IgG1 Fc region was conducted in the pFUSE-hIgG1-Fc2 vector (Invivogen). This vector contains an IL-2 signal sequence, an multiple cloning site which translates to a seven-amino acid sequence (ISAMVRS), and the native IgG Fc sequence consisting of Asp221–Lys447. The Fc sequence is identical to the UniProt sequence P01857 except for two natural variants: D239E and L241M. Mutagenesis was conducted using the Quikchange Lightning Site-Directed Mutagenesis kit with the primers listed in Table 2.6. All inserts in the pFUSE vectors were verified by sequencing (Source Bioscience, UK) using the primers hEF1-HTLV_fwd: 5'–CCACGCTTTGCCTGACCCTGCTTGC–3 and SV40pAn_rev: 5'–GCTTATAATGGTTACAAATAAAGC–3'. All wild type and single mutant Fc proteins used for SPR binding analysis to IdeS were expressed from this vector without using the MCS; these proteins therefore contain the ISAMVRS sequence at their N-termini.

Table 2.3: Molecular cloning of non-fusion proteins used in this thesis.

Protein	Source of sequence	Cloned region	Source plasmid		Differences to UniProt	Amplification primers (5' – 3')	Expression plasmid
			Name	Vector			
Bavituximab, heavy chain	IMGT [®] 8734	Entire	Custom-ordered	pMK-RQ	GeneArt, Invitrogen	Fwd: GTAGCTGAAACCGGTGAAGTGCAG Rev: GTGGTGCTTGGTACCCCTTGCCGGG	pHLSec
Bavituximab, light chain	IMGT [®] 8734	Entire	Custom-ordered	pMA-T	GeneArt, Invitrogen	Fwd: GTAGCTGAAACCGGTGACATCCAG Rev: GTGGTGCTTGGTACCCACTCGCC	pHLSec
IgG1 Fc, hingeless	UniProt P01857	Ser239–Lys447	pFUSE-hlgG1-Fc2	pFUSE-hlgG1-Fc2	Invivogen	Fwd: CGTGAATTCGTCAGTCTTCCTCTT-CCCCC Rev: CGTGCTAGCACTCATTTACCCGGA-GACAG	pFUSE-hlgG1-Fc2
TIM-4	GenBank AAH08988.1	24–153	Human TIMD4 Gene cDNA Clone	pGEM-T	Sino Biological Inc, PRC	Fwd: CGTACCGGTAGCGAGACTGTTGT-GACGGAG Rev: CGATGGTACCGCTTGTGTT-GT-TGTTGTG	pHLSec

The name of the protein as given in the first column is the name as referred to in the relevant results sections — for example, 'TIM-4' is denoted as such to distinguish it from the shorter 'hTIM4XII' construct used in the fusion proteins (Table 2.4).

The cloned region refers to amino acid residues.

IMGT[®], International IMmunoGeneTics Information System[®]

Agel (ACCGGT), KpnI (GCTACC), EcoRI (GAATTC) and NheI (GCTAGC) restriction sites are underlined.

In primer sequences, the nucleic acid sequence upstream of each restriction site is flanking junk DNA.

'Natural variants' here refer to human polymorphisms.

Table 2.4: Molecular cloning of fusion proteins used in this thesis.

Name	Protein A details		Protein B details		PCR primers (5'–3')	
	Name	Cloned region	Name	Cloned region	Protein A	Protein B
TIM-4(cryst)–Fc	TIM-4	25–153	IgG1 heavy chain	107–330	Fwd: <u>CGTACCGGTGAGACTGTTGTG</u> -ACGGAGGTT Rev: GCACGGTGGGCATGTGTG-GCTTGTGTGTTGTTCTG	Fwd: CAGAACAAACAACAAGCCA-CACATGCCCCACCGTGC Rev: <u>CGATGGTACCACCCGGGAGACAG</u> -GGAGAGG
TIM-4(EC)–Fc	TIM-4	25–281	IgG1 heavy chain	107–330	Fwd: <u>CGTACCGGTGAGACTGTTGTG</u> -ACGGAGGTT Rev: GCACGGTGGGCATGTGTG-TCCAGGCTGAGGAGAAGAC	Fwd: GTCTTCTCTCAGCCTGGACA-CACATGCCCCACCGTGC Rev: <u>CGATGGTACCACCCGGGAGACAG</u> -GGAGAGG
hTIM4XII–hIgG1II	TIM-4	24–134	IgG1 heavy chain	104–330	Fwd: <u>CGTACCGGTTCAGAGACTGTTG</u> -TGACGGAG Rev: GGGCATGTGTGAGTTTGTGCG-GCTCTGTAGATTCAGGCG	Fwd: CGCCTGAATCTACAGAGAGCC-GACAAAATCACACATGCCCC Rev: <u>CGATGGTACCACCCGGGAGACAG</u> -GGAGAGG
hIgG1II–hTIM4XII	IgG1 heavy chain	104–330	TIM-4	24–134	Fwd: <u>CGTACCGGTGACAAAAC</u> TCACA-CATGCC Rev: CCTCCGTCACAAACAGTCTCT-GATTACCCGGAGACAGGGAGAGG	Fwd: CCTCTCCCTGTCTCCGGGTAAATCA-GAGACTGTTGTACGGAGG Rev: <u>CGTGGTACCGGCTCTCTGTAGAT</u> -TCAGGC

Agel (ACCGGT) and KpnI (GGTACC) restriction sites are underlined.

For the second stage of overlap PCR, the forward primer for protein A and reverse primer for protein B was used.

The nucleic acid sequence upstream of each restriction site is flanking junk DNA.

Table 2.5: DNA constructs that were cloned by others.

Protein	UniProt ID	Region cloned (aa)	Differences from UniProt seq.		Vector	Source
			Mutation	Effect		
FcγRIIIA	P08637	17–208	F176V	Natural variant; higher affinity binding to IgG1, IgG3 and IgG4	pHLSec	Dr Ben Yu, University of Oxford
IdeS WT (M1 serotype)	Q7DAM2	38–339	A195S	Natural variant		
IdeS C94A (M1 serotype)	Q7DAM2	38–339	None	Enzyme inactivation	pET303/CT-His	Dr Emma Beale, University of Oxford

Aa, amino acids; WT, wild-type.

Table 2.6: List of primers for Fc mutagenesis

Mutation	Forward primer (5'–3')	Reverse primer (5'–3')
C226A	GGGCACGGTGGGGCTGTGTGAGTTTGTGATCTAA	TTAGATCTGACAAAACCTCACACAGCCCCACCGTGCCC
P228A	GCTGGGCACGCTGGGCATGTGTGAG	CTCACACATGCCCAGCGTGCCCAAGC
C229A	GTTCAGGTGCTGGGGCCGGTGGGCATGTGT	ACACATGCCCACCGGCCCCAGCACCTGAAC
P230A	GTTCAGGTGCTGCGCACGGTGGGCA	TGCCCACCGTGCGCAGCACCTGAAC
A231T	CAGGAGTTCAGGCGTTGGGCACGGTGGGC	GCCCACCGTGCCCAACGCCTGAACTCCTG
P232A	CCAGGAGTTCAGCTGCTGGGCACGG	CCGTGCCCAGCAGCTGAACTCCTGG
E233A	CCCCCAGGAGTGCAGGTGCTGGGC	GCCCAGCACCTGCACTCCTGGGGGG
L234A	GGTCCCCCAGGGCTTCAGGTGCTGGGC	GCCCAGCACCTGAAGCCCTGGGGGGACC
L235A	CTGACGGTCCCCCGCGAGTTCAGGTGCTG	CAGCACCTGAACTCGCGGGGGGACCGTCAG
G236A	GAAGACTGACGGTCCCCGCCAGGAGTTCAGG	CCTGAACTCCTGGCGGGACCGTCAGTC
G237A	GAAGACTGACGGTGCCCCCAGGAGTTC	GAACTCCTGGGGGGACCGTCAGTCTTC
P238A	GGAAGACTGACGCTCCCCCAGGAGTT	AACTCCTGGGGGGAGCGTCAGTCTTCC
S267A	CAGGGTCTTCGTGGGCCACGTCCACCACCA	TGGTGGTGGACGTGGCCCCACGAAGACCCTG
H268A	CCTCAGGGTCTTCGGCGCTCACGTCCACCA	TGGTGGACGTGAGCGCCGAAGACCCTGAGG
P271A	TGAACTTGACCTCAGCGTCTTCGTGGCTCAC	GTGAGCCACGAAGACGCTGAGGTCAAGTTCA
K274A	CCACGTACCAGTTGAACGCGACCTCAGGGTCTTCGT	ACGAAGACCCTGAGGTGCGGTCAACTGGTACGTGG
N276A	GCCGTCCACGTACCAGGCGAACTTGACCTCAGGG	CCCTGAGGTCAAGTTCGCCTGGTACGTGGACGGC
T289A	CCTCCCGCGGCTTTGCCTTGGCATTATGCAC	GTGCATAATGCCAAGGCAAAGCCGCGGGAGG
K290A	CCTCCCGCGGCGCTGTCTTGGCATTATGCACCTC	GAGGTGCATAATGCCAAGACAGCGCCGCGGGAGG
P291A	GCTCCTCCCGCGCCTTTGTCTTGGCATTATGC	GCATAATGCCAAGACAAAGGCGCGGGAGGAGC
E293A	TGTTGTACTGCTCCGCCCGCGGCTTTGTC	GACAAAGCCGCGGGCGGAGCAGTACAACA
E294A	GCTGTTGTACTGCGCCTCCCGCGGCT	AGCCGCGGGAGGCGCAGTACAACAGC
P329A	TCGATGGGGGCTGCGAGGGCTTTGTTG	CAACAAAGCCCTCGCAGCCCCCATCGA
P331A	GGTTTTCTCGATGGCGGCTGGGAGGGCT	AGCCCTCCAGCCGCCATCGAGAAAACC
I332A	GAGATGGTTTTCTCGGCGGGGGCTGGGAGGG	CCCTCCAGCCCCCGCCGAGAAAACCATCTC
E333A	TGGAGATGGTTTTCGCGATGGGGGCTGGG	CCCAGCCCCCATCGCGAAAACCATCTCCA

Hingeless Fc and C γ 2

IgG1 Fc DNA encoding amino acid residues Ser239–Lys447 ('hingeless Fc') was PCR-amplified from the pFUSE-hIgG1-Fc2 vector (Table 2.3). This vector template was chosen to minimise

the sequence variation between Fc constructs used for IdeS binding analysis. Primers used for PCR-amplification contained the EcoRI and NheI restriction sites and the resulting insert was ligated into a pFUSE-hIgG1-Fc2 vector digested with the same enzymes. The hingeless Fc construct therefore does not contain the ISAMVRS amino acid sequence found at the N-terminus of the single mutant Fcs.

IgG1 C γ 2 DNA encoding residues Ser239–Lys338 was PCR-amplified from the pFUSE-hIgG1-Fc2 vector to incorporate AgeI and KpnI restriction sites. The insert was subsequently ligated into pHLSec for expression.

2.2 Protein analytical techniques

2.2.1 Determination of protein concentration

Absorbance of protein solutions was measured at a wavelength of 280 nm (A₂₈₀) using a Nanodrop 1000 (Thermo Scientific, UK). This was then corrected using the extinction coefficient (ϵ) for the primary sequence as given by ProtParam on the ExPASy server [70]. A list of extinction coefficients and the corresponding absorbances of a 0.1% solution is given in Table 2.7.

2.2.2 Protein gel electrophoresis

Gel electrophoresis of proteins ('SDS-PAGE') was conducted using Novex[®] NuPAGE[®] 4-12% Bis-Tris gels with NuPAGE[®] MES (2-(N-morpholino) ethane sulfonic acid) SDS (sodium dodecyl sulphate) buffer, both from Invitrogen, UK. NuPAGE[®] LDS Sample Buffer, and NuPAGE[®] Reducing Agent in the case of reduced samples, were added according to manufacturer's recommendations. Gels were run at 180 V for ~45 mins, or ~55 mins in the case of IdeS activity assays to promote separation of the singly-cleaved and uncleaved antibody bands. Gels were stained with a Coomassie stain containing 0.3% w/v Coomassie Brilliant

Blue R-250 in 50% v/v methanol and 10% v/v acetic acid. Destaining was carried out using a solution of 30% v/v methanol, 10% v/v acetic acid.

2.2.3 Western blotting

Proteins were transferred from SDS-PAGE gels onto PVDF membranes (Bio-Rad #1704156) using a Trans-Blot[®] Turbo[™] Transfer system (Bio-Rad). The 'High MW' protocol was used for transfer of all antibodies. Membranes were blocked for 1 h at room temperature with gentle agitation, using 5% milk in PBS containing 0.05% Tween-20 (PBST). Following removal of the blocking buffer, membranes were incubated with a solution of the primary antibody diluted 1:10,000–1:30,000 in PBS. Membranes were then washed in PBST (three short washes plus three 5-minute washes with agitation). For unconjugated primary antibodies, a horseradish peroxidase- (HRP) conjugated secondary antibody was then added at a 1:30,000 dilution. The washing steps were repeated before the signal was developed with Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare #RPN2232). Protein bands were visualised for 1–60 s using a FUJIFILM LAS-1000 camera connected to a FUJIFILM IDX2 Intelligent Dark Box II (Fuji).

2.3 Mammalian protein expression

Bavituximab (wild-type and IdeS-resistant forms; Bavi_WT and Bavi_IdeS-R respectively), SerTIM-4, the fusion proteins and Fc γ R1IIIA were expressed by transient transfection of either HEK 293T adherent cells (ATCC, UK) or FreeStyle[™] 293-F suspension cells (Invitrogen Life Technologies, UK). All cell culture reagents were supplied by Invitrogen Life Technologies, UK, unless otherwise specified. All cells were grown at 37°C in a humidified atmosphere with 5% CO₂. Transfected cells were incubated at the conditions specified for 4–7 days; the culture supernatant was then harvested, centrifuged (3,200 g, 15 mins) to remove cellular debris and sterile-filtered (0.22 μ m).

2.3.1 Large-scale DNA preparation

DNA for mammalian cell transfection was prepared using maxiprep or megaprep kit (Qiagen, UK). A colony from a freshly streaked antibiotic selection plate was used to inoculate 100 mL (maxiprep) or 500 mL (megaprep) of LB medium containing 100 µg/mL carbenicillin (pHLSec plasmids) or Fast-Media® Zeo TB (pFUSE plasmids). Cultures were incubated for 15–16 h at 37°C with shaking at 250 rpm, after which bacteria were pelleted, cells lysed and plasmid DNA extracted according to manufacturer's protocol. The purity and yield of DNA was measured using a Nanodrop 1000 (Eppendorf, UK), with a ratio of absorbance at 260 nm and 280 nm being considered pure.

2.3.2 HEK 293T

Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and penicillin-streptomycin solution ('P/S': final concentrations 1,000 U/ml penicillin and 0.1 mg/ml streptomycin). Prior to transfection, spent media was replaced by DMEM without FBS and containing 1 X P/S ('serum-free DMEM'). Transfection was carried out at ~90–100% cell confluence using a mixture of DNA and polyethylenimine (PEI) 'max' (Polysciences GmbH, Germany) at a mass ratio of 1:3.5 or 1:3.6. The quantity of DNA used per 225 cm² of cells was 65–78 µg. For production of bavituximab, the mass ratio of heavy:light chain DNA was 1:1. DNA and PEI were mixed in pre-warmed serum-free DMEM for 10–15 minutes at room temperature before addition to cells.

2.3.3 HEK 293F

293F cells were maintained in FreeStyle™ 293 Expression Medium at a density of 0.1–2 x 10⁶ cells/mL at 135 rpm, 37°C and 8% CO₂. Transfection was carried out according to the supplier's protocol, using a ratio of 250 µg DNA and 250 µL FreeStyle™ MAX reagent per

250 mL culture at 1×10^6 cells/mL. For production of bavituximab, the mass ratio of heavy:light chain DNA was 1:1.

2.4 Insect protein expression

Bavituximab wild-type and IdeS-resistant forms used in the IdeS resistance assays (Section 4.4.4) were kindly expressed by Michael Birnbaum (Stanford School of Medicine, USA). The two heavy chains (WT and IdeS-R) and the WT light chain were cloned into the pAcGP67a insect expression vector. These vectors were mixed with baculovirus DNA and used to cotransfect SF9 cells to create recombinant baculovirus stocks. Large-scale expression was then carried out in High Five cells infected with an empirically determined ratio of virus carrying heavy and light chains. After 48 h, the media was harvested and bavituximab purified using Ni-NTA resin (Qiagen). The resin was washed with 20 mM imidazole and protein eluted with 200 mM imidazole. Both Bavi_WT and Bavi_IdeS-R were further purified using size exclusion.

2.5 Bacterial protein expression

2.5.1 Bacterial transformation and culture

IdeS wild type, serotype M1 ('IdeS WT') and IdeS C94A, serotype M1 ('IdeS C94A') were expressed in *Escherichia coli* (*E. coli*) cells. pET303/CTHis vectors containing the desired coding sequences were transformed into the BL21(DE) strain (NEB) according to the manufacturer's protocol. Transformed cultures were streaked onto selection plates containing 100 µg/mL carbenicillin and incubated overnight at 37°C.

A single colony was used to inoculate a seed culture of 50 mL Lysogeny Broth (LB) with 100 µg/mL carbenicillin, which was incubated for 16 h at 30°C with agitation at 250 rpm. The seed culture was diluted 1:40 into 500 mL MagicMedia™ *E. coli* expression medium (Life

Technologies, UK) containing 100 µg/mL carbenicillin and pre-warmed at 30°C. This was then incubated at 30°C at 300 rpm for 7 h before the temperature was lowered to 25°C for a further 24 h. As MagicMedia™ is an autoinduction system, additional inducer was not required. Bacterial cells were harvested by centrifugation at 4,000 g for 30 mins, and the pellets stored at -20°C before lysis.

2.5.2 Bacterial lysis

All steps were carried out on ice or at 4°C. The pellet from 250 mL culture was resuspended in 80 mL lysis buffer (30 mM sodium phosphate pH 8.0, 300 mM NaCl, containing lysozyme (Sigma) and deoxyribonuclease (DNAse) I (Roche). Following 30 mins incubation on ice, cells were lysed by sonication (70% duty cycle in 1 min bursts) then cell debris removed by centrifugation at 17,600 g for 30 mins. The supernatant was filtered (0.22 µm) before protein purification.

2.6 Protein purification

Proteins were purified from culture supernatants using affinity chromatography followed by gel filtration (GF). Details for individual proteins are provided in the following subsections. All buffers were filtered through a 0.22 µm nitrocellulose membrane (Millipore) before use. Columns were obtained from GE Healthcare, UK. Chromatography systems for gel filtration were either Biologic DuoFlow, or ÄKTA Start running Unicorn 1.0 software (both GE Healthcare, UK). In both cases, the presence of protein was continuously monitored using absorbance at a wavelength of 280 nm (A_{280}). Following purification procedures, fraction(s) corresponding to the desired protein were pooled and concentrated using Vivaspin concentrators. Protein concentration was calculated using a Nanodrop and purity assessed by SDS-PAGE.

2.6.1 Mammalian-expressed hexahistidine-tagged proteins

TIM-4, Fc γ RIIIA, bavituximab, and the fusion proteins all contained a hexahistidine tag derived from the pHLSec vector, which enabled nickel-affinity purification using a HisTrap FF 5 mL column. Binding/equilibration buffer was 50 mM sodium phosphate buffer pH 7.4, containing 300 mM sodium chloride ('HisTrap buffer'). Concentrations of imidazole for washing and elution were optimised for each protein purified and are detailed in Table 2.8. Clarified cell culture supernatant was supplemented with 5 X concentrated HisTrap buffer to a final 1 X concentration and imidazole, if used, to a final concentration of 20–40 mM. The column was pre-washed with water to remove storage ethanol and pre-equilibrated with HisTrap buffer prior to loading the sample (flow rate 2.5 mL/min). 10 column volumes of HisTrap buffer containing 20–50 mM imidazole were used to remove weakly binding protein contaminants before elution (1 mL/min) with 7–10 column volumes of 300 mM imidazole in HisTrap buffer.

TIM-4 and Fc γ RIIIA were further purified using a HiLoad Superdex 75 pg 16/600, equilibrated in PBS. Fusion proteins were further purified using a HiLoad Superdex 200 pg 16/600, equilibrated in PBS.

2.6.2 Fc-containing proteins

Bavituximab and IgG1 Fc fragments were purified using nProtein A Sepharose 4 Fast Flow beads (GE Healthcare). Culture supernatants were diluted 1:1 by volume with 20 mM sodium phosphate buffer, pH 7.0 ('Protein A buffer'). Approximately 1 mL of packed beads, pre-washed with water and pre-equilibrated with buffer, was added to each 250 mL culture supernatant. The mixture was incubated for ~3 h at room temperature with shaking at 200 rpm then transferred into an empty Econo-Column (Bio-Rad, UK) to retain the Protein A beads. The media was allowed to drain by gravity flow then the beads were washed with at least 40 volumes Protein A buffer before elution with 3 x 10 volumes of 0.1 M sodium citrate buffer,

pH 3.0. To prevent acid hydrolysis of the antibody or Fc fragments, the eluate was immediately neutralised to pH ~7 with an appropriate quantity of 1 M Tris-HCl, pH 9.0.

All Fc fragments underwent a further gel filtration step using HiLoad Superdex 200 pg 16/600 equilibrated with 10 mM HEPES pH 7.4, 150 mM NaCl. Initial batches of bavituximab were also purified in this manner; however, it was found that this did not increase the purity as assessed by SDS-PAGE. In the interests of reducing protein loss, as bavituximab yields from mammalian cell culture were relatively low, later batches were purified using Protein A only.

2.6.3 IdeS WT and IdeS C94A

All samples and buffers were kept on ice throughout purification. Each 80 mL post-lysis supernatant was topped up to 200 mL with binding buffer (100 mM HEPES pH 7.5, 150 mM NaCl) containing 5 mM imidazole. This was applied to a pre-equilibrated HisTrap FF 5 mL column then the column washed with 200 mL binding buffer containing 10 mM imidazole. IdeS was eluted using a step gradient of imidazole at 20 mM, 40 mM, 100 mM and 300 mM. Elutions were checked by SDS-PAGE and the appropriate fractions pooled and concentrated using a Vivaspin 20 concentrator (10 kDa MWCO). The sample was mixed thoroughly at intervals during concentration to prevent aggregation.

Both IdeS proteins were further purified using a HiLoad Superdex 75 pg 16/600, equilibrated with 10 mM HEPES pH 8.0, 150 mM NaCl. Fractions corresponding to eluted protein were pooled and concentrated as above then stored as aliquots at -20°C .

2.7 Production of Semliki Forest Virus

Baby hamster kidney (BHK)-21 cells (University of Helsinki, Finland) were maintained in Glasgow Minimum Essential Medium (GMEM; Sigma-Aldrich) containing 5% FBS, 20 mM HEPES pH 7.4 and 10% tryptose phosphate broth ('GMEM+++'). Cells were grown at 37°C

in a humidified atmosphere with 5% CO₂. For virus cultivation, concentrated Semliki Forest Virus (SFV) stocks were diluted in GMEM containing 20 mM HEPES pH 7.4 and 10% tryptose phosphate broth, without FBS ('GMEM++'). This was used to infect 70–90% confluent BHK-21 cells, pre-washed in GMEM++, at an M.O.I. of ~0.1. After 1 h incubation, the virus inoculum was replaced with GMEM++ and the cells incubated for 24 h before harvesting the virus.

All following centrifugation steps were carried out at 4°C. The buffer used for sucrose cushion, sucrose density gradient and virus resuspension was 20 mM Tris-HCl, 100 mM NaCl, pH 7.5 ('T20N100'). Spent media was cleared of cell debris by centrifugation at 3,000 g for 30 mins before virus was pelleted through a 20% sucrose cushion for 2 h at 100,000 g. The virus pellet was resuspended in 100–200 µL of T20N100 and allowed to resolve for 1 h. For ELISA, the virus underwent further purification using a 15–45% sucrose density gradient with centrifugation for 2.5 h at 100,000 g. Purified virus was used for ELISA within 4 days of preparation and otherwise stored at –80°C.

Purified virus was quantified by gel electrophoresis. A known volume of virus solution was run on Bolt 4–12% Bis-Tris Plus gels (Novex, Life Technologies) with NuPAGE MOPS SDS Running Buffer (Novex, Life Technologies), alongside bovine serum albumin (BSA) standards. Band pixel densities were quantitated using Image Lab 5.0 software (Bio-Rad) and the mass of the band corresponding to SFV E1 glycoprotein (50 kDa) calculated by interpolation from the BSA calibration curve.

2.8 IdeS resistance assays

The rationale behind the design of IdeS resistance assays is detailed in Section 4.4.4. The following procedure was used to compare the resistance to IdeS of Bavi_IdeS-R and Bavi_WT.

IdeS WT (Section 2.6.3) was diluted one-hundred- or one-thousand-fold from 3.9 mg/mL stock at pH 8.0 to working concentrations, using PBS at pH 7.4. Each IdeS aliquot underwent no more than one freeze-thaw and protein integrity was found to be unaffected, as determined by

SDS-PAGE (Fig. 4.12). Bavi_WT and IdeS-R were generously provided by Michael Birnbaum (Stanford School of Medicine, USA), for which cloning and expression methods are outlined in Section 2.4. Both antibodies were in PBS, pH 7.4, and used within 2 weeks of expression and purification.

IdeS was mixed with 10 μg of Bavi_WT or Bavi_IdeS-R at IdeS:antibody mass ratios of 1:1000, 1:600 and 1:100. Reactions were made up to a total volume of 20 μL with PBS, pH 7.4, then incubated at 37°C for 0, 2, 4, 8, 16 or 24 h. A PBS-only control was included for each timepoint. Cleavage was stopped with the addition of 7 μL NuPAGE LDS sample buffer (Life Technologies, UK). For the 0 h timepoint, addition of LDS sample buffer directly preceded the addition of IdeS. All reaction products were loaded onto NuPAGE 4–12% Bis-Tris gels and run at 180 V for 55 mins, then stained with Coomassie Blue.

2.9 Enzyme-linked immunosorbant assay (ELISA)

ELISAs were conducted with the coating ligand consisting of either protein or intact virus, as given in the following sections. All ELISA data was analysed using GraphPad Prism (GraphPad Software, Inc., USA). The baseline, defined as 0 $\mu\text{g}/\text{mL}$ analyte, was averaged from the replicates for each condition and subtracted from the results; curves were produced using non-linear least-squares fitting (one-site, specific binding with Hill slope).

2.9.1 ELISA with protein ligand

The following protocol was used for ELISAs described in chapter 4 testing the interaction of immobilised Fc γ RIIIA with either monoclonal antibody or human serum IgG as the analyte. All steps were carried out at room temperature with gentle agitation at ~75–80 rpm unless otherwise specified. Each step was followed with three quick washes and three 5-minute washes with agitation, using PBS containing 0.05% Tween-20 ('PBST'). Analyte was serially

diluted in 96-well round-bottom microplates (Fisher Scientific, # 12078528) before addition to the receptor. Assay measurements were conducted in duplicate or triplicate.

High-binding half-area 96-well microplates (Costar, # 3690) were coated with 5 µg/mL of FcγRIIIA in PBS, overnight at 4°C without agitation. The FcγRIIIA solution was then removed and plates washed before blocking with 3% bovine serum albumin (BSA) in PBS for 2 h. After blocking, plates were incubated with antibody dilutions for 2 h followed by detection antibody (goat F(ab)₂ anti-mouse F(ab)₂, abcam, # 98659) at a 1:10,000 dilution in PBS for 1 h. TMB substrate (Thermo Scientific) was added and colour allowed to develop before reaction was stopped by addition of 5 M sulphuric acid. Absorbance was read at 450 nm using a SpectraMax plate reader.

2.9.2 ELISA with virus ligand

TIM-4 and bavituximab proteins were expressed in HEK-293T cells and purified as detailed in sections 2.3 and 2.6. TIM-1 was kindly donated by Xiangxi Wang (Division of Structural Biology, University of Oxford). TIM-1 and TIM-4 were diluted in 20 mM Tris-HCL pH 7.2, 100 mM NaCl, 5 mM CaCl₂ (‘T20N100C5’). Bavituximab was diluted in T20N100C5 containing 10% FBS to provide a source of β2GP1. Serial dilutions of analyte were made in 96-well tissue culture plates (BD Falcon, # 353077). Blocking and incubation of virus with analyte were carried out either at room temperature or 37°C, as specified in results section 2.9.2. Assay measurements were conducted in duplicate.

Virus corresponding to 5 pmoles of glycoprotein was plated in Nunc-Immuno™ Microwell™ 96-well Polysorp® plates (Thermo Scientific, # 456529) and allowed to adsorb overnight at 4°C. Bovine serum albumin (BSA) was plated at the same concentration as a negative control. Following removal of unbound virus, the plate was blocked for 2 h using 5% milk in T20N100C5. The virus was then incubated with serial dilutions of protein in 5% milk in T20N100C5. Protein solutions were then removed, the plates washed once with T20N100C5 and incubated with anti-His (C-term)-HRP detection antibody (Invitrogen, # 46-07-07) for

1 h. Plates were then washed four times with T20N100C5 containing 0.05% Tween-20. The ELISA was developed using ABTS substrate (Vector Laboratories, # SK-4500) according to manufacturer's instructions. The reaction was stopped by the addition of an equivalent volume of 5% sodium dodecyl sulphate (SDS) and absorbance measured at 405 nm using a Tecan Infinite F200 plate reader and Tecan i-control 1.5 software.

2.10 Surface plasmon resonance

The binding affinity of Fc mutants to an inactive form of IdeS (IdeS C94A) was assessed using surface plasmon resonance (SPR) using a BIAcore T200 instrument (GE). IdeS C94A was coupled onto a CM5 sensor chip (BIAcore) at 0.3 μ M in 10 mM sodium acetate, pH 5.5, by using *N*-hydroxysuccinimide/1-ethyl-3-(3-dimethylaminopropyl) carbodiimidehydrochloride (NHS/EDC). The level of immobilisation was approximately 1,000 response units. Flow cell 1 was mocked with NHS/EDC as a blank control.

Fc mutants were serially diluted to 0–40 μ M in 10 mM HEPES, 150 mM NaCl ('HEPES'), and a single duplicate sample of 5 μ M was made up separately for each mutant. An Fc wild type control dilution series was included in every BIAcore run to monitor the integrity of the sample chip over time. All experiments were conducted using HEPES containing 3 mM EDTA and 0.05% surfactant P20 as running buffer, at a flow rate of 10 μ L/min and a temperature of 25°C. The contact time was 120 s and the dissociation time was 200 s. After each injection, the sensor chip was regenerated for 30 s with 50 mM glycine, pH 9.5. Equilibrium dissociation constants (K_D) were derived from a plot of response against concentration and calculated using non-linear fitting using BIAEvaluation software (GE).

2.11 Analysis of N-linked oligosaccharides

2.11.1 Release of N-linked oligosaccharides from proteins

N-linked glycans were released from proteins (20 µg in solution) using peptide N-glycosidase F, glycerol-free (PNGase F) (NEB, # P0705S) following manufacturer's instructions with an extended reaction time of 16 h. Released glycans were separated from remaining protein using a 96-well protein-binding membrane (Millipore MAIPS4510). Membrane wells were pre-wetted with 100 µL ethanol, 100 µL water and 100 µL ethanol before addition of the reaction solution. Sample tubes were washed with 100 µL water and the washes added to the membrane before pulling through with vacuum. Samples were dried using vacuum centrifugation at 30°C and stored at 4°C before fluorescent labelling.

2.11.2 Fluorescent labelling

Each glycan sample was then resuspended in 30 µl water and incubated with 80 µl 2-anthranilic acid (2-AA) labelling mixture (4% w/v sodium ethanoate, 2% w/v hydrogen borate, 3% w/v 2-AA and 4.5% w/v sodium cyanoborohydride) at 80°C for 1 h. After addition of 1 ml 97% acetonitrile, the samples were loaded onto Spe-ed Amide-2 cartridges (100 mg/ml, Applied Separations), pre-washed with 1 ml acetonitrile, 1 ml water and 1 ml acetonitrile, for removal of non-carbohydrate contaminants and unconjugated 2-AA. Columns were washed with 2 x 1 ml 95% acetonitrile before elution of oligosaccharides with 2 x 0.75 ml water and storage at 4°C.

2.11.3 Liquid chromatography

35 µL labelled, purified glycans (2.11.2) were mixed with 65 µL acetonitrile prior to loading onto either a high-performance liquid chromatography (HPLC) or an ultra-performance liquid chromatography (UPLC) system, as detailed below. In both cases, fluorescence is

reported as Relative Fluorescence (RF) and peak assignment indicated using the Oxford Glycan Nomenclature with the colours of the Consortium for Functional Glycomics [78].

High performance liquid chromatography (HPLC)

An XBridge Amide column (4.6 x 250 mm, Waters, Elstree, UK) was connected to a Waters Alliance[®] HPLC W2690/5 system. Glycans were separated using a gradient of 50 mM ammonium formate pH 4.4 (solvent A) and 100% acetonitrile (solvent B) (2.9) at 35°C. Fluorescence was detected with a Waters W2475 instrument using an excitation wavelength of 360 nm and an emission wavelength of 425 nm. Spectra were analysed using Empower Pro software (Waters).

Ultra-performance liquid chromatography (UPLC)

An Acquity BEH 2.1 mm x 10 mm column (1.7 µm particle size, Waters) was connected to a Waters Acquity UPLC instrument. Glycans were separated using a gradient of 40 mM ammonium formate pH 4.4 (solvent A) and 100% acetonitrile (solvent B) (2.9) at 35°C. Fluorescence was detected using an excitation wavelength of 250 nm and an emission wavelength of 428 nm. Spectra were analysed using Empower 3 software (Waters).

Table 2.7: Parameters used for determination of protein concentration.

Protein	ExPASy MW [†] (g mol ⁻¹)	Apparent MW [‡] (kDa)	ϵ^* (M ⁻¹ cm ⁻¹)	Abs 0.1%
TIM-4	15,612.5	30	22,835	1.463
TIM4(EC)	28,874.3	~70 [#]	39,335	1.362
TIM4(cryst)—Fc	40,614.9	55	58,620	1.443
TIM4(EC)—Fc	53,963.8	95	75,120	1.392
hTIM4XII—IgG1II	38,982.1	44	58,620	1.504
hIgG1II—hTIM4XII	39,110.3	44	58,620	1.499
Bavituximab WT (IE)	149,719.5	160	218,360	1.458
Bavituximab WT (MB)	148,061.6	160	218,360	1.475
Bavituximab IdeS-R (IE)	147,362.8	160	218,360	1.482
Bavituximab IdeS-R (MB)	147,923.3	160	218,360	1.476
Fc γ R1IIA	22,912.6	37-45	41,160	1.796
IdeS WT (M1 serotype)	35,257.5	35	49,390	1.401
IdeS C94A (M1 serotype)	35,225.4	35	49,390	1.402

[†] ExPASy MW is the value given by the ExPASy server using the primary sequence.

[‡] Apparent MW is approximate and derived from SDS-PAGE analysis of proteins expressed in-house or by collaborators.

* All extinction coefficients were generated under the assumption that all pairs of cysteine residues form cystines (pairs of cysteine residues joined by a disulphide bond). In the case of IdeS WT, the single cysteine in the active site would remain unbonded and therefore have no impact on the extinction coefficient.

[#] Integrity of expression poor: see Figure 5.2, panels A and B.

Table 2.8: Imidazole concentrations used for the washing and elution steps during nickel-affinity purification of TIM-4, the fusion proteins and Fc γ R1IIA. SN: culture supernatant.

Protein	Imidazole concentration (mM)				
	SN	Equilibration	Wash 1	Wash 2	Elution
TIM-4	20	20	20	40	300
TIM4(EC)	20	20	20	40	300
Fc γ R1IIA	20	20	20	40	300
Bavituximab	20	20	30	30	300
TIM4(cryst)—Fc	20	20	20	N/A	300
TIM4(EC)—Fc	20	20	20	N/A	300
hTIM4XII—hIgG1XII	20	20	20	N/A	300
hIgG1XII—hTIM4XII	20	20	20	N/A	300

Table 2.9: Solvent gradients for HPLC (Xbridge column) and UPLC (BEH column) analysis of N-linked glycans.

Column	Time (mins)	Flow rate (mL/min)	%A	%B
XBridge	0.0	1.0	35.0	65.0
	6.0	1.0	35.0	65.0
	46.0	1.0	46.0	54.0
	48.0	1.0	80.0	20.0
	52.0	1.0	35.0	65.0
	75.0	1.0	35.0	65.0
BEH	0.0	0.50	22.0	78.0
	38.5	0.50	44.1	55.9
	39.5	0.25	100.0	0.0
	44.5	0.25	100.0	0.0
	46.5	0.25	22.0	78.0
	46.6	0.50	22.0	78.0
	48.0	0.50	22.0	78.0

Chapter 3

Mapping the IgG–IdeS interface

3.1 Rationale

The bacterial enzyme IdeS cleaves human IgG in the hinge region (Section 1.10). It is produced as an immune evasion factor by *Streptococcus pyogenes* but has therapeutic potential, both as an immunosuppressive agent to facilitate kidney transplantation [200] and to enhance the efficacy of targeted therapeutic antibodies (Chapter 4). The exact binding site of IdeS on IgG is still unknown. Although IdeS is known to hydrolyse the peptide bond between Gly236 and Gly237, peptides matching the IgG hinge region were not cleaved without the presence of the rest of the Fc domain [191]. It is therefore hypothesised that a secondary binding site, or exosite, exists on either the C γ 2 or C γ 3 regions.

Detailed knowledge of the IgG amino acid residues interacting with IdeS would facilitate the production of IdeS-resistant therapeutic antibodies for use in the receptor refocusing approach (Chapter 4). This is particularly important as the IdeS binding site overlaps with those of the classical Fc γ receptors, which are important for many antibody effector functions (Sections 1.8.3 and 1.10). Work on the creation of an IdeS-resistant antibody found that certain mutations, especially S239D and I332E, both conferred IdeS resistance and improved binding to Fc γ RIIIA [101].

We therefore sought to map the binding site of IdeS onto IgG by site-directed mutagenesis. Surface-exposed residues in the hinge and C γ 2 regions were systematically mutated to alanine and the affinity of each mutant to IdeS was determined using surface plasmon resonance (SPR). The aim was to gain a comprehensive picture of IgG residues involved in binding, and in so doing to resolve the question of the secondary binding site.

3.2 Prediction of IgG residues involved in interactions

It is known that IdeS can bind to and hydrolyse papain-cleaved IgG1 Fc. Papain cleavage results in an Fc domain with the N-terminal amino acid residue corresponding to His224 (IgG1 Eu numbering [60]). The binding site(s) of IdeS must therefore be in the Fc region between residues His224 and Lys447. Although no X-ray crystal structure of an IdeS–IgG complex is available, SAXS analysis of an IdeS–Fc complex showed the IdeS located on top of the Fc in the hinge region. It was therefore hypothesised that the probable exosite would be located in the C γ 2 domain, in addition to a main binding site in the hinge region.

Residues in the C γ 2 domain that are potentially involved in protein–protein interactions residues were identified using three methods. Firstly, surface-exposed residues were manually selected using an X-ray crystal structure of IgG1 Fc in complex with Fc γ RIIIB (PDB 1T83). Secondly, the server meta-PPISP (meta-Protein–Protein Interaction Site Prediction) was used [144]. This is a structure- and sequence-based prediction method combining the results from three individual servers: cons-PPISP, Promate and PINUP. Interaction sites are predicted based on sequence profiling, secondary structure and solvent accessibilities. The input file used in this case was the structure of the fucosylated IgG1 Fc (PDB 3AVE). Thirdly, another prediction tool known as Sppider (Solvent accessibility based Protein–Protein Interface iDentification and Recognition), version 2, was included [142]. The Fc structure 3AVE was again used as the input, and the offset between sensitivity and specificity was set at 0.5. The residues identified by each of these methods are listed in Table 3.1. There is some, but not total, consensus among the methods with regard to the particular residues identified. As expected, the prediction

servers gave substantially fewer results than the manual method due to their incorporation of interaction profiling. It should be noted that neither metaPPISP or Sppider identified Ile332 as a potential interaction site.

Table 3.1: List of potentially interacting residues on IgG1 Fc, identified manually and by two prediction methods.

Residue	Eu number	Manual	metaPPISP	Sppider	Selected
H	224				
T	225	X			X
C	226	X			X
P	227	X			X
P	228	X			X
C	229	X			X
P	230	X			X
A	231	X			X
P	232	X			X
E	233	X			X
L	234	X	X	X	X
L	235	X	X	X	X
G	236	X	X	X	X
G	237	X	X	X	X
P	238	X	X		X
S	239	X	X	X	X
V	240				
F	241		X	X	

Table 3.1 continued: potentially interacting residues on IgG1 Fc.

Residue	Eu number	Manual	metaPPISP	Sspider	Selected
L	242				
F	243		X		
P	244				
P	245				
K	246				
P	247				
K	248				
D	249				
T	250				
L	251				
M	252				
I	253			X	
S	254			X	
R	255				
T	256			X	
P	257				
E	258				
V	259				
T	260				
C	261				
V	262				

Table 3.1 continued: potentially interacting residues on IgG1 Fc.

Residue	Eu number	Manual	metaPPISP	Sspider	Selected
V	263				
V	264		X		
D	265	X	X	X	X
V	266				
S	267	X			X
H	268	X			X
E	269	X	X	X	X
D	270		X		
P	271	X			X
E	272	X	X		X
V	273				
K	274	X			X
F	275		X		
N	276	X			X
W	277				
Y	278	X			X
V	279				
D	280				
G	281				
V	282				
E	283				

Table 3.1 continued: potentially interacting residues on IgG1 Fc.

Residue	Eu number	Manual	metaPPISP	Sspider	Selected
V	284				
H	285				
N	286				
A	287				
K	288				
T	289	X			X
K	290	X			X
P	291	X			X
R	292	X			X
E	293	X			X
E	294	X			X
Q	295		X		
Y	296	X		X	
N	297	X	X		
S	298	X	X		
T	299	X	X		
Y	300	X			
R	301				
V	302				
V	303				
S	304				

Table 3.1 continued: potentially interacting residues on IgG1 Fc.

Residue	Eu number	Manual	metaPPISP	Sspider	Selected
V	305				
L	306				
T	307				
V	308				
L	309				
H	310				
Q	311				
D	312				
W	313				
L	314				
N	315				
G	316				
K	317				
E	318				
Y	319				
K	320	X			
C	321				
K	322	X	X		
V	323				
S	324		X		
N	325				

Table 3.1 continued: potentially interacting residues on IgG1 Fc.

Residue	Eu number	Manual	metaPPISP	Sspider	Selected
K	326	X			
A	327	X		X	
L	328				
P	329	X		X	X
A	330	X		X	X
P	331	X			X
I	332	X			X
E	333	X			X
K	334	X			
T	335	X			
I	336				
S	337				
K	338				
Total identified		46	20	15	35

Taking all this into consideration, it was decided to focus primarily on the residues in the hinge, at the N-terminal end of the C γ 2 and the region of the C γ 2 surrounding the I332 residue (column ‘Selected’ in Table 3.1). Selected residues, the locations of which are shown on the structure in Figure 3.1, were mutated individually to alanine. In addition to the point mutations, two hingeless Fc constructs (Pro238–Lys447 and Ser239–Lys447) and a hingeless C γ 2 (Ser239–Lys338) were also created, to ascertain which of the C γ 2 or C γ 3 domains contains the putative exosite.

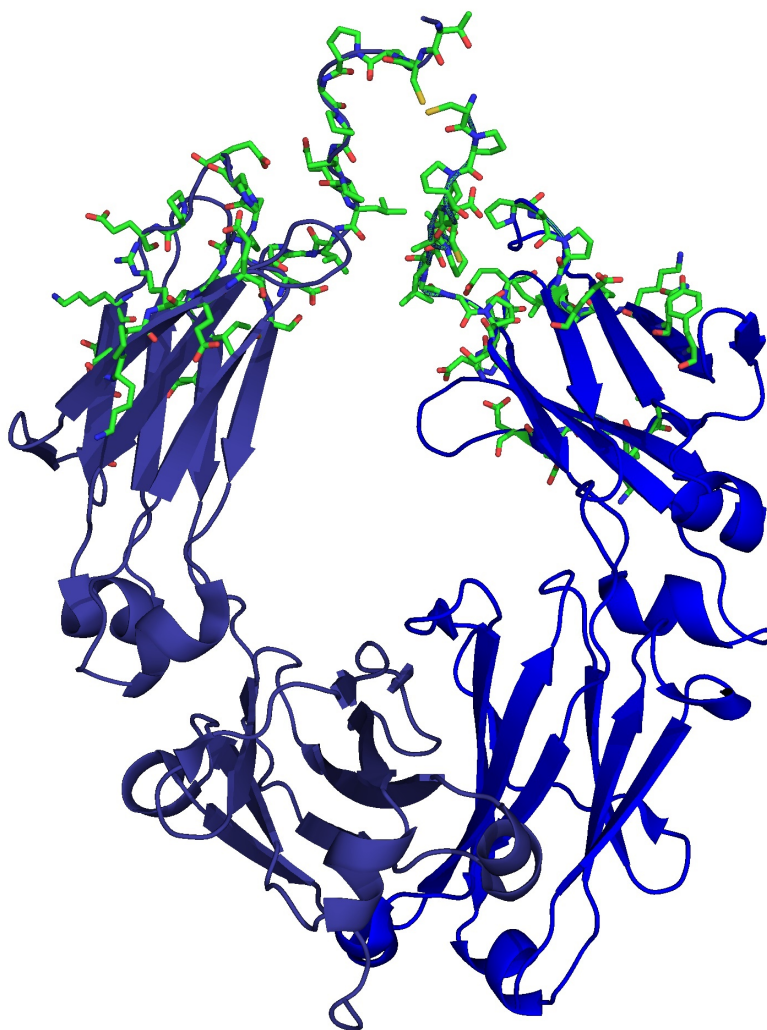


Figure 3.1: Cartoon representation of IgG1 Fc, cropped from the crystal structure of B12 (PDB 1HZH). Chain A is coloured dark blue and chain B in blue. The residues mutated to alanine as part of this study are shown on both chains in stick representation and coloured by atom (carbons in green, oxygens in red, nitrogens in green and sulphurs in yellow). For a detailed list of these residues, please refer to Table 3.1.

3.3 Production of a series of Fc mutants

3.3.1 Cloning and expression

Single amino acid mutations to alanine were carried out on the human IgG1 Fc domain encoded in the pFUSE-hIgG1-Fc2 vector, as detailed in Section 2.1.6. Wild type and mutant Fc proteins were expressed from this vector in mammalian HEK 293T cells and affinity-purified by Protein A followed by gel filtration (Sections 2.3 and 2.6). Each of these wild type and single mutant proteins contained the IgG1 residues Asp221 to Lys447, plus an N-terminal ISAMVRS sequence from the vector backbone. The hingeless Fc and hingeless C γ 2 were cloned and expressed as described in Sections 2.1.6 and 2.3.2. The wild type Fc was expressed multiple times to use as a control for each BIAcore run (Section 3.5).

All Fc fragments were expressed by transient transfection of HEK 293T cells and purified by Protein A affinity followed by gel filtration. The purification procedure for wild type Fc is shown in Figure 3.2; the gel filtration profile in panel B is representative of every wild type and single mutant Fc, all of which eluted at a retention volume of between 62 mL and 72 mL. Proteins were assessed by SDS-PAGE following purification. All wild type and single mutant Fc proteins had an apparent molecular weight of ~50 kDa, except for I332A which ran at ~55 kDa. As the glycan analysis showed no substantial alteration in oligosaccharide structure, this variation was attributed to a gel artefact. The majority of mutants expressed and purified to a sufficiently high level for subsequent analysis (Figure 3.3) — the exceptions to this were one of the hingeless Fc constructs (Pro238–Lys447), the hingeless C γ 2 and certain Fc single point mutation constructs that are identified in Table 3.2.

3.3.2 Glycan analysis

IdeS cleavage was found to be glycan-independent [191]; however, analysis of the N-linked glycans from each Fc fragment was carried out for two reasons. Firstly, it enabled

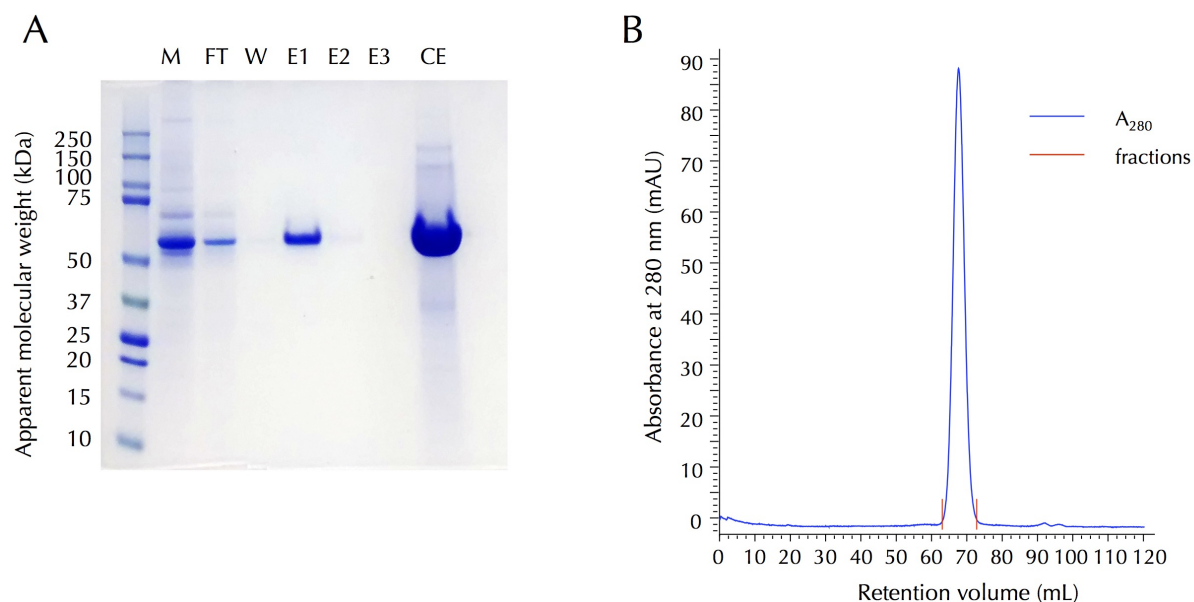


Figure 3.2: Purification of wild type IgG1 Fc by Protein A and gel filtration. **A** | Fractions from Protein A purification stages were subjected to non-reducing SDS-PAGE, using a 4–12% Bis-Tris gel, and stained with Coomassie Blue. In this case, the culture supernatant was harvested 72 h after transfection. M, media 48 h following transfection; FT, flow-through (unbound) from Protein A-Sepharose; W, wash fraction; E1–E3, eluates (each 10 bead volumes of 0.1 M citrate buffer); CE, pooled and concentrated eluates. **B** | Gel filtration chromatogram of Protein A-purified wild type Fc using a Superdex 200 pg 16/600 column. Blue trace indicates absorbance at 280 nm, red lines indicate the start and end of fraction collection. Panels A and B are representative samples from separate expression batches.

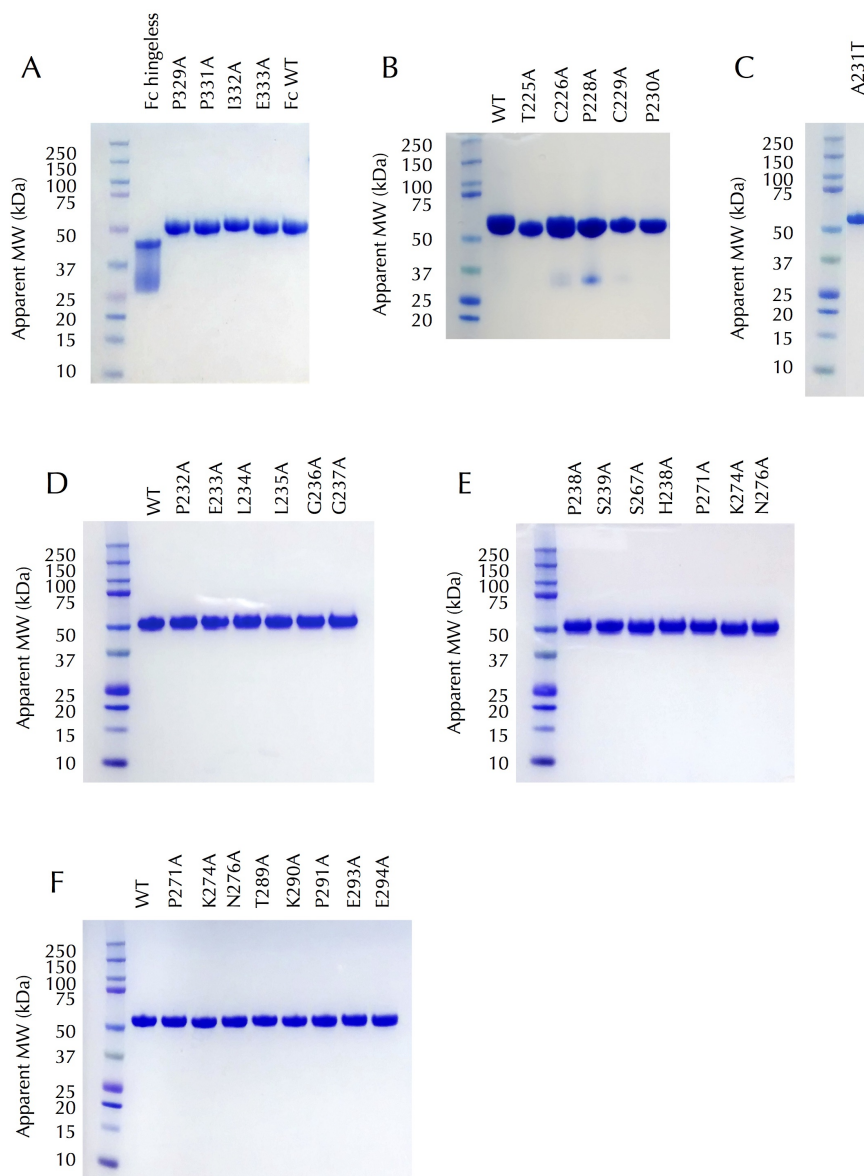


Figure 3.3: Non-reducing SDS-PAGE analysis of Fc constructs following Protein A purification and gel filtration. This figure includes all constructs that expressed to a sufficient level for glycan analysis and SPR. The mutation for each construct is specified above the lanes and the first lane of each gel contains molecular weight (MW) markers (10–250 kDa). A wild type Fc construct was regularly expressed to monitor batch-to-batch variation in the expression system and to provide a control for each SPR run. **A]** Wild type control, hingeless Fc containing residues Ser239–Lys447, and Fc fragments with mutations surrounding the I332 residue. **B]** Wild type and mutants T225A–P230A. **C]** Mutant A230T. **D]** Wild type and mutants P232A–G237A. **E]** Mutants P238A–N276A. **F]** Wild-type plus mutants P271A–E294A.

a more accurate estimation of the molecular weight of each Fc construct by combining amino acid sequence-based prediction with calculation of the glycan mass. Secondly, protein conformational changes may alter the accessibility of the oligosaccharide to processing enzymes, and therefore be reflected in the glycan profile. The activity of an enzyme with two separate binding sites may well be affected by slight changes in protein conformation. The glycan analysis provided a straightforward and reproducible initial factor by which to assess the consistency of protein expression; any substantial differences to the wild type would then have been investigated further. The glycan assignments for each Fc construct is shown in Figures 3.4 to 3.9.

3.3.3 Determination of molecular weight and concentration

Concentration of each Fc fragment was measured using a Nanodrop and the average of three readings taken. For the molecular weight, the peptide mass was calculated theoretically using the ExPASy server. A weighted average of the glycan mass for each expressed construct was also calculated based on the oligosaccharide assignments (Appendix C), and added to the peptide calculation to give the total estimated mass (Table 3.2). For an initial expression of Fc wild type, the molecular weight was measured using multiple angle light scattering (MALS) following elution from a Superdex 200 column. The chromatogram in Figure 3.10, panel A, shows a single peak with a consistent molecular weight of $54.2 \text{ kDa} \pm 0.3\%$. ExPASy-calculated peptide mass and glycan-estimated mass were 52.6 kDa and 1.6 kDa respectively; the total calculated mass of 54.2 kDa is in agreement with the MALS data. This provided confidence that summation of peptide and glycan masses could be used to estimate molecular weight for all the Fc fragments.

Figure 3.10, panel B, shows MALS analysis of the hingeless Fc containing residues Ser239–K447. This protein produced a diffuse band between $\sim 30 \text{ kDa}$ and $\sim 45 \text{ kDa}$ on SDS-PAGE (Figure 3.3, panel A) and was therefore subjected to MALS to ascertain the molecular weight and the integrity of the sample. Gel filtration produced a single peak with a molecular weight

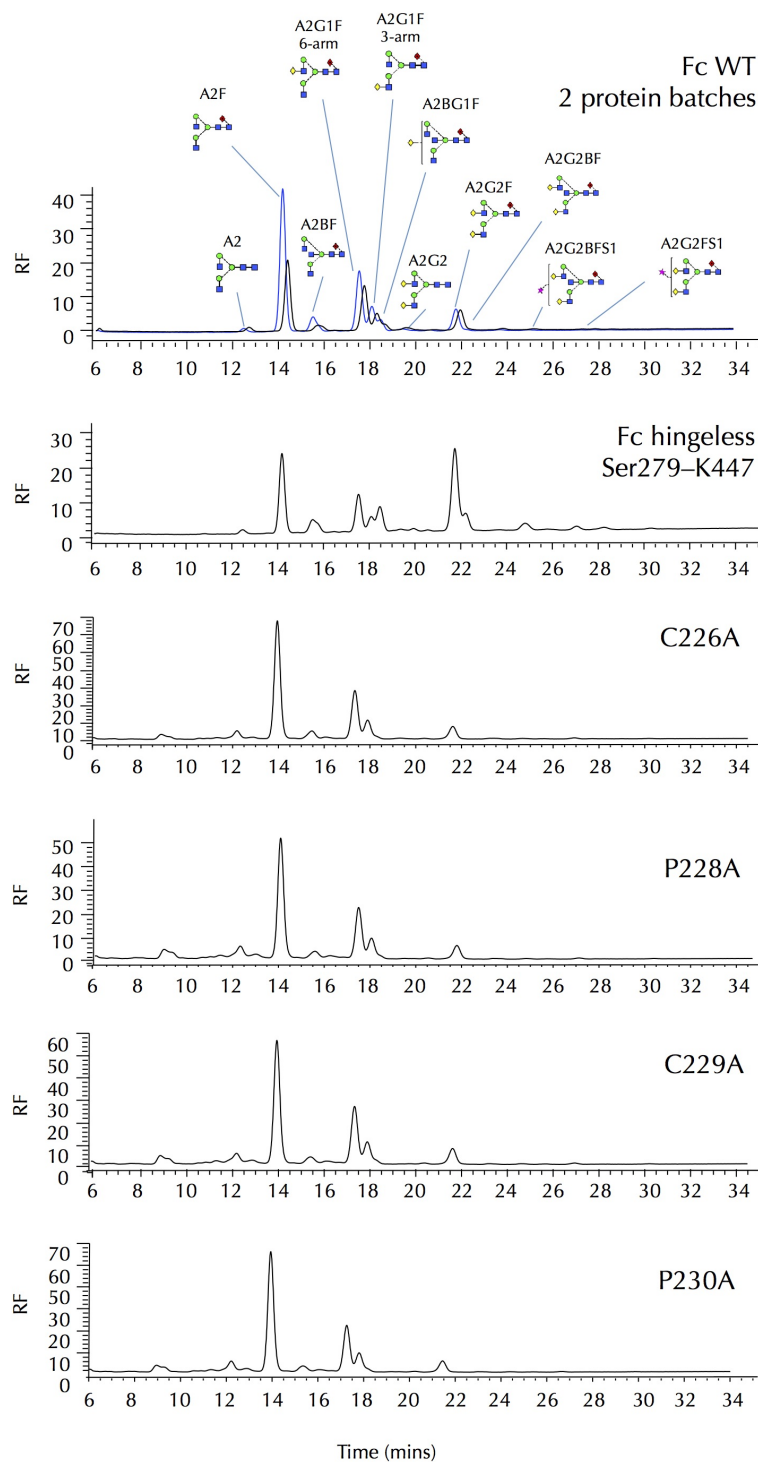


Figure 3.4: Glycan profiles for Fc wild type, hingeless Fc (Ser239–Lys447) and Fc mutants C226A, P228A, C229A and P230A. Glycans were separated by HPLC using an Xbridge Amide column and assigned with reference to [55]. Glycan structure diagrams follow the Oxford Glycan Nomenclature with the colours of the Consortium for Functional Glycomics [78]. The Fc wild type includes an overlay of profiles from two separate protein expressions. Fluorescence is reported as relative fluorescence units (RF).

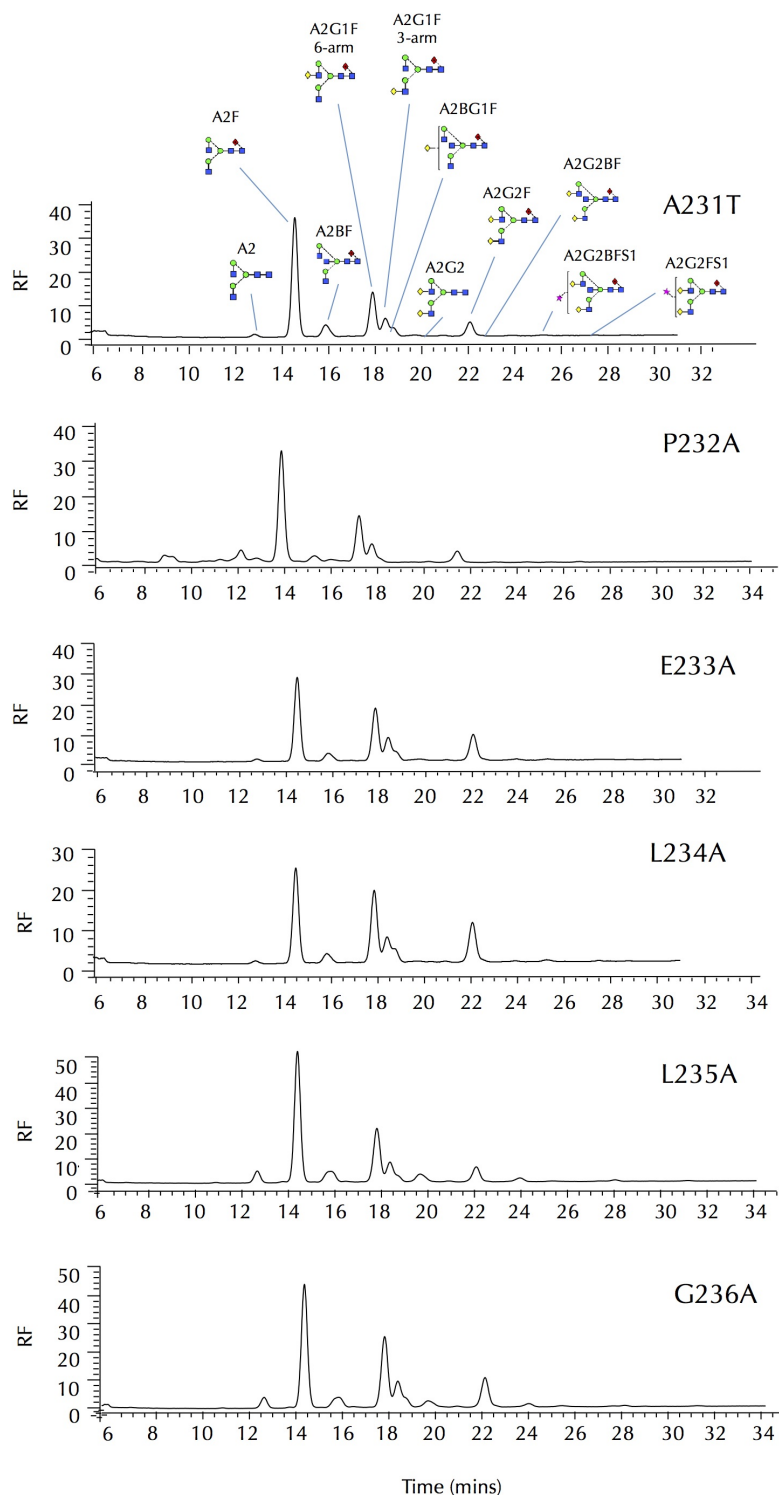


Figure 3.5: Glycan profiles for Fc mutants A231T, P232A, E233A, L234A, L235A and G236A. Glycans were separated by HPLC using an Xbridge Amide column and assigned with reference to [55]. Glycan structure diagrams follow the Oxford Glycan Nomenclature with the colours of the Consortium for Functional Glycomics [78]. Fluorescence is reported as relative fluorescence units (RF).

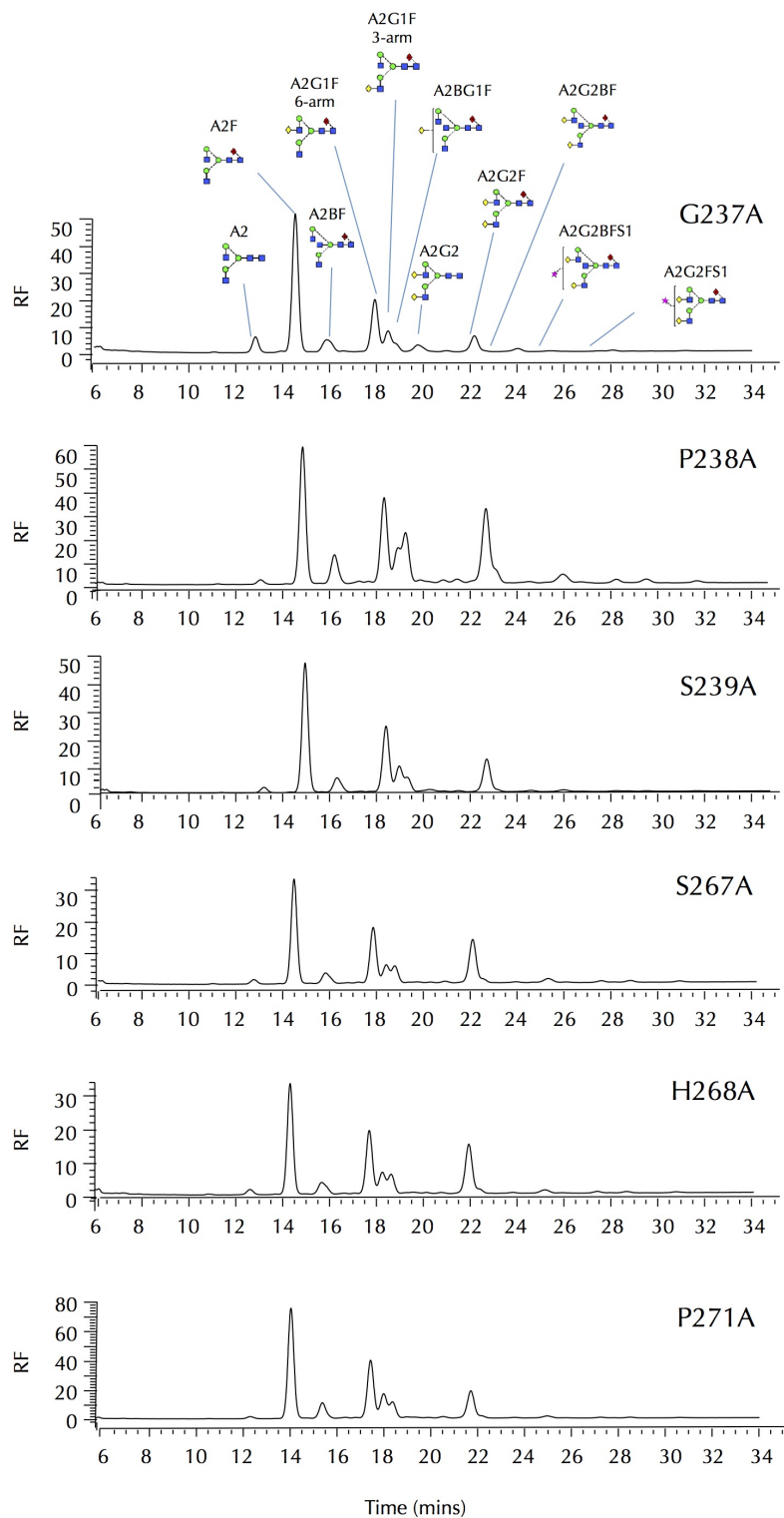


Figure 3.6: Glycan profiles of Fc mutants G237A, P238A, S239A, S267A, H268A and P271A. Glycans were separated by HPLC using an Xbridge Amide column and assigned with reference to [55]. Glycan structure diagrams follow the Oxford Glycan Nomenclature with the colours of the Consortium for Functional Glycomics [78]. Fluorescence is reported as relative fluorescence units (RF).

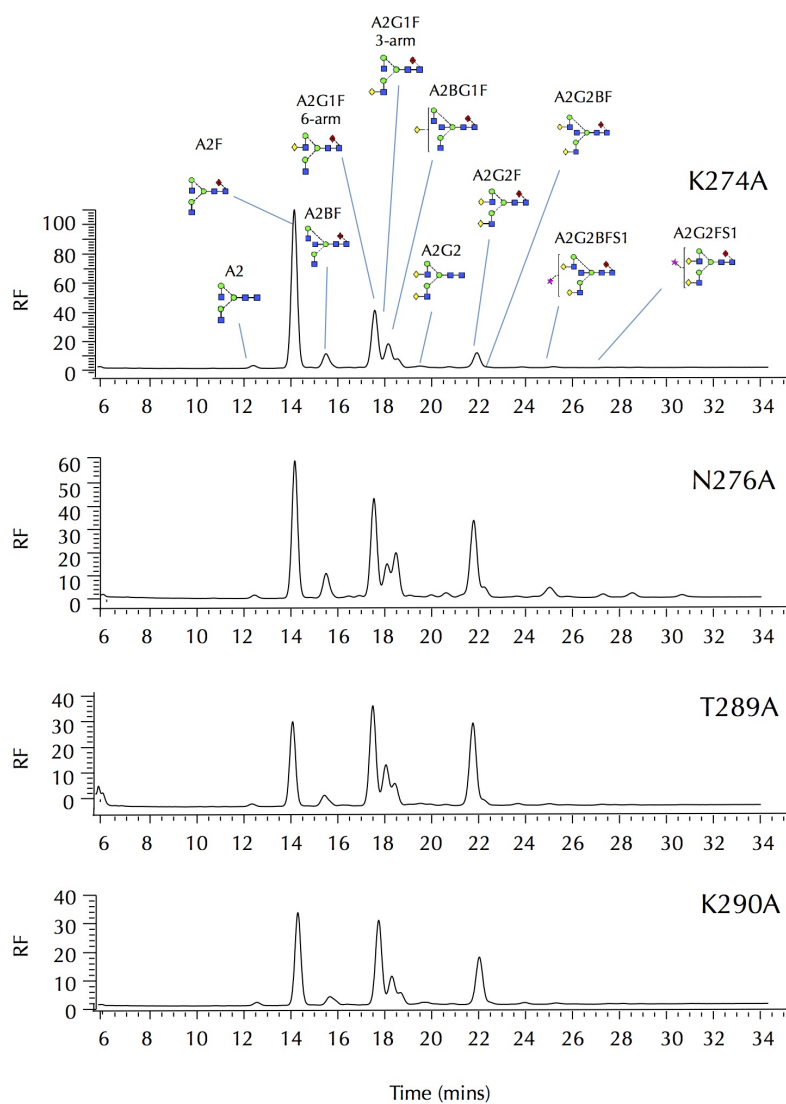


Figure 3.7: Glycan profiles of Fc mutants K274A, N276A, T289A and K290A. Glycans were separated by HPLC using an Xbridge Amide column and assigned with reference to [55]. Glycan structure diagrams follow the Oxford Glycan Nomenclature with the colours of the Consortium for Functional Glycomics [78]. Fluorescence is reported as relative fluorescence units (RF).

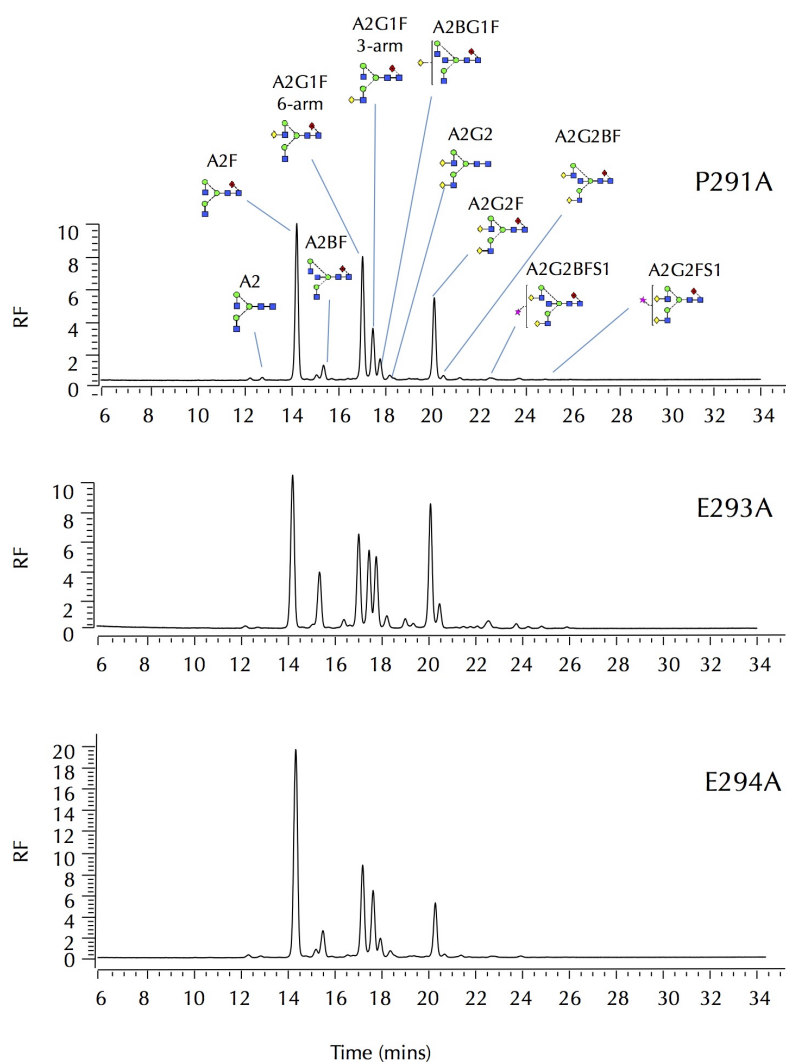


Figure 3.8: Glycan profiles of Fc mutants P291A, E293A and E294A. Glycans were separated by UPLC using an Aquity BEH column and assigned with reference to [55]. Glycan structure diagrams follow the Oxford Glycan Nomenclature with the colours of the Consortium for Functional Glycomics [78]. Fluorescence is reported as relative fluorescence units (RF).

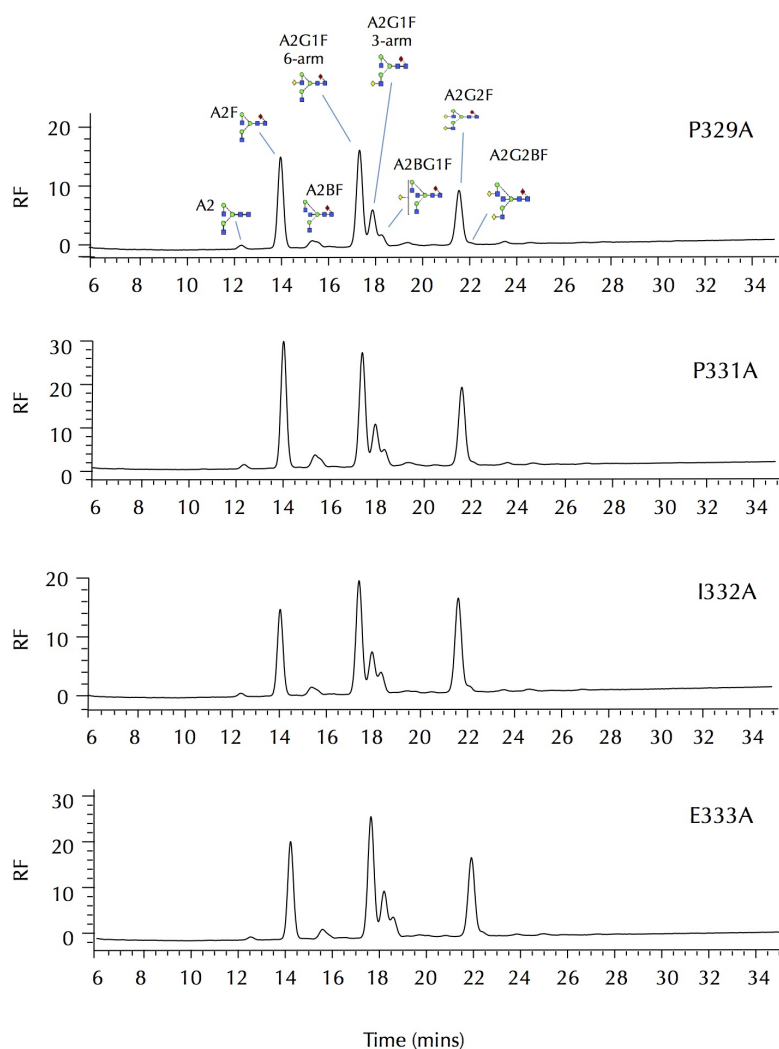


Figure 3.9: Glycan profiles of Fc mutants P329A, P331A, I332A and E333A. Glycans were separated by HPLC using an Xbridge Amide column and assigned with reference to [55]. Glycan structure diagrams follow the Oxford Glycan Nomenclature with the colours of the Consortium for Functional Glycomics [78]. Fluorescence is reported as relative fluorescence units (RF).

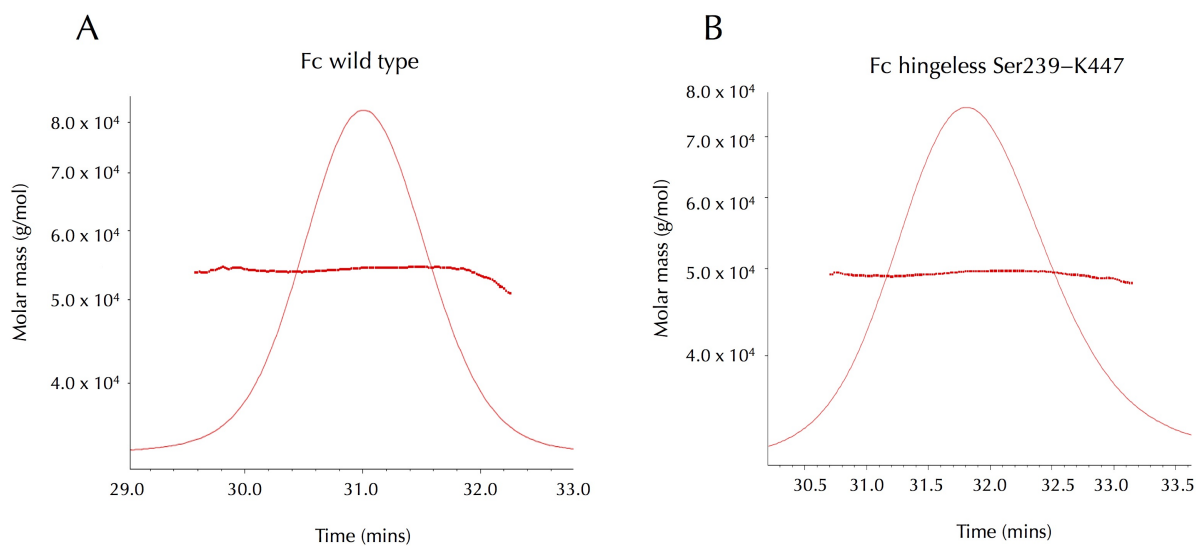


Figure 3.10: Multiple angle light scattering (MALS) analysis of **A** wild type Fc and **B** hingeless Fc (Ser239–K447), following elution from a Superdex 200 10/300 column. The red dotted line indicates the molar mass in g/mol, while the solid red trace represents UV absorption.

of $49.4 \text{ kDa} \pm 0.3\%$, in agreement with the calculated result of 49.2 kDa (Table 3.2). The latter value was therefore used for further analysis.

3.4 Production of inactive IdeS

For assessment of Fc binding to IdeS, it was necessary to use an inactive form of IdeS which retains binding ability but does not cleave Fc. The Cys94 residue is located in the IdeS active site, and mutation of this residue to alanine produces a catalytically inactive form that still binds IgG. This mutant ('IdeS C94A') was therefore chosen for subsequent SPR assays. The mutant enzyme was expressed in *E. coli* cells as detailed in Section 2.5 and purified using a nickel-affinity column. A step gradient of imidazole was used to elute the enzyme from the column; the resulting fractions were concentrated and assessed by SDS-PAGE as shown in Figure 3.11. The 100 mM and 300 mM imidazole eluates were then pooled and concentrated to 25 mL. One-fifth of the concentrate was further purified by gel filtration (Figure 3.11, panel

C). Fractions eluting between the retention times of 55 and 68 minutes were pooled and left unconcentrated for SDS-PAGE (Figure 3.11, panel B, lane 2). The final yield from the equivalent of 50 mL culture was 33.5 mg. Purified enzyme was divided into 1 mL aliquots and stored at -20°C before use.

3.5 Affinity of Fc mutants for IdeS

The affinity of IdeS for wild type Fc, the hingeless Fc constructs and each single point mutant Fc was measured by surface plasmon resonance. IdeS C94A was immobilised onto a CM5 sensor chip by amide coupling. The Fc fragments were flowed over the sensor chip at concentrations ranging from $0.3\ \mu\text{M}$ to $40\ \mu\text{M}$. This range was chosen in order to bracket the previously published dissociation constant (K_D) of IdeS C94A binding to Fc, which was $5.6\ \mu\text{M}$ [3]. Binding of each sample rapidly reached a steady state, allowing equilibrium analysis of the K_D for each mutant. To achieve this, the reference-subtracted response, from a report point at stable binding, was plotted against concentration and the data fitted to a global 1:1 interaction model. Sensorgrams and equilibrium binding isotherms are given in Figures 3.12 to 3.17. The resulting dissociation constants for all mutants tested are listed in Table 3.2.

Binding of Fc wild type was $3.5 \pm 0.5\ \mu\text{M}$; this figure is an average of measurements from three separate protein expression batches and two individual sensor chips, both immobilised with IdeS C94A under the same conditions, which gave dissociation constants of $2.9\ \mu\text{M}$, $3.8\ \mu\text{M}$ and $3.9\ \mu\text{M}$. The consistency of the Fc wild type measurements over time demonstrated the reliability of results across protein expression batches.

Table 3.2: Affinity of Fc mutants for IdeS C94A as measured by surface plasmon resonance.

Mutant	Expressed protein molecular mass (Da)			Binding to IdeS C94A	
	ExPASy	Glycan	Total	K_D (μM)	SE (K_D) ^c
Wild type	52632	1596	54228	3.5	0.5

Table 3.2 continued: Affinity of Fc mutants to IdeS C94A

Mutant	Expressed protein molecular mass (Da)			Binding to IdeS C94A	
	ExPASy	Glycan	Total	K_D (μ M)	SE (K_D) ^c
Fc P238-K447	poor expression				
Fc S239-K447	47512	1702	49214	7.9	1.2
C γ 2 S239-K338	poor expression				
T225A	precipitated prior to SPR				
C226A	52568	1542	54109	5.8	0.9
P227A	cloning unsuccessful				
P228A	52580	1545	54125	4.4	0.8
C229A	52568	1551	54118	6.8	0.1
P230A	52580	1533	54113	5.2	0.8
A231T	52692	1568	54260	4.1	0.7
P232A	52580	1541	54121	8.5	0.2
E233A	52516	1597	54112	9.7	0.9
L234A	52548	1621	54168	9.5	0.9
L235A	52548	1557	54105	15	1
G236A	52660	1578	54238	9.7	0.7
G237A	52660	1551	54211	15	2
P238A	52580	1662	54242	2.6	0.5
S239A	low yield				
S267A	52600	1620	54220	7.5	0.5
H268A	52500	1627	54126	3.1	0.6
E269A	cloning unsuccessful				
P271A	52580	1605	54184	3.3	0.4
E272A	cloning unsuccessful				

Table 3.2 continued: Affinity of Fc mutants to IdeS C94A

Mutant	Expressed protein molecular mass (Da)			Binding to IdeS C94A	
	ExPASy	Glycan	Total	K_D (μ M)	SE (K_D) ^c
K274A	52518	1554	54072	4.6	0.2
N276A	52546	1661	54207	4.0	0.2
Y278A	poor expression				
T289A	52572	1706	54277	4.2	0.5
K290A	52518	1691	54209	3.3	0.5
P291A	52580	1615	54194	3.7	0.5
R292A	cloning unsuccessful				
E293A	52516	1643	54159	4.9	0.8
E294A	52746	1582	54328	4.3	0.7
P329A	52580	1616	54196	17 ^a	1
A330T	low yield				
P331A	52580	1619	54198	14 ^a	1
I332A	52548	1644	54192	130 ^b	20
E333A	52516	1631	54147	3.2	0.4

^a Plot of response against concentration did not reach a plateau.

^b Calculated K_D inaccurate: greater than half the highest concentration tested

^c SE, standard error of the K_D , an indication of the closeness of fit of the model.

3.6 Discussion

The aim of this chapter was to map the contact amino acid residues for IdeS on IgG1 Fc, and in so doing to determine whether there is a secondary binding site in addition to the hinge region.

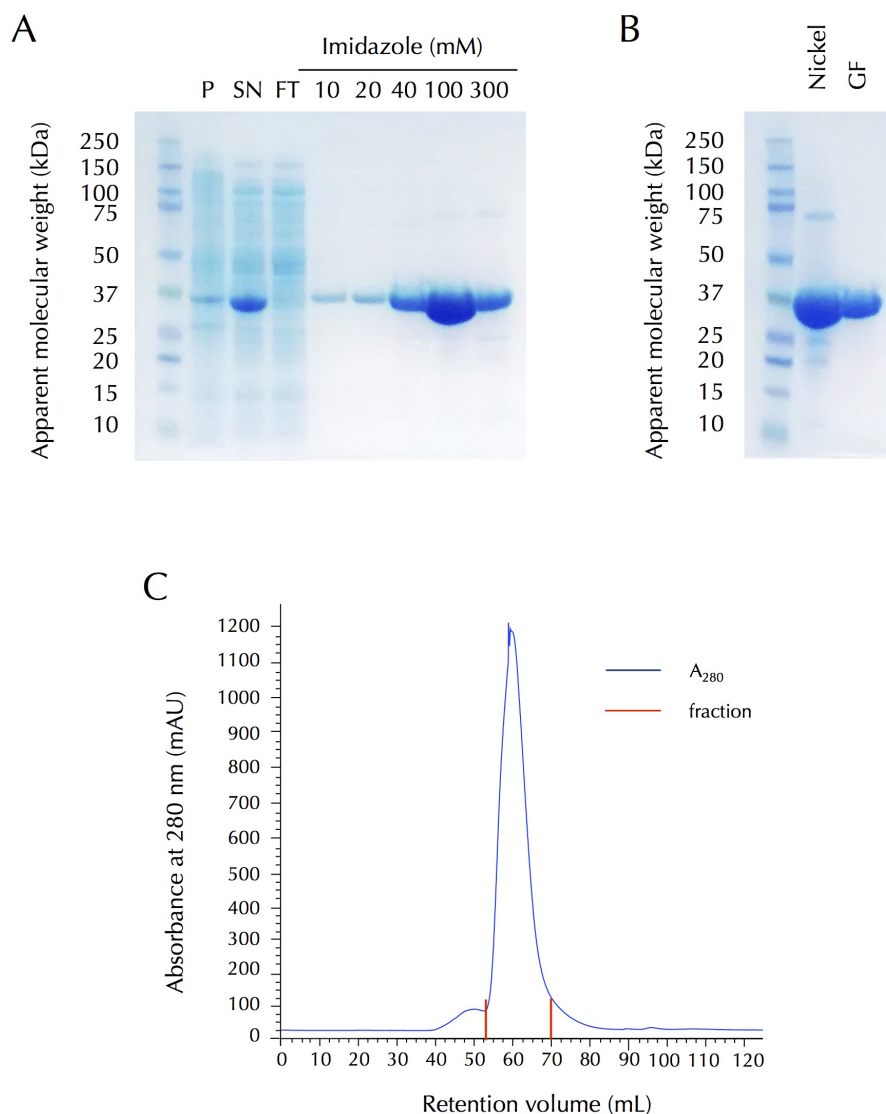


Figure 3.11: IdeS C94A expression and purification. **A** | Non-reducing SDS-PAGE assessment of IdeS C94A eluates from a nickel-affinity column. P, post-lysis bacterial pellet; SN, post-lysis bacterial supernatant; FT, flow-through from column; 10/20/40/100/300, step gradient of imidazole elutions with concentrations given in mM. The first lane contains molecular weight markers. **B** | Non-reducing SDS-PAGE comparison of IdeS C94A purity following nickel-affinity alone ('Nickel') and nickel affinity followed by gel filtration ('GF'). The first lane contains molecular weight markers. **C** | Gel filtration profile of IdeS C94A. The 100 mM and 300 mM eluates were combined and a portion of the pooled eluates injected onto a Superdex 75 pg 16/60 column. The blue trace indicates absorbance at 280 nm; red lines indicate the start and end of fraction collection.

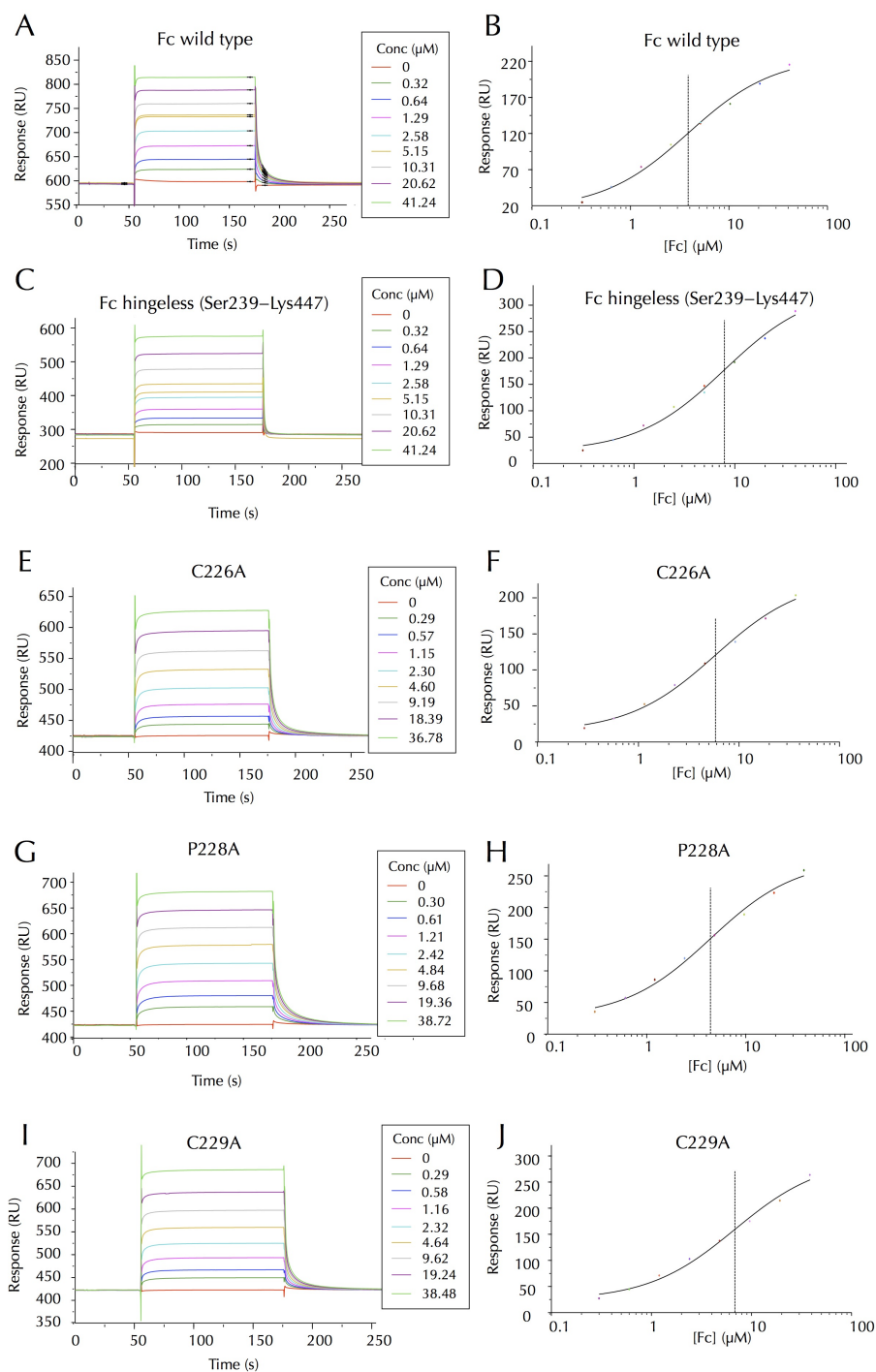


Figure 3.12: SPR analysis of IgG1 Fc variants binding to IdeS C94A: Fc wild type (panels A & B), hingeless Fc (Ser239-Lys447) (C & D), C226A (E & F), P228A (G & H) and C229A (I & J). Left-hand panels contain the sensorgrams for each Fc concentration tested. Right-hand panels give the equilibrium binding isotherms (solid black line) derived from a plot of binding response against Fc concentration; the vertical dotted line indicates the K_D derivation. In both cases the binding response is given in resonance units (RU).

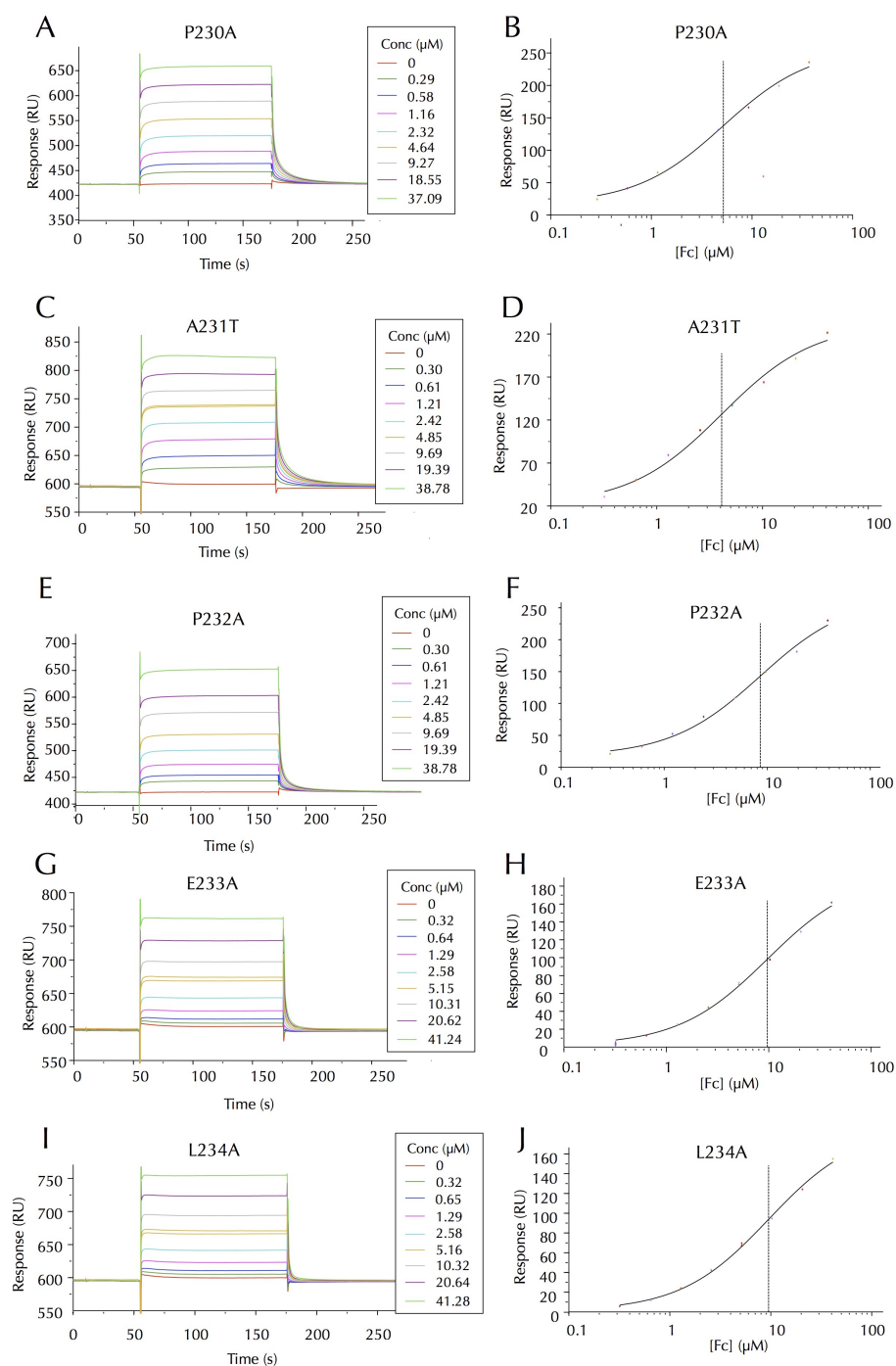


Figure 3.13: SPR analysis of IgG1 Fc variants binding to IdeS C94A: P230A (panels A & B), A231T (C & D), P232A (E & F), E233A (G & H) and L234A (I & J). Left-hand panels contain the sensorgrams for each Fc concentration tested. Right-hand panels give the equilibrium binding isotherms (solid black line) derived from a plot of binding response against Fc concentration; the vertical dotted line indicates the K_D derivation. In both cases the binding response is given in resonance units (RU).

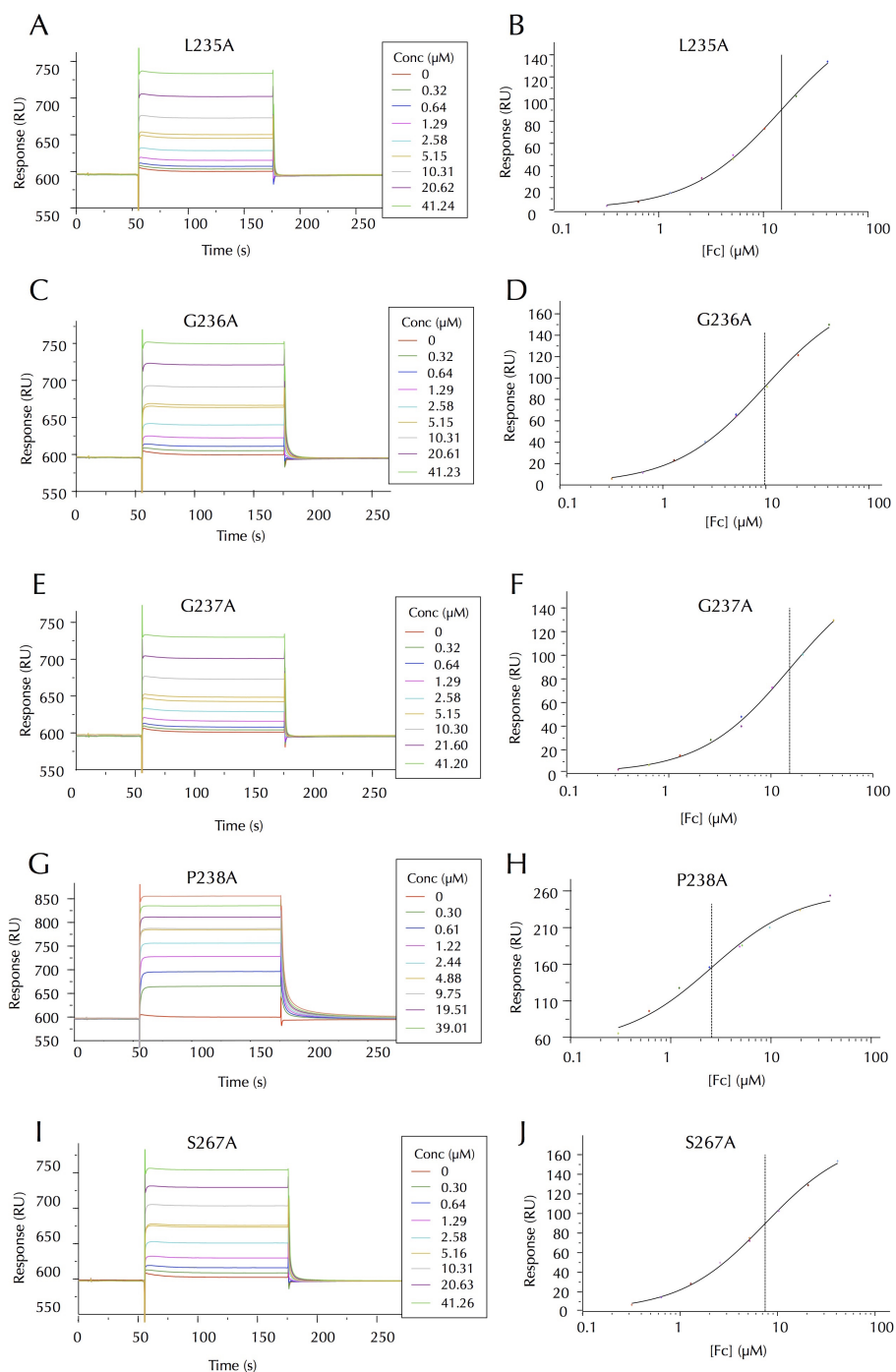


Figure 3.14: SPR analysis of IgG1 Fc variants binding to IdeS C94A: L235A (panels A & B), G236A (C & D), G237A (E & F), P238A (G & H) and S267A (I & J). Left-hand panels contain the sensorgrams for each Fc concentration tested. Right-hand panels give the equilibrium binding isotherms (solid black line) derived from a plot of binding response against Fc concentration; the vertical dotted line indicates the K_D derivation. In both cases the binding response is given in resonance units (RU).

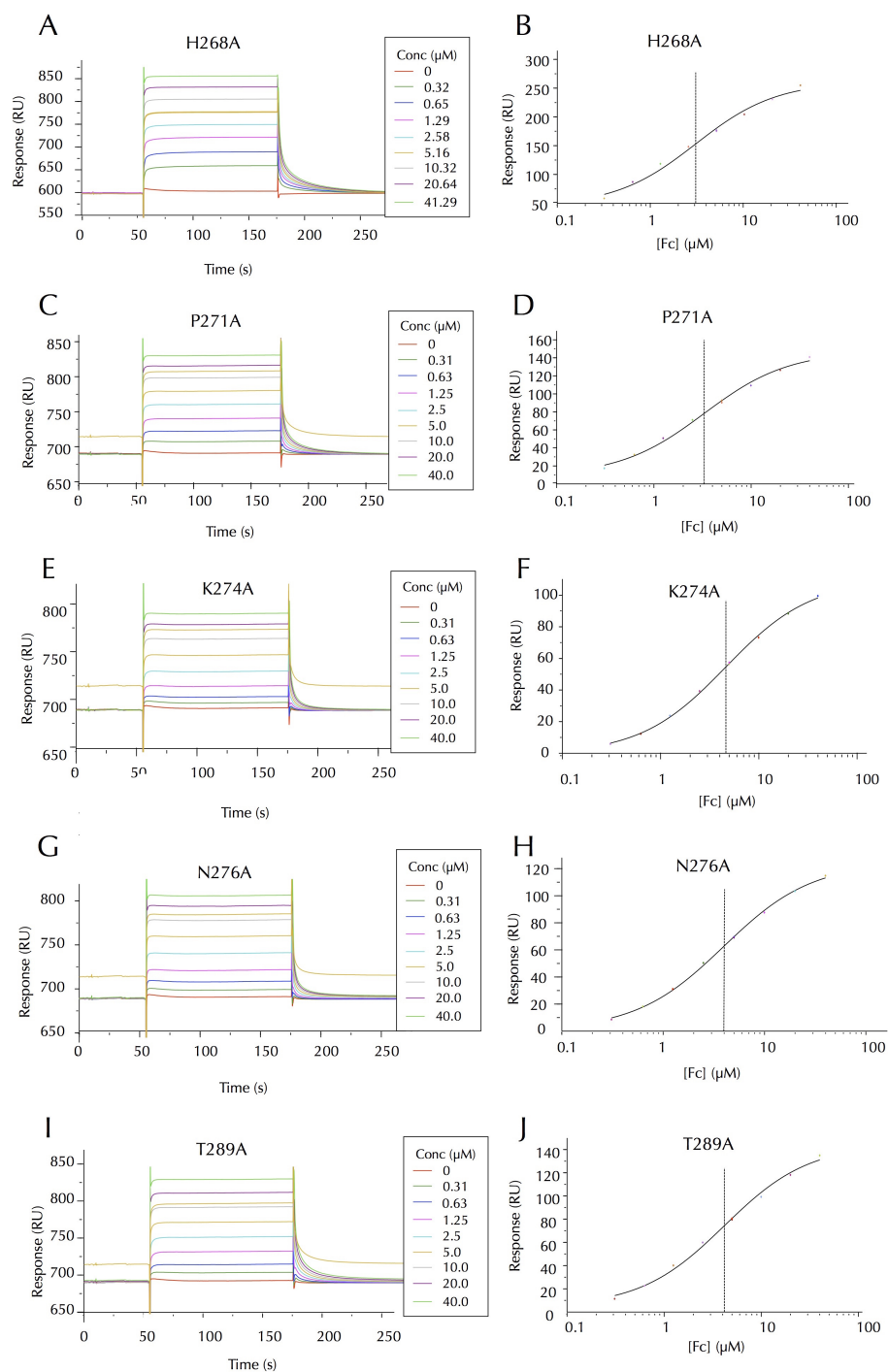


Figure 3.15: SPR analysis of IgG1 Fc variants binding to IdeS C94A: H268A (panels A & B), P271A (C & D), K274A (E & F), N276A (G & H) and T289A (I & J). Left-hand panels contain the sensorgrams for each Fc concentration tested. Right-hand panels give the equilibrium binding isotherms (solid black line) derived from a plot of binding response against Fc concentration; the vertical dotted line indicates the K_D derivation. In both cases the binding response is given in resonance units (RU).

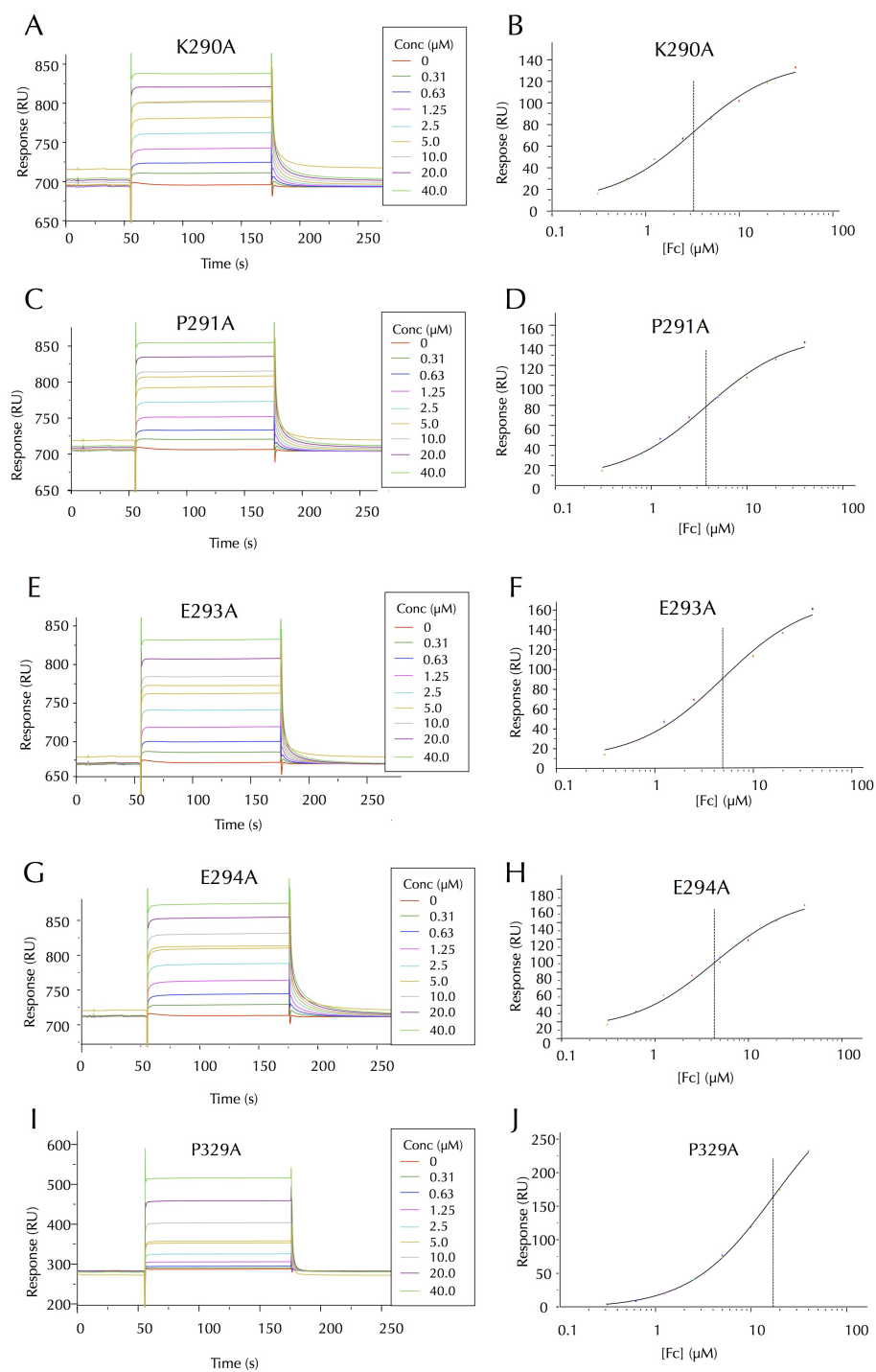


Figure 3.16: SPR analysis of IgG1 Fc variants binding to IdeS C94A: K290A (panels A & B), P291A (C & D), E293A (E & F), E294A (G & H) and P329A (I & J). Left-hand panels contain the sensorgrams for each Fc concentration tested. Right-hand panels give the equilibrium binding isotherms (solid black line) derived from a plot of binding response against Fc concentration; the vertical dotted line indicates the K_D derivation. In both cases the binding response is given in resonance units (RU).

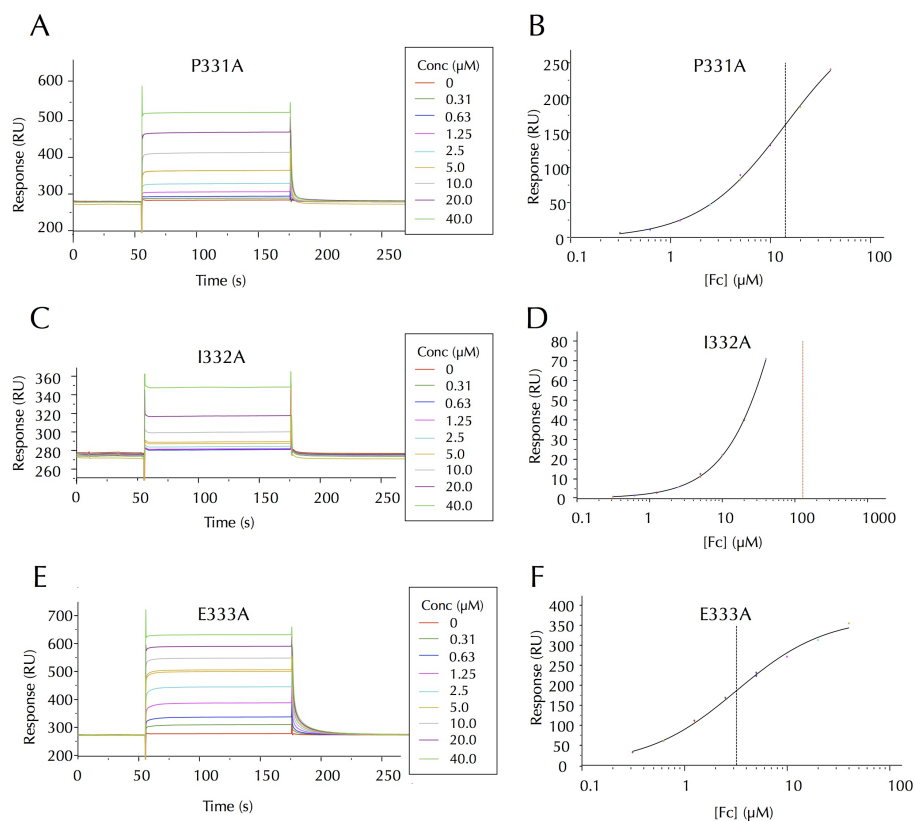


Figure 3.17: SPR analysis of IgG1 Fc variants binding to IdeS C94A: P331A (panels A & B), I332A (C & D) and E333A (E & F). Left-hand panels contain the sensorgrams for each Fc concentration tested. Right-hand panels give the equilibrium binding isotherms (solid black line) derived from a plot of binding response against Fc concentration; the vertical dotted line indicates the K_D derivation. In both cases the binding response is given in resonance units (RU).

IdeS C94A, a catalytically inactive mutant was used to test binding of a variety of Fc mutants using surface plasmon resonance. The K_D of the wild type Fc measured using this method was 3.5 μM , which is similar to the previously published figure of 5.6 μM . The majority of Fc mutants bound to IdeS C94A with a similar affinity to the wild type Fc. As expected, mutation of the lower hinge region (Pro232 to Gly237) produced the expected decrease in affinity for IdeS C94A, although the resulting affinities remained within the same order of magnitude as that of the wild type Fc.

Crucially, the hingeless Fc containing residues Ser239 to Lys447 bound IdeS C94A with only a slightly reduced affinity of 7.9 μM . This finding strongly supports the presence of a secondary binding site in the Fc domain, in addition to the hinge region. The secondary binding site could not be delineated to either the $C\gamma 2$ or the $C\gamma 3$ domain as very poor expression levels of the hingeless $C\gamma 2$ domain (residues Ser239–Lys338) prevented investigation of its binding affinity. However, it seems likely that the secondary binding site involves residues Pro329 to Ile332. This hypothesis is supported by the binding studies presented in this chapter which show a IdeS C94A affinity upon alanine mutation of these residues. The I332A mutation in particular increased the K_D from 3.5 μM to ~130 μM , although the latter figure should be treated with caution as the concentration range of Fc I332A used here did not allow a reliable calculation of the K_D . Previous work on the creation of enzyme-resistant antibodies found that mutation of Ile332 to glutamate conferred almost complete resistance to IdeS in a 24 h assay [101]. The mutation S239D also conferred substantial resistance to IdeS cleavage, although not to the same extent as I332E. Finally, Ser267 may also be involved in IdeS binding: the mutation S267A slightly reduces Fc affinity for IdeS C94A (Table 3.2 above). The locations of all residues currently known to be interact with IdeS are mapped on the structure in Figure .

There is substantial overlap on IgG1 between the binding site for $\text{Fc}\gamma\text{Rs}$ and the binding site for IdeS (as far as has been elucidated), not only in the hinge region but also in the $C\gamma 2$ domain (Table 3.3). To complete this study, the additional residues identified as potentially involved in protein–protein interaction in Table 3.1, but not further explored, could also be assessed using SPR in the same manner. Double or triple mutations, particularly in the hinge region, and to

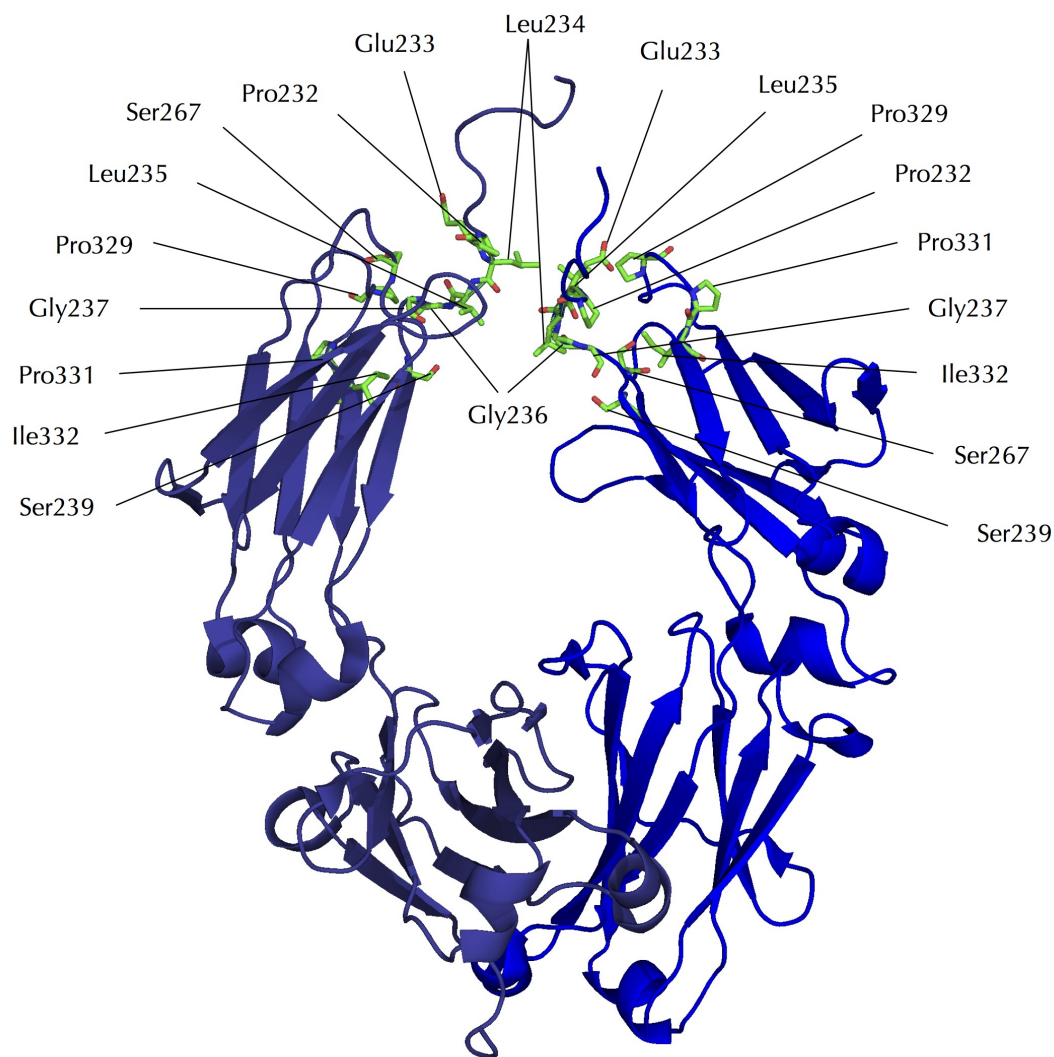


Figure 3.18: Cartoon representation of IgG1 Fc, cropped from the crystal structure of B12 (PDB 1HZH). Chain A is coloured in dark blue and chain B in blue. Residues known to interact with IdeS are shown in stick representation on both chains and coloured by atom (carbons in green, oxygens in red, nitrogens in blue).

residues other than alanine, may result in a more substantial effect on binding than the single mutations to alanine created in this chapter.

Table 3.3: List of IgG1 amino acid residues involved in interaction with Fc γ RI, Fc γ RIIA/B, Fc γ RIIIA and IdeS.

Residue	Fc γ RI	Fc γ RIIA/B	Fc γ RIIIA	IdeS
Pro232				X
Glu233	X	X	X	X
Leu234	X	X	X	X
Leu235	X	X	X	X
Gly236	X	X	X	X
Gly237				X
Pro238	X	X	X	
Ser239			X	X
Asp265	X	X	X	
Ser267				X
His268			X	
Glu269			X	
Asp270		X	X	
Arg292		X		
Glu293			X	
Gln295		X	X	
Tyr296			X	
Asn297	X	X	X	
Asp298		X		

Table 3.3 continued

Residue	Fc γ RI	Fc γ RII	Fc γ RIIIA	IdeS
Arg301			X	
Val303			X	
Lys322			X	
Ala327	X	X	X	
Pro329	X	X	X	X
Pro331				X
Ile332				X
Lys338			X	
Asp376			X	
Lys414		X		

Information regarding residues involved in Fc γ R binding was obtained from ref. [166].

Information regarding residues involved in IdeS binding was obtained from the data presented in this chapter and ref. [101].

The location of the putative exosite on IdeS is still to be defined. Mutation of the amino acid residues Phe129 and Met134 were found by Agniswamy *et al* to reduce binding affinity to IgG1 [4]. While M134L increased the K_D almost five-fold but had little effect on the kinetics of catalysis, F129I increased the K_D almost one hundred-fold and markedly decreased the k_{cat} . These residues are located on the C-terminal lobe removed from the active site (Figure 3.19) and were originally thought to be involved in dimerisation [4] or protein structural integrity

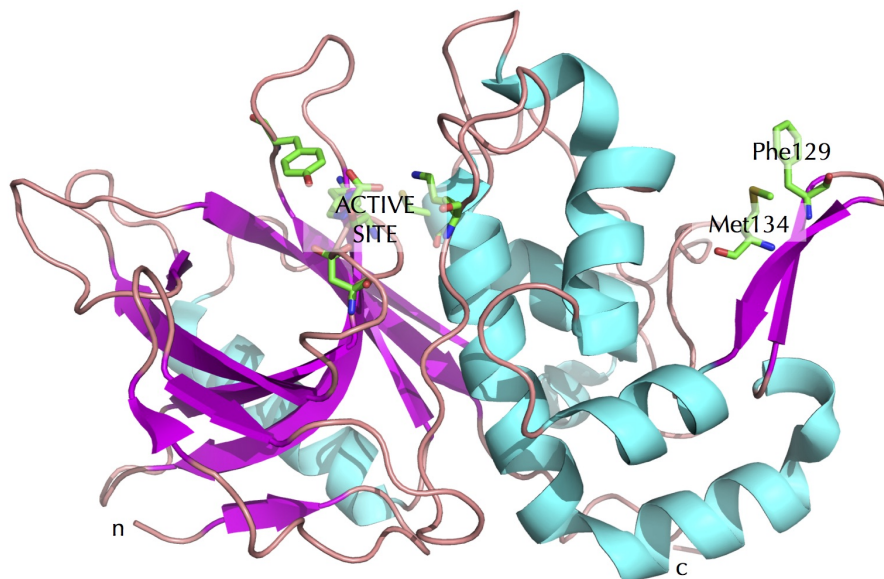


Figure 3.19: Crystal structure of wild type IdeS (PDB 2AU1), showing the location of residues Phe129 and Met134 relative to the enzyme active site. The enzyme is given in cartoon representation coloured according to secondary structure: α -helices in blue, β -sheets in dark pink and loops in light pink. The catalytic residues in the active site, Phe129 and Met134 are labelled and shown in stick representation; these residues are coloured according to element: carbon in green, nitrogen in blue and oxygen in red. The N- and C-termini are marked in lower-case letters.

[192]. It may be that these residues and/or others are involved in the additional binding to IgG Fc; however, there is currently no direct evidence to support this speculation.

The location of the IdeS exosite and the IgG secondary binding site would ideally be resolved by X-ray crystallographic analysis of the complex. This was the subject of previous studies by Emma Dixon (Oxford Glycobiology Institute) using IdeS C94A and IgG1 Fc but selective crystallisation of the Fc prevented analysis of the complex [55]. In the course of work presented in this chapter, we also attempted to create a complex of IdeS C94A with the broadly neutralising anti-HIV antibody 2G12. 2G12 was chosen due to the unique domain-exchanged structure of its V_H regions [32]. It was hoped that this antibody would possess greater rigidity than a non-domain-exchanged antibody and therefore be more likely to form a stable complex with IdeS. Unfortunately, gel filtration assessment indicated that was not the case under the

conditions used. Further methods to explore the IdeS–IgG binding site include hydrogen-deuterium exchange (HDX), which can be used as a tool to quantify the relative level of surface exposure of the complex relative to the individual components. A second alternative is covalent crosslinking using bidirectional linkers followed by fragmentation and mass spectrometry to identify the joined peptide fragments. A combination of these biophysical methods may be required to fully elucidate locations of the IdeS–IgG interfaces.

Chapter 4

Receptor refocusing of therapeutic antibodies using IdeS

4.1 Rationale

Binding to Fc receptors (FcRs) is important for the efficacy of certain classes of therapeutic antibody (Section 1.9). For instance, baviximab, a PS-targeting antibody currently in Phase III clinical trials for non-small cell lung cancer, is thought to produce its anti-tumour effects mainly by antibody-dependent cell-mediated cytotoxicity (ADCC) [171]. This is primarily mediated by the binding of the Fc γ RIIIA receptor, expressed by natural killer (NK) cells, to the Fc region of IgG molecules immobilised on the surface of target cell [134].

High serum trough levels are frequently required for therapeutic antibodies to be effective *in vivo*. This contrasts with pre-clinical studies which showed maximal cell lysis by ADCC at concentrations several orders of magnitude lower. Work by Preithner *et al* [143] identified serum IgG as the main inhibitor of ADCC *in vivo*, due to its competition with the therapeutic mAb for binding to Fc γ RIIIA. Serum concentration of IgG in humans is ~10 mg/mL [177]; removal of this competing factor thus represents a potential strategy for improving clinical response rates to antibody treatments.

IdeS is a bacterial enzyme that specifically cleaves all subclasses of IgG in the hinge region [196]. The cleavage products, which consist of singly-cleaved IgG (scIgG), F(ab)₂ and/or ½ Fc, have a much lower affinity to FcγRIIIA than the intact IgG (iIgG). If administered in conjunction with a therapeutic mAb, IdeS could be used to reduce serum iIgG concentrations and increase binding of the mAb to target receptors. The clearance of IdeS from the bloodstream is rapid [200] and this enzyme could therefore be administered 1–3 days prior to antibody treatment. However, mAb efficacy is likely to be maximal when endogenous IgG concentrations are lowest and an IdeS-resistant therapeutic mAb would allow for simultaneous administration of mAb and IdeS. We therefore designed an IdeS-resistant antibody based on the results of Chapter 3 and previously published studies [101]. Its binding to FcγRIIIA in the presence of serum and IdeS was investigated by ELISA, with a view to testing this concept in a future cellular assay.

4.2 Serum inhibition of FcγRIIIA binding to antibody *in vitro*

As proof of principle, serum inhibition of the FcγRIIIA interaction with bavituximab, a PS-binding monoclonal antibody, was demonstrated by ELISA. The extracellular region of FcγRIIIA was expressed in HEK 293T cells and purified by nickel affinity followed by gel filtration (Figure 4.1). Bavituximab was diluted with various concentrations of human serum and incubated with FcγRIIIA-coated microwell plates. The result is shown in Figure 4.2. Concentrations of serum as low as 10% were sufficient to reduce the maximal binding of bavituximab by approximately half compared to the PBS-only control. Similar levels of inhibition were seen with serum concentrations of 10%, 20%, 50% and 100%. Only with 1% serum did the binding begin to approach that of control levels, although it seems that higher bavituximab concentrations than those used here would be required to restore maximal binding.

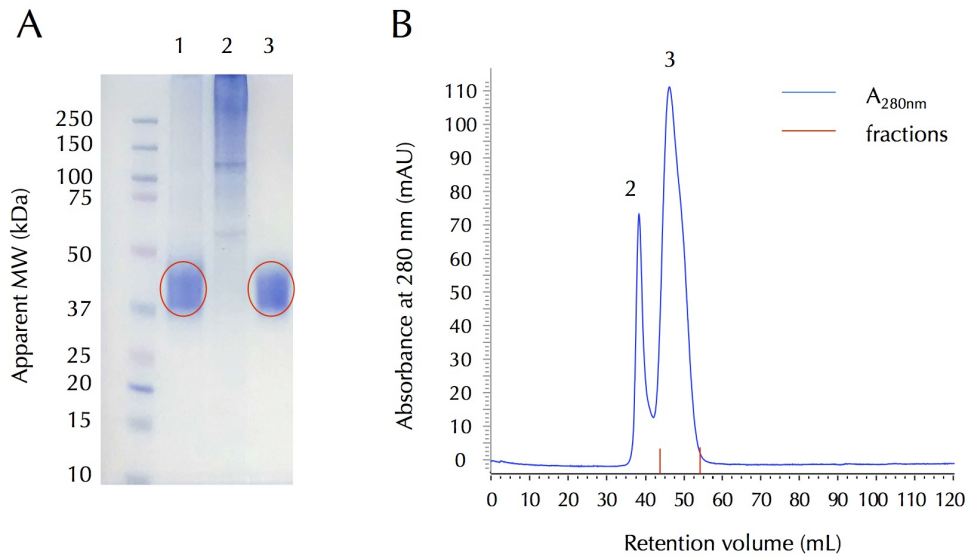


Figure 4.1: Expression and purification of FcγRIIIA. **A|** Non-reducing SDS-PAGE of FcγRIIIA following nickel affinity purification (lane 1) and gel filtration (lanes 2 & 3). Each lane contains 10 µg protein. FcγRIIIA is circled in red. Molecular weight (MW) markers are given on the left-hand side. Lanes 2 and 3 on the gel refer to peaks as labelled in panel B. **B|** Gel filtration chromatogram of nickel-purified FcγRIIIA eluted from a Superdex 75 pg 16/600 column. Blue trace indicates UV absorbance at 280 nm; red vertical lines indicate the start and end of fraction collection.

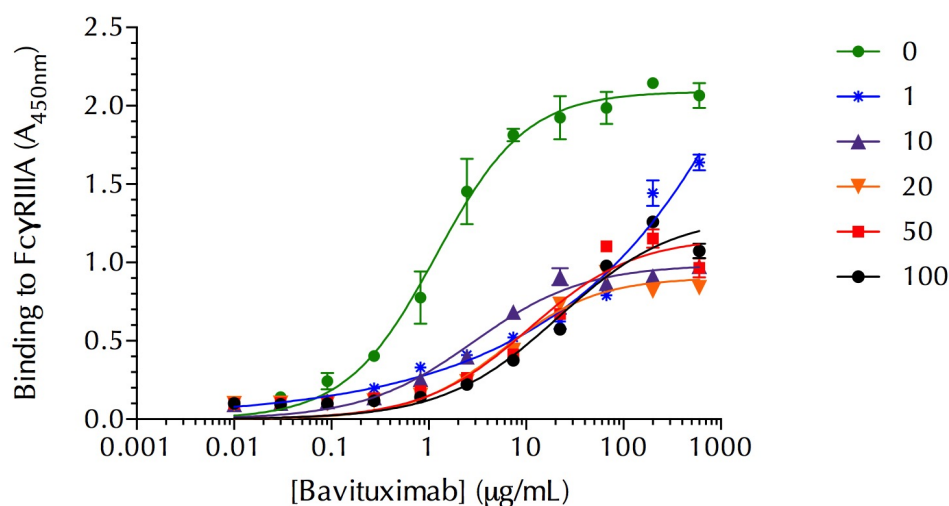


Figure 4.2: Inhibition of the binding of bavituximab to FcγRIIIA by human serum. Serum was mixed with PBS to the percentage concentrations shown and the mixtures used to create dilution series of bavituximab. Data are plotted as the mean $\pm$ standard deviation of the mean (SEM) of triplicate measurements. Curves were produced using non-linear least-squares fitting with a one-site total binding model.

4.3 Production of IdeS

4.3.1 Cloning and expression

IdeS (M1 serotype, without the signal sequence) was expressed in *E. coli* as detailed in Section 2.5. Following lysis of bacterial cells, IdeS was purified from the supernatant by nickel-affinity using a step gradient of imidazole at concentrations of 20 mM, 40 mM, 100 mM and 300 mM. The 100 mM and 300 mM fractions were concentrated, combined and further purified by gel filtration (Figure 4.3).

4.3.2 IdeS activity assays

The activity of the purified IdeS was tested against polyclonal human IgG at the following pH conditions:

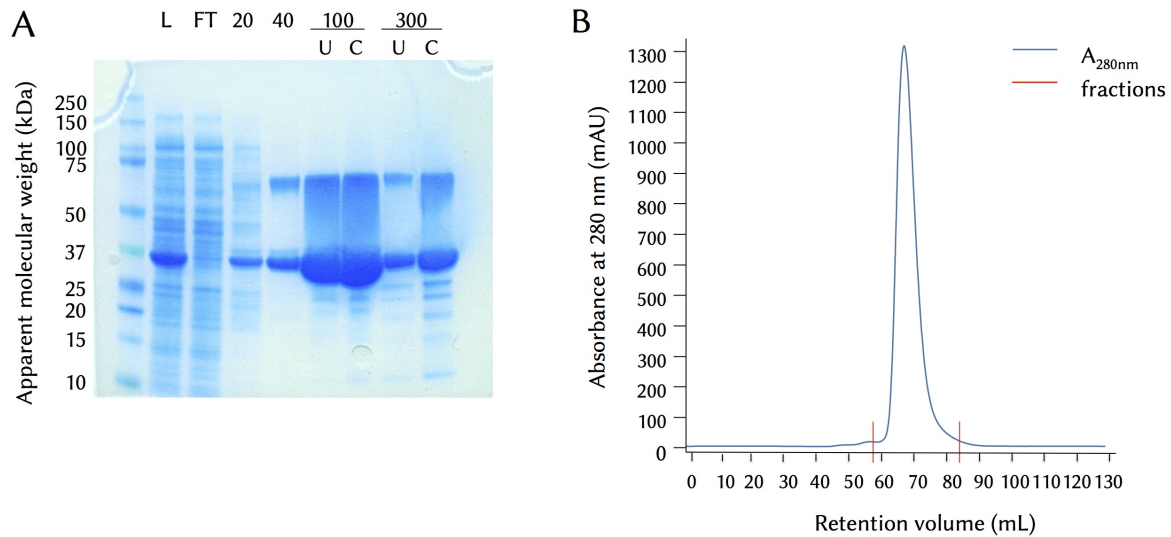


Figure 4.3: Purification of IdeS WT from bacterial culture. **A** | Non-reducing SDS-PAGE of fractions following nickel-affinity purification of post-lysis supernatant. Protein was eluted using imidazole at concentrations of 20 mM, 40 mM, 100 mM and 300 mM as indicated above the lanes. L, lysate; FT, flow-through; U, unconcentrated; C, concentrated. **B** | Gel filtration chromatogram of pooled 100 mM and 300 mM imidazole fractions eluted from a Superdex 75 pg 16/600 column. Blue line represents UV absorbance at A280; red lines indicate the start and end of fraction collection. Fractions were subsequently pooled, concentrated and stored at -20°C in 1 mL aliquots.

- pH 6.6, previously found to be the optimal pH for IdeS activity [191]
- pH 7.4, which closely corresponds to the pH of human blood (7.35–7.45) [137] and is therefore more applicable to *in vivo* use of IdeS.

Comparing Figures 4.4 and 4.5, IgG cleavage at pH 7.4 is notably slower than at pH 6.6. For example, at pH 6.6 and a mass ratio of 1:100 IdeS:IgG, the majority of IgG was fully cleaved to the F(ab')₂ and ½Fc products. At pH 7.4 and the same ratio of enzyme to substrate, a substantial amount of IgG remains in the singly-cleaved form even after 14 h. This is consistent with previous studies which found that the cleavage rate of the first heavy chain progresses one-hundred-fold faster than the cleavage rate of the second heavy chain [155, 192].

4.3.3 Demonstration of IdeS activity against serum

An ELISA was performed to determine the efficacy of IdeS at reducing serum IgG binding to FcγRIIIA. Assuming the serum concentration of IgG to be ~10 mg/mL, IdeS was added to undiluted serum to final IdeS:IgG mass ratios of 1:100, 1:600 and 1:1000 (final IdeS concentrations 96 µg/mL, 16 µg/mL and 10 µg/mL, respectively). Cleavage was allowed to proceed overnight at 37°C before the serum was serially diluted and added to microplates pre-coated with FcγRIIIA. IdeS at all concentrations dramatically reduced serum–FcγRIIIA binding to very low levels (Figure 4.6).

4.4 Engineering an IdeS-resistant mAb

4.4.1 Design of an IdeS-resistant antibody

The initial research for creation of an IdeS-resistant antibody was carried out by Susan Mattick (University of Oxford, UK). Mutations to confer resistance were chosen based on work by Kinder *et al* [101], which has also been patented [23]. It had previously been found that out

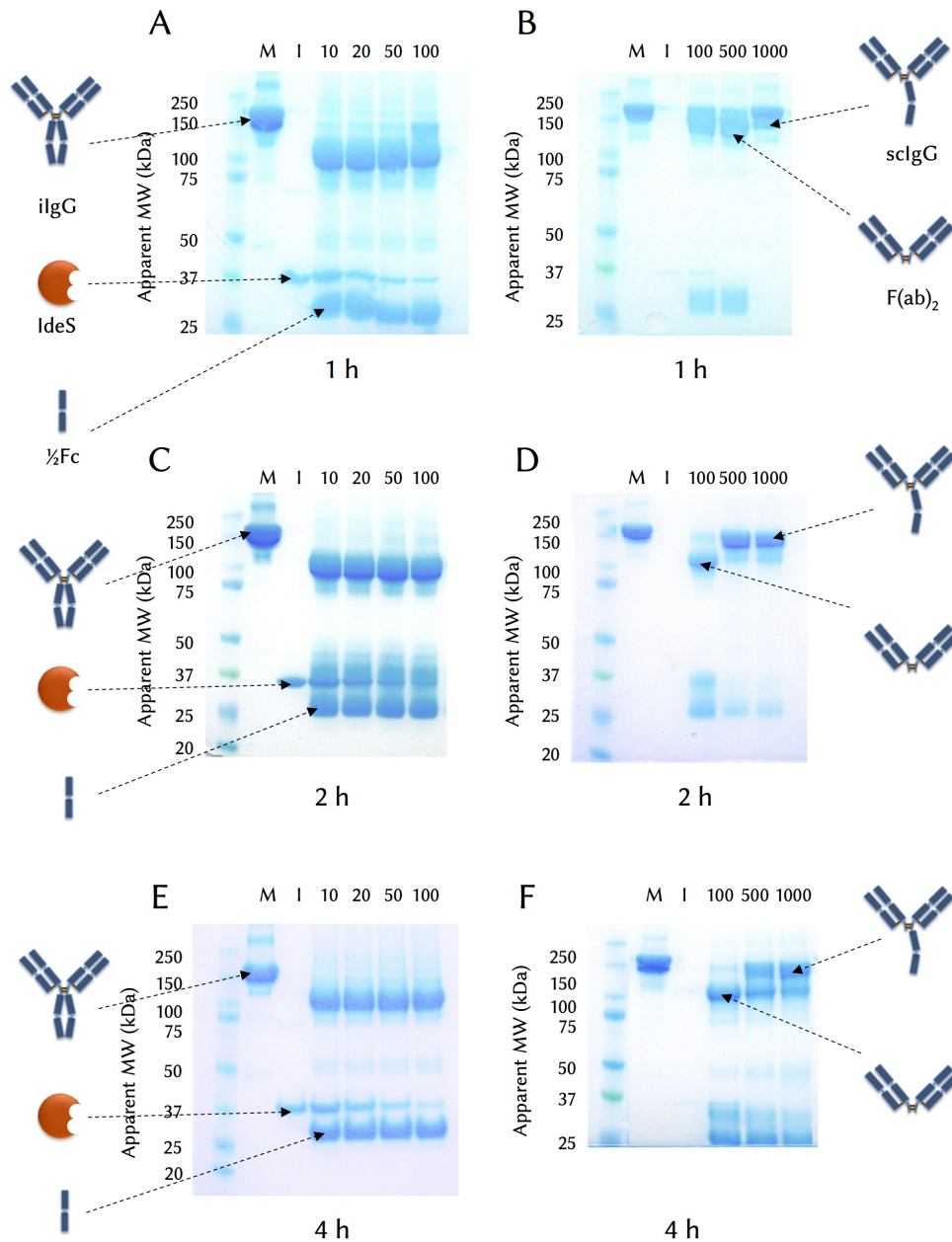


Figure 4.4: Cleavage of pooled serum IgG by IdeS at pH 6.6. IdeS was incubated with IgG at 37°C for 1 h (panels A and B), 2 h (panels C and D) or 4 h (panels E and F), then reactions stopped by the addition of buffer containing LDS. Reaction products were assessed by non-reducing SDS-PAGE on 4–12% Bis-Tris gels and stained with Coomassie Blue. M, mock reaction; I, IdeS alone; numbers 10/20/50/100/500/1000 refer to IgG:IdeS mass ratios of 1:10, 1:20, 1:50, 1:100, 1:400 and 1:1000. In all cases, IgG concentration was assumed to be 10 mg/mL for the purposes of calculating ratios. Cleavage products were assigned with reference to [95]. Molecular weight (MW) markers are provided in the first lane of each gel.

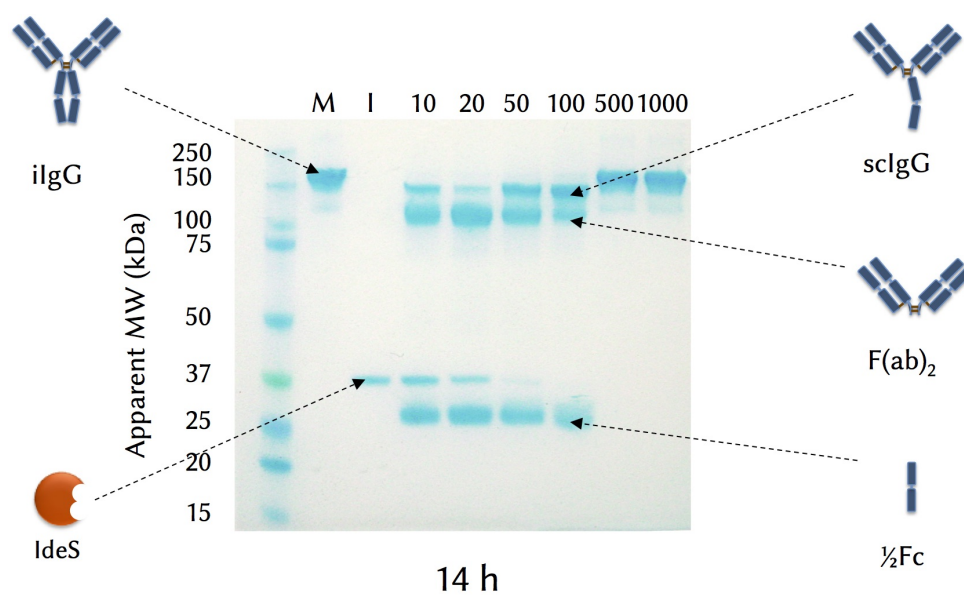


Figure 4.5: Cleavage of pooled serum IgG by IdeS at pH 7.4. IdeS was incubated with IgG at 37°C for 14 h then reactions stopped by the addition of buffer containing LDS. Reaction products were assessed by non-reducing SDS-PAGE on 4–12% Bis-Tris gels and stained with Coomassie Blue. M, mock reaction; I, IdeS alone; numbers 10/20/50/100/500/1000 refer to IgG:IdeS mass ratios of 1:10, 1:20, 1:50, 1:100, 1:400 and 1:1000. IgG concentration was assumed to be 10 mg/mL for the purposes of calculating ratios. Cleavage products were assigned with reference to [95]. Molecular weight (MW) markers are provided in the first lane of the gel.

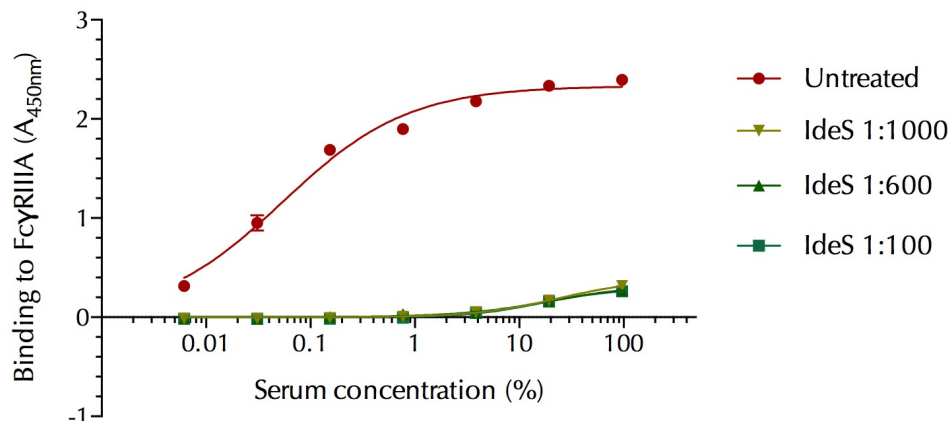


Figure 4.6: IdeS activity against serum IgG as measured by ELISA. Serum was incubated with IdeS overnight at 37°C before serial dilution and incubation with FcγRIIIA. Binding was detected using Protein A-HRP with TMB substrate. A₄₅₀ readings are plotted as the mean ± SEM of triplicate measurements.

of the four human IgG subclasses, IgG2 was the least susceptible to IdeS proteolysis [196]. This is attributable to substantial differences in the amino acids surrounding the IdeS cleavage site in the IgG2 sequence compared to IgG1, IgG3 and IgG4 (Figure 1.12).

The group led by Brezski therefore created an initial IgG variant by domain exchange of the lower hinge region to that of IgG2, which involved the substitutions E233P, L234V, L235A and the deletion of G236 (G236X). This was sufficient to confer resistance to proteases such as MMP-3 and MMP-12 but not to IdeS. Additionally, this exchange results in over a hundred-fold lower binding to FcγRIIIA [166] which translates into reduced NK-cell-mediated ADCC [101]. Complement activation via C1 is also lower with IgG2 [89].

To restore FcγR binding, additional mutations were made in the Cγ2 region. The mutations of S239A and I332E, in particular, had the dual effect of uniquely conferring IdeS resistance and also enhancing the ADCC activity ~11-fold relative to the wt IgG1. Therefore, the mutations chosen for creation of an IdeS-resistant antibody were E233P, L234V, L235A, G236X, S239D and I332E.

4.4.2 Bavituximab expression & purification

The chosen antibody for engineering of IdeS-resistance was bavituximab, which is a chimeric antibody containing mouse variable and human constant regions. The presence of the murine variable region enables specific detection in an ELISA despite the presence of competing human IgG, which was crucial for later assay design. Mutagenesis of the human IgG1 constant region was carried out in the pFUSEss-CHlg-hG1 vector, into which the bavituximab variable region was subsequently cloned. Initial expression tests of the wild type form of bavituximab were performed in HEK 293T cells, using heavy:light chain DNA ratios of 1:1, 1:2, 1:3 and 1:4 (Figure 4.7), followed by nickel affinity purification. Very little difference in expression levels was observed for the ratios tested, therefore a 1:1 ratio was used for all subsequent work. For the majority of experiments, including serum/IdeS ELISAs (Section 4.5) and virus ELISA (Chapter 5, Section 5.2), both wild type ('Bavi_WT') and IdeS-resistant ('Bavi_IdeS-R') forms of bavituximab were expressed from HEK 293T and 293F cells and purified using Protein A Sepharose, as detailed in the Materials and Methods.

Yields for both proteins were usually, but not always, higher from 293F cells compared to 293T, and were in the range 1.1–6.7 mg/L for the wild type form and 0.8–2.5 mg/L for the resistant form. The variability may be partly attributed to an unpredictable rate of degradation in cell culture: Figure 4.8 shows Western blots from three separate transfections (A–C) of 293F cells that provide clear evidence of the breakdown of protein over time. The rate of degradation differed for each transfection studied. For the blot in panel A, bands of a lower molecular weight (MW) are already present on the second day following transfection, whereas for panels B and C most of the antibody was still intact after this time. For expression C, only a slight amount of degradation is evident by day 4 and when the protein was subsequently harvested and purified, the yields obtained were 5.9 mg/L for the wild type form and 2.2 mg/L for the IdeS-resistant form.

A minor amount of fragmented protein was usually co-purified with the intact antibody when using Protein A affinity (Figure 4.9); however, further purification by gel filtration

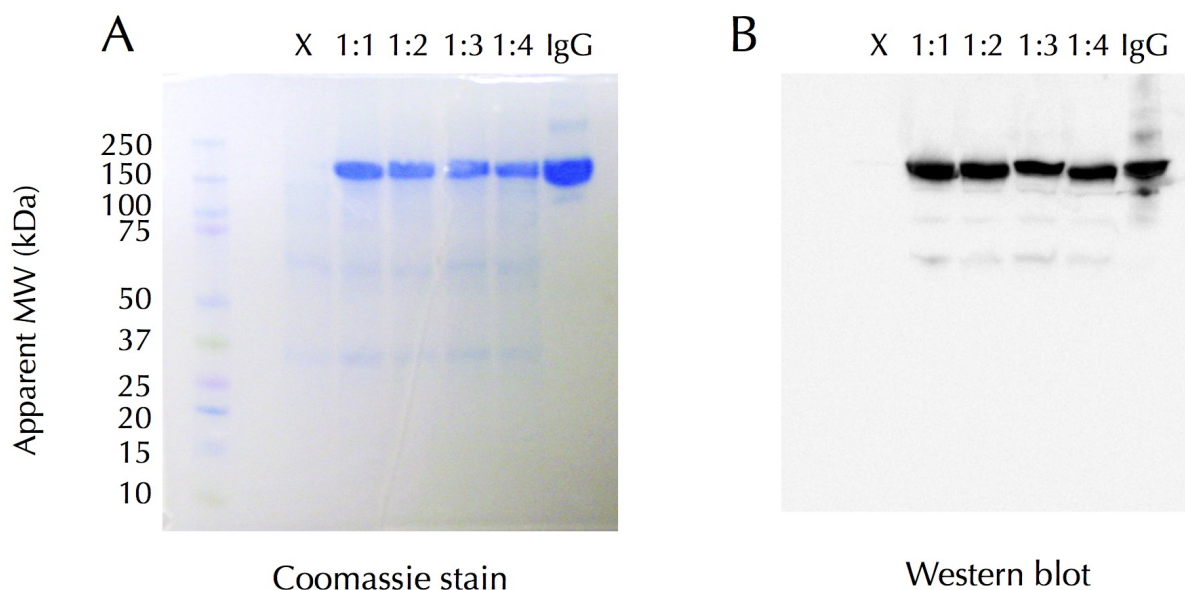


Figure 4.7: Assessment of the optimal ratio of heavy:light chain DNA for bavituximab expression. HEK 293T cells were transfected with heavy:light chain DNA ratios of 1:1, 1:2, 1:3 and 1:4, and protein was purified from the culture supernatant using nickel affinity chromatography. A mock transfection of PEI only ('X') was conducted in parallel. **A**| Samples of purified and concentrated protein were run on a non-reducing 4–12% Bis-Tris gel before **A**| staining with Coomassie blue or **B**| transferring to a PVDF membrane and probing with Protein A-HRP (1:30,000). Polyclonal human IgG was used as a positive control.

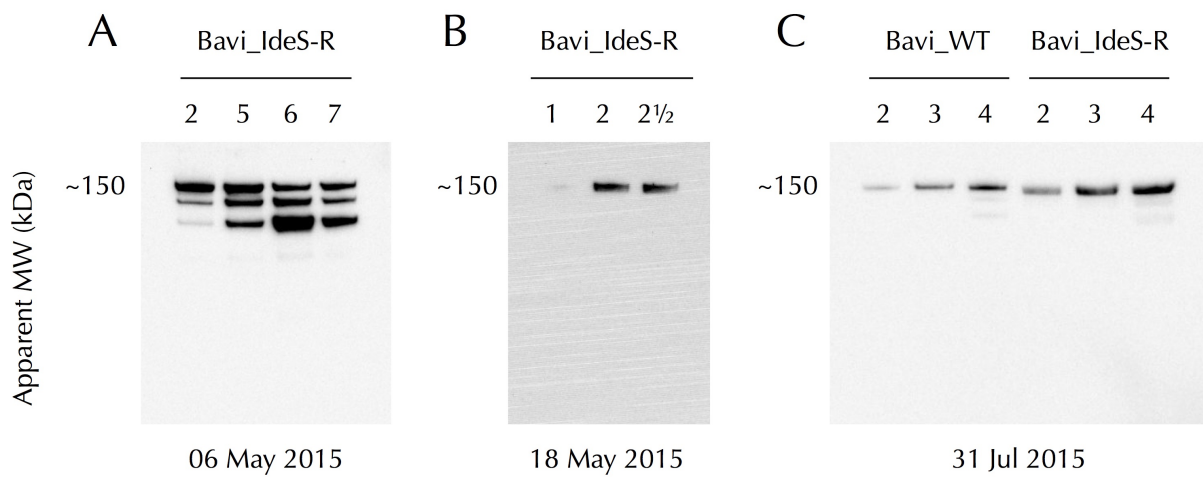


Figure 4.8: Variable rate of degradation of Bavituximab in cell culture following transfection. Samples of culture supernatant were removed, centrifuged to remove cell debris and, in some cases, stored at -20°C until gel was run. Following non-reducing SDS-PAGE using a 4–12% Bis-Tris gel, protein was transferred to a PVDF membrane and probed with Protein A-HRP (1:30,000). Dates below blots indicate date of transfection: **A**| 06 May 2015; **B**| 18 May 2015; **C**| 31 Jul 2015. Numbers above lanes indicate days after transfection. Bavi_WT and Bavi_IdeS-R refer to wild type and IdeS-resistant bavituximab respectively.

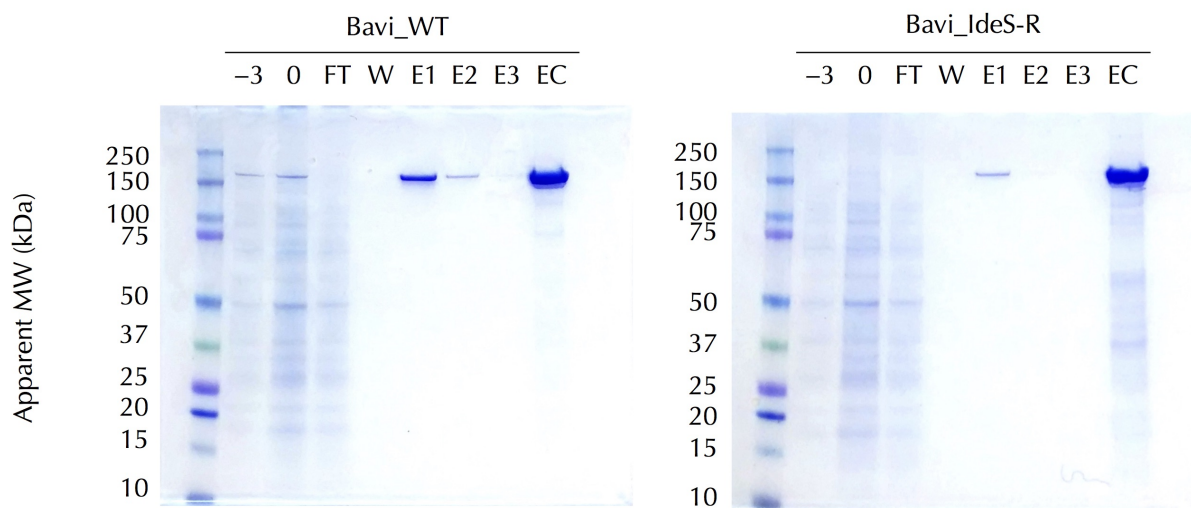


Figure 4.9: Protein A purification of wild type and IdeS-resistant bavituximab. Fractions from each purification stage were run on a 4–12% Bis-Tris gel and stained with Coomassie Blue. –3, media 3 days before harvest; 0, media on day of harvest; FT, flow-through (unbound); W, wash; E1–E3, elution series; EC, pooled and concentrated eluates. Gels show a representative batch of protein from a single harvest.

caused substantial protein loss of 50–70%. Given the low yields from cell culture and marginal improvement in purity from gel filtration, most of the protein used in the following experiments was purified by Protein A alone.

The protein batch used to test IdeS resistance of Bavi_IdeS-R was produced in High Five insect cells by Michael Birnbaum (Stanford School of Medicine, USA), according to the method in Section 2.4.

4.4.3 Bavituximab stability and storage conditions

The IdeS-resistant form of bavituximab is more stable when stored at 4°C than wild type bavituximab. However, both forms deteriorate within 28 days of harvest. Figure 4.10 compares the integrity of wild type and resistant proteins following storage at 4°C. The wild type protein produced in 293F cells was purified on 15 June 2015 and the wild type and resistant proteins from High Five cells were purified on 22 June 2015. The 293F wild type sample appears slightly

more stable than its equivalent expressed in the High Five system, as only a slight amount of degradation is seen 35 days following purification (Figure 4.10, panel B). By contrast, the same gel shows the High Five proteins 28 days following purification, at which point a substantial amount of the wild type form now runs at ~120 kDa and ~30 kDa. The resistant antibody from High Five cells appears to be largely intact after the same time period, with only small amount of protein running at ~110 kDa in addition to the full-length antibody.

The degraded wild type and resistant proteins from High Five cells both eluted as a single peak from a Superdex 200 pg column (Figure figure 4.10, panels D & E). Their subsequent appearance on the gel remained unaltered (Figure 4.10, panel C), indicating that the antibody fragments remained non-covalently associated in solution. An ELISA comparing these antibody samples showed that greater functionality, as assessed by binding to Fc γ RIIIA, was retained by Bavi_IdeS-R compared to Bavi_WT, although binding of the former was diminished compared to 'fresh' antibody as used in Section 4.5.

When bavituximab was expressed in 293T cells, similar observations were made regarding the greater stability of the R form over the WT. However, both WT and IdeS-R forms were found to be unaffected by a single freeze-thaw, as assessed by SDS-PAGE (Figure 4.12).

4.4.4 Assays of IdeS resistance

Wild type and resistant forms of bavituximab used in these resistance assays were generously provided by Michael Birnbaum (Stanford School of Medicine, USA), for which cloning and expression methods are outlined in Section 2.4. The resistance of Bavi_IdeS-R was compared to Bavi_WT using a timecourse assay with the IdeS produced in Section 4.3. The results of published studies of IdeS activity *in vitro* and *in vivo* [131, 95] were used to determine conditions for resistance assays. Previous studies *in vitro* used a high concentration of IdeS relative to IgG, usually at a mass ratio of 2:5 [4, 15] or 1:20 [16]. Reactions were incubated at 37°C and terminated by the addition of SDS-PAGE sample buffer or iodoacetamide.

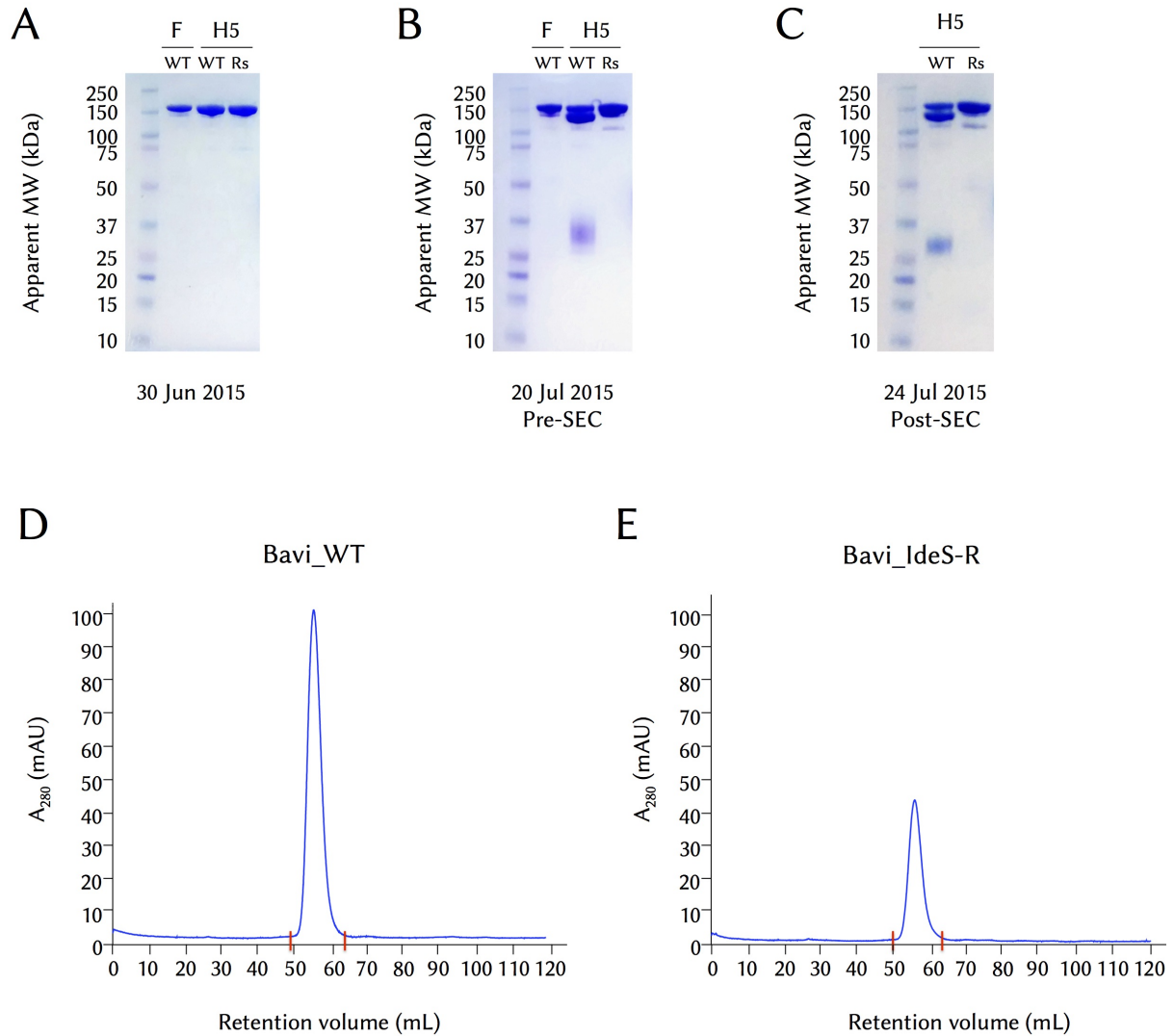


Figure 4.10: Comparison of wild type and resistant bavituximab protein stability following storage at 4°C. The 293F ('F') sample was purified by Protein A on 15 June 2015 and the High Five ('H5') samples by Ni-NTA resin on 22 June 2015. Panel **A** shows SDS-PAGE analysis of all samples, 8–14 days following purification. Panel **B** shows the same proteins after storage at 4°C for a further 20 days. The High Five samples from panel **B** were loaded onto a Superdex 200 pg 16/600 column to produce the chromatograms in panels **D** and **E**. Gel filtration fractions (outer boundaries indicated with red lines) from **D** and **E** were pooled, concentrated and reassessed using SDS-PAGE as shown in panel **C**. All SDS-PAGE was conducted with 10 µg of each protein on non-reducing 4–12% Bis-Tris gels, followed by Coomassie Blue stain. WT, wild type bavituximab; Rs, IdeS-resistant bavituximab. Dates for SDS-PAGE are given below each gel.

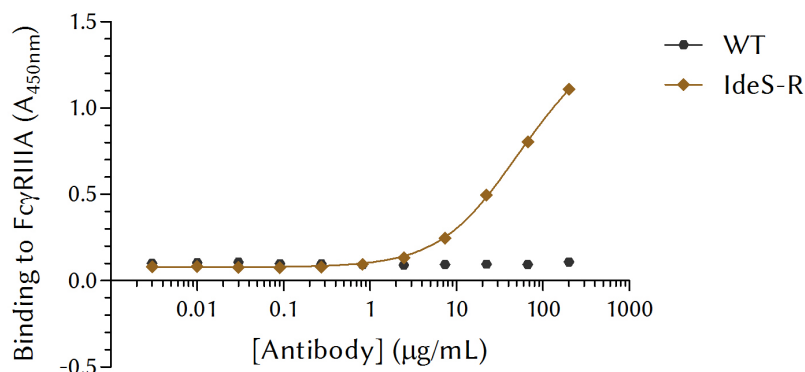


Figure 4.11: ELISA to test FcγRIIIA binding of bavituximab WT (grey/closed hexagons) and IdeS-R (brown/closed diamonds) after 28 days storage at 4°C. Data are plotted as the mean ± SEM of duplicate measurements. Curves were produced using non-linear least-squares fitting with a one site total binding model.

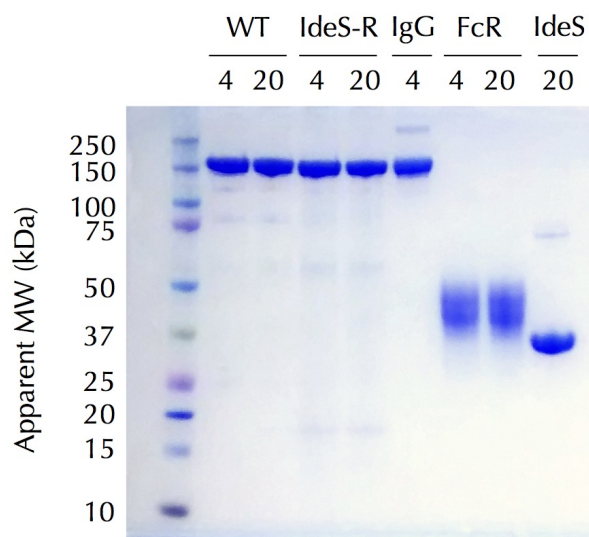


Figure 4.12: Confirmation of protein integrity following freeze-thaw, prior to use in ELISA. 10 μg of each protein was run on a 4–12% Bis-Tris gel and stained with Coomassie Blue. WT and IdeS-R refer to the wild type and IdeS-resistant bavituximab; in this case both were produced in HEK 293F cells. IgG, human IgG from Sigma resuspended in PBS and used in the competition ELISA; FcR, FcγRIIIA; IdeS, IdeS from Section 4.3. Samples labelled 4 were stored continuously at 4°C; B, samples labelled 20 were stored at -20°C then thawed before analysis.

Previous *in vivo* work, on the other hand, used much lower concentrations of IdeS relative to IgG. Nandakumar *et al* [131] used 9 mg of an anti-CII IgG2a antibody cocktail to induce arthritis in B10-RIII mice. This was either preceded or followed by injection of 10 µg or 100 µg IdeS per mouse; if administered within 48 h of the onset of arthritis, IdeS prevented progression of the disease, indicating successful cleavage of the IgG2a antibody cocktail. This gives an IdeS:IgG2a mass ratio of 1:900 and 1:90 for treatment with 10 µg and 100 µg IdeS respectively. Another study used a rabbit model [95], for which all subclasses of IgG are cleaved by IdeS. An injection of 5 mg GST-tagged IdeS, amounting to 10 µg/mL blood, achieved complete clearance of IgG from the blood within 6 h. In the rabbit for which data were shown, the IgG concentration was 8 mg/mL and 6 mg/mL at the time of the first and second injections respectively, which gives ratios of 1:800 and 1:600 GST-IdeS:IgG by mass. This calculation does not take into account the mass of the 26 kDa GST tag—the IdeS, at 35 kDa, contributes only 57% of the MW of the tagged protein and therefore 5.7 µg IdeS per mL blood volume was injected. Using this value, the IdeS:IgG ratio was 1:1394 at the time of the first injection and 1:1045 at the time of the second injection. However, preliminary work by the same group showed that rabbit IgG was cleaved more effectively than human IgG *in vitro*. Complete cleavage of 1 mg of rabbit IgG required 1.6 µg IdeS, in a 15 minute assay at 37°C, whereas 3.9 µg IdeS was required for complete cleavage of 1 mg human IgG under the same conditions.

Taking all of the above into consideration, the resistance assay used here was designed as follows. IdeS was mixed with 10 µg of Bavi_WT or Bavi_IdeS-R using IdeS:antibody mass ratios of 1:1000, 1:600 and 1:100. All reactions were carried out in phosphate-buffered saline (PBS) at pH 7.4 to match physiological conditions, and a PBS-only control was included for each timepoint. Reaction mixtures were incubated at 37°C for 0, 2, 4, 8, 16 or 24 h before termination with the addition of LDS sample buffer. Products were analysed with non-reducing SDS-PAGE and are shown in Figure 4.13.

Bavi_IdeS-R clearly possesses greater resistance to IdeS cleavage than Bavi_WT. Even after 24 h at the highest IdeS concentration used (1:100 mass ratio, corresponding to 5 µg/mL), almost all Bavi_IdeS-R was intact; only one faint additional band was observed that corresponds to

the F(ab')₂ fragment (Figure 4.13, panel F). By contrast, under the same conditions the majority of Bavi_WT was cleaved to F(ab')₂ and Fc or ½Fc products (Figure 4.13, panel F). Under all conditions and timepoints, a substantial amount of non-specific cleavage was observed, seen as diffuse bands between 25 kDa and 50 kDa (Figure 4.13, panels B to F).

4.5 Application of IdeS *in vitro*

4.5.1 IdeS efficacy in the presence of IgG

Based on the improved resistance of Bavi_IdeS-R compared to Bavi_WT, the IdeS-based receptor-refocusing approach was tested using ELISA as follows. Purified human IgG at concentrations of 2 mg/mL and 5 mg/mL were treated for 16 h at 37°C with IdeS at a mass ratio of 1:600 IdeS:IgG. As controls, IgG was incubated with PBS only ('0 mg/mL IgG') and with 3.3 µg/mL and 8.3 µg/mL IdeS, to match the concentrations used for IgG cleavage. The IgG/IdeS mixtures were then used to create dilution series of Bavi_WT and Bavi_IdeS-R, which were added to multiwell plates pre-coated with FcγRIIIA. Results are given in Figure 4.14.

The IdeS-resistant bavituximab has a greater affinity for FcγRIIIA than the wild type: the former saturates FcγRIIIA at ~100 µg/mL while the latter required at least 600 µg/mL to reach saturation. This is consistent with published data showing that incorporation of the S239D and I332E mutations into IgG1 increased ADCC potency almost eleven-fold over the IgG1 WT [101]. For both the wild type and resistant forms, the presence of IgG dramatically reduced FcγRIIIA binding. As expected from the similar effects of 20% and 50% serum in Figure 4.2, 2 mg/mL and 5 mg/mL IgG were almost equally effective at competing for FcγRIIIA binding. IdeS-resistant bavituximab achieved greater levels of binding under these conditions, due to its higher affinity for FcγRIIIA; this is a particular advantage for a therapeutic mAb that depends largely on ADCC for its tumour killing function. In the presence of both 2 mg/mL and 5 mg/mL IgG, IdeS was highly effective at restoring the binding of the resistant protein to control levels.

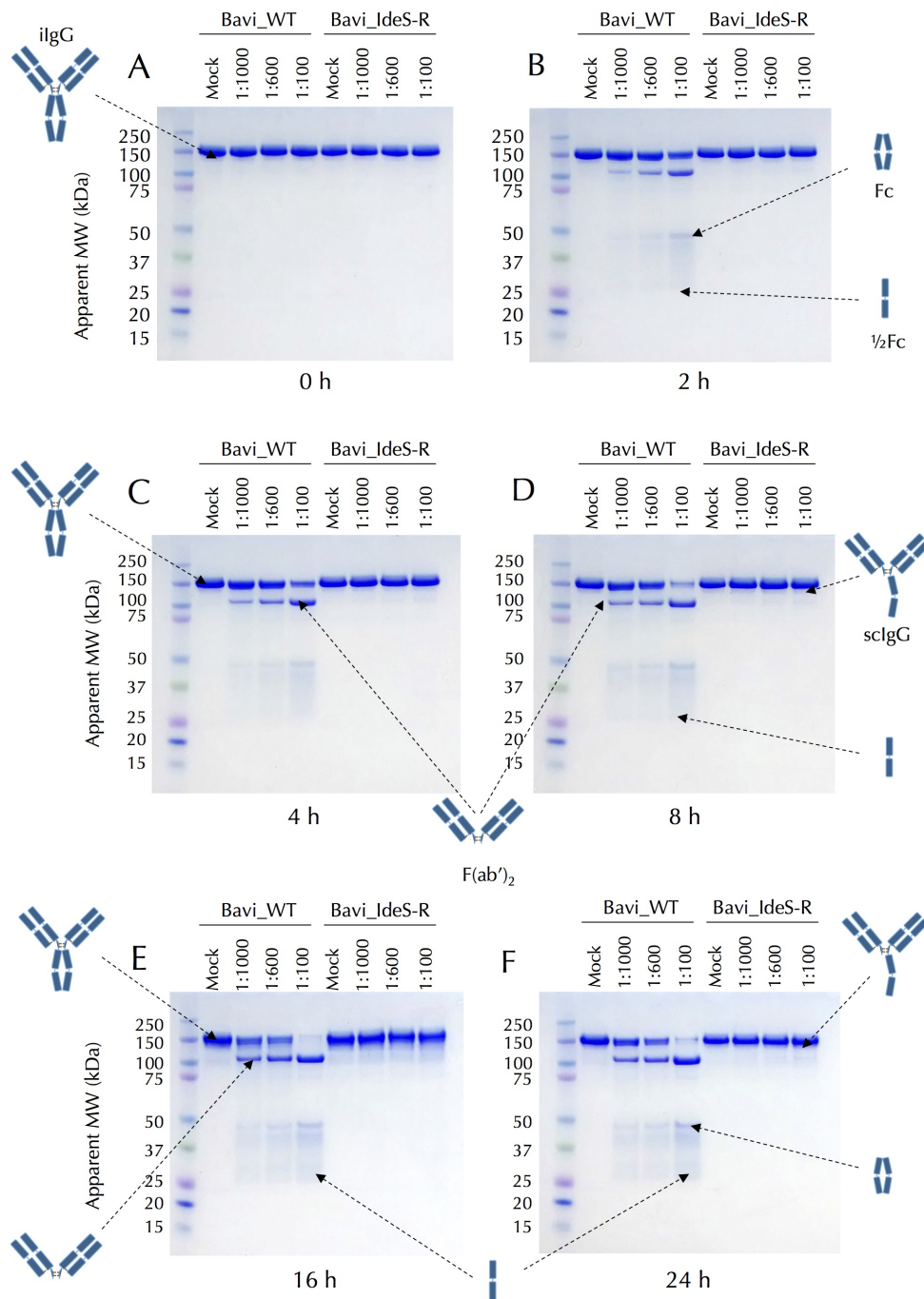


Figure 4.13: IdeS activity with wild-type bavituximab and resistant bavituximab. Bavituximab was incubated with IdeS for varying timepoints before reaction was terminated with LDS sample buffer. Each panel shows a separate timepoint: A| 0 h; B| 2 h; C| 4 h; D| 8 h; E| 16 h and F| 24 h. Products were analysed with non-reducing SDS-PAGE and stained with Coomassie Blue. Gel lanes represent reactions: mock was PBS-treated, 1:1000, 1:600 and 1:100 refer to IdeS:bavituximab mass ratios. Intact IgG (ilgG), singly-cleaved IgG (sclgG), F(ab')₂, Fc and ½Fc products are arrowed.

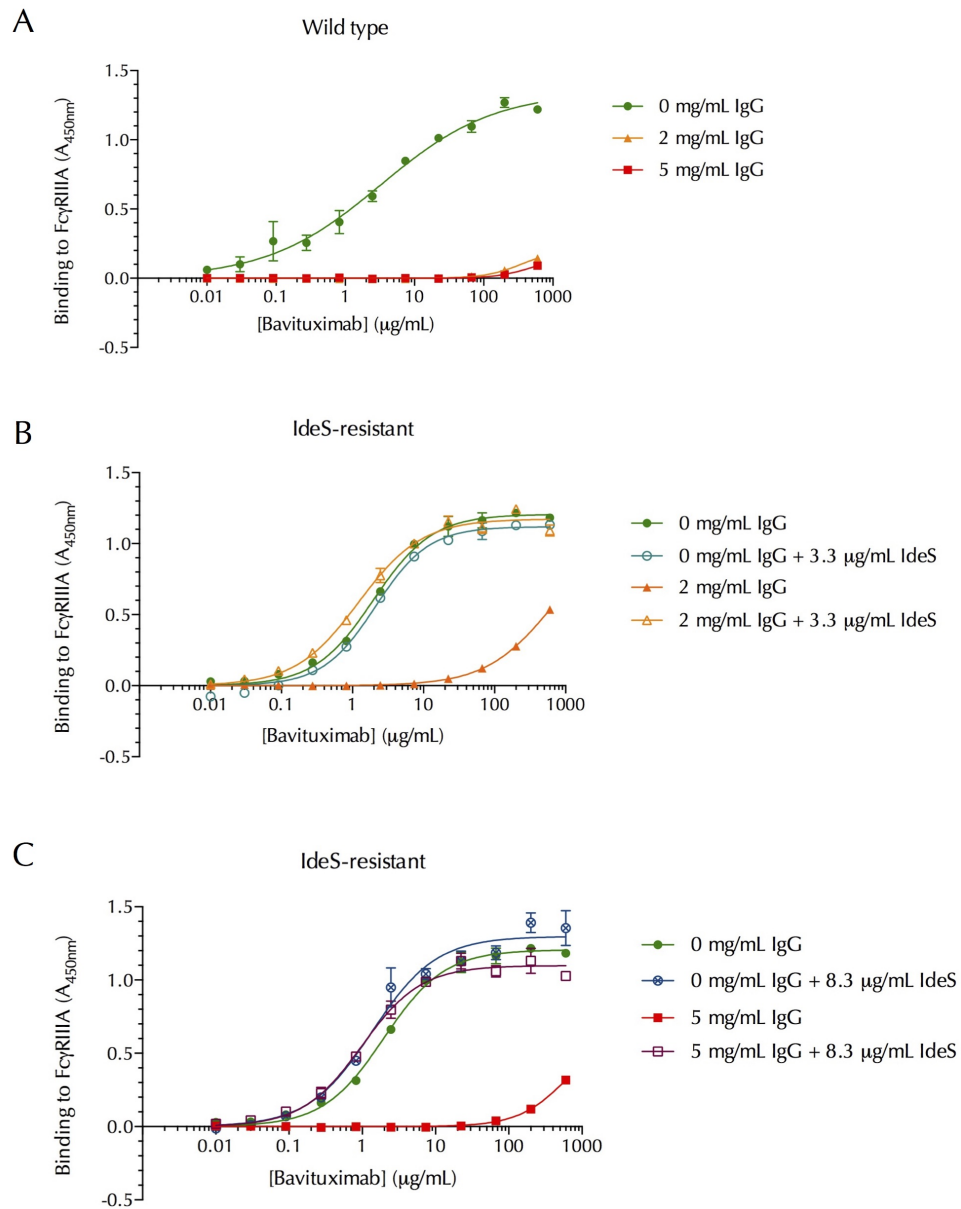


Figure 4.14: Binding of wild type and IdeS-resistant bavituximab to FcγRIIIA in presence of competing IgG and IdeS. Bavituximab was bound to immobilised FcγRIIIA in the presence of PBS only ('0 mg/mL IgG', green/filled circles), PBS + 3.3 $\mu\text{g/mL}$ IdeS (light blue/open circles), PBS + 8.3 mg/mL IgG (dark blue/crossed circle), 2 mg/mL IgG (orange/closed triangles), 2 mg/mL IgG + 3.3 $\mu\text{g/mL}$ IdeS (yellow/open triangles), 5 mg/mL IgG (red/closed squares), 5 mg/mL IgG + 8.3 $\mu\text{g/mL}$ IdeS (maroon/open squares). **A)** Wild type bavituximab in the presence of 2 mg/mL and 5 mg/mL competing IgG. **B)** IdeS-resistant bavituximab in the presence of 2 mg/mL IgG. **C)** IdeS-resistant bavituximab in the presence of 5 mg/mL IgG. In all cases, binding was detected using an antibody specific to the murine F(ab')₂ domain of bavituximab. Data are plotted as the mean $\pm$ SEM of duplicate measurements, following baseline correction. Curves are produced using non-linear least squares fitting with a one-site specific binding, Hill slope model.

4.5.2 IdeS efficacy in the presence of serum

The effects of IdeS appear more complicated in the presence of serum than in the presence of purified IgG. When 20% and 50% human serum was used in place of competing IgG and treated with IdeS as before, IdeS was less effective at restoring binding of bavituximab to levels of the PBS-only control (Figure 4.15). Despite IdeS treatment, bavituximab binding in the presence of serum reached less than half of control levels. Moreover, at bavituximab concentrations below 5 $\mu\text{g/mL}$, the activity of IdeS combined with serum increased binding over control levels. This effect is observed only when both IdeS and serum are both present, and only at the previously stated lower concentrations of bavituximab. Thus additional, as yet unknown, factor(s) in the serum are interacting with IdeS to influence bavituximab binding.

4.6 Discussion

In this chapter, an IdeS-resistant form of bavituximab was successfully created by incorporation of specific mutations in the hinge and C γ 2 domains. This IdeS-resistant antibody is not only substantially less susceptible to IdeS-mediated proteolysis than its wild type equivalent, but (in agreement with previous studies) also has greater affinity to Fc γ RIIIA even in the absence of any competing factors, and a prolonged shelf life at 4°C. At a concentration 600-fold less than that of its IgG substrate, IdeS effectively increases the binding of IdeS-resistant bavituximab to Fc γ RIIIA *in vitro*. Pre-treatment of IgG by IdeS for 16 h was sufficient to entirely remove competition and restore receptor binding to control levels. However, there appear to be additional factors in serum that interact with IdeS and prevent restoration of maximal antibody binding — further work is required to investigate this.

Quantities of IdeS used in the Phase I clinical trial, which was published as these experiments were being completed, resulted in an IdeS:IgG mass ratio lower than that used in the ELISAs presented in this chapter. Trial data shows the IdeS concentration reaching a peak of ~9.5 mg/L in serum one minute before the end of infusion, for one individual receiving the highest dose

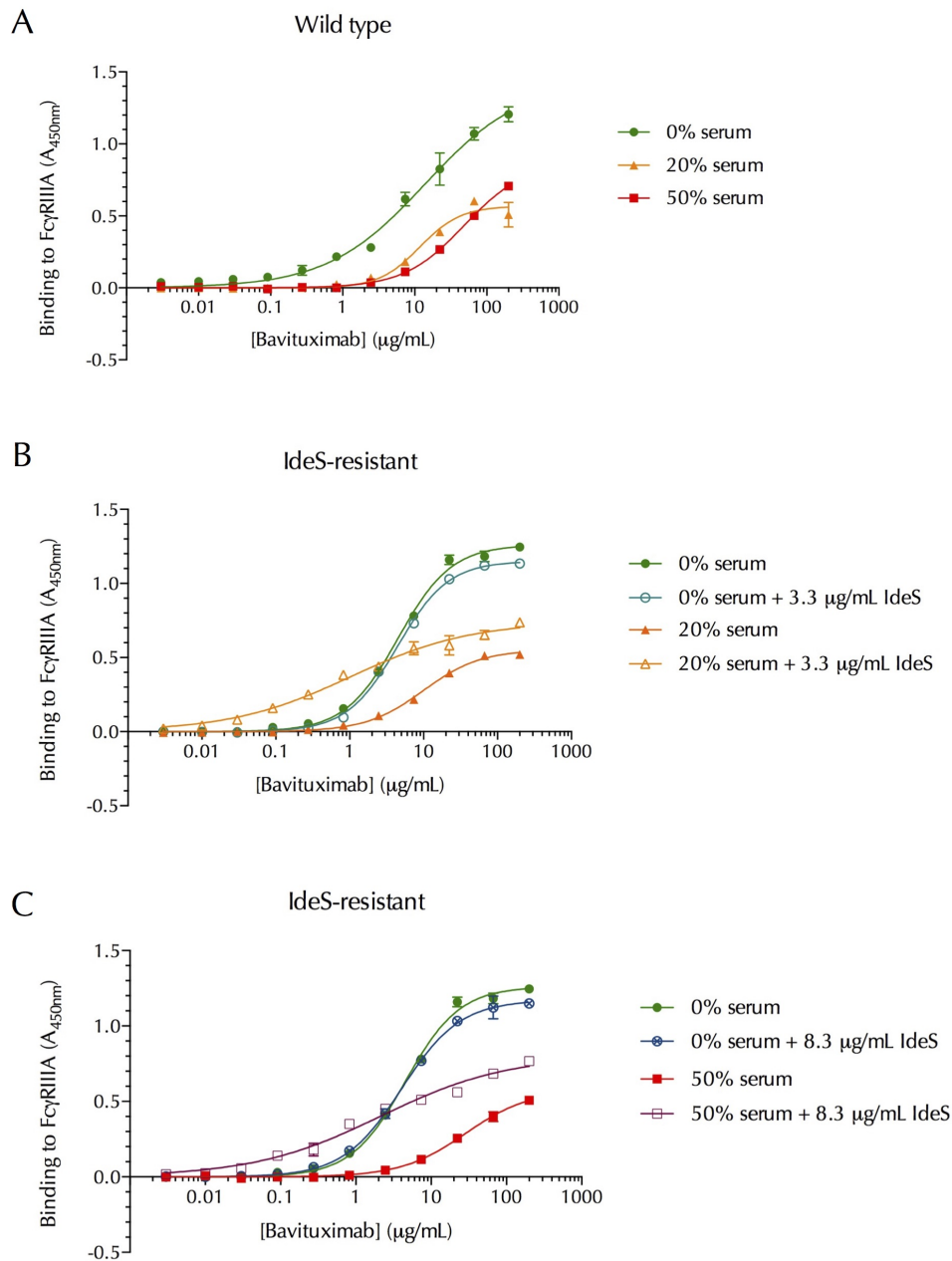


Figure 4.15: Binding of WT and IdeS-resistant bavituximab to FcγRIIIA in presence of human serum and IdeS. Bavituximab was bound to immobilised FcγRIIIA in the presence of PBS only ('0 mg/mL IgG', green/filled circles), PBS + 3.3 $\mu\text{g/mL}$ IdeS (light blue/open circles), PBS + 8.3 mg/mL IgG (dark blue/crossed circle), 20% serum (orange/closed triangles), 20% serum + 3.3 $\mu\text{g/mL}$ IdeS (yellow/open triangles), 50% serum (red/closed squares), 50% serum + 8.3 $\mu\text{g/mL}$ IdeS (maroon/open squares). **A|** Wild type bavituximab in the presence of 20% and 50% serum. **B|** IdeS-resistant bavituximab in the presence of 20% serum. **C|** IdeS-resistant bavituximab in the presence of 50% serum. In all cases, binding was detected using an antibody specific to the murine F(ab')₂ domain of bavituximab. Data are plotted as the mean $\pm$ SEM of duplicate measurements, following baseline correction. Curves are produced using non-linear least squares fitting with a one-site specific binding, Hill slope model.

of 0.24 mg/kg. Assuming a serum IgG concentration of 10 mg/mL, the IdeS:IgG mass ratio used in this instance was therefore 1:1053. The lowest peak concentration in this dose group, sampled at the same timepoint, was ~5.7 mg/L, which equates to an IdeS:IgG mass ratio of 1:1754. SDS-PAGE analysis of plasma from a single individual in this dose group showed that all IgG had been cleaved within 14 mins after infusion of IdeS, mostly to scIgG. By 2 h after infusion, only F(ab')₂ and ½Fc fragments remained. Degradation was slightly slower for an individual receiving half this dose: although there was again no intact IgG 14 mins after infusion, it took 2–6 h for the remaining scIgG to be completely cleaved to F(ab')₂ and ½Fc.

The resistance assays presented above show a slower rate of degradation than in the Phase I study. Most of the polyclonal IgG used in Figure 4.5 remained uncleaved even after 14 h when IdeS was used at a mass ratio of 1:1000. Similar results were obtained with wild type bavituximab: even after 24 h incubation with 1:1000 IdeS, less than half of the bavituximab had been completely cleaved to F(ab')₂ and ½Fc. Only at a ratio of 1:100 or greater does the reaction go almost to completion within this time, although a small amount of intact bavituximab can still be seen. In both these cases, the assay pH was chosen to reflect that of human plasma. Therefore, the disparity in IdeS activity between previously published work and the assays presented here could be due to differences in expression of IdeS or a reduction in enzyme activity following freeze-thaw. The former seems unlikely, as both the IdeS used in this study and that used in published papers were produced by overexpression of recombinant IdeS in *E. coli*. The only difference within published work as compared to the present study was the use of regular media and IPTG, coupled with a significantly shorter expression time of 3 h. The latter possibility of freeze-thaw effects has not yet been tested, as all the IdeS used here was stored in aliquots at –20°C following expression and purification, brought to 4°C as necessary and used within 2 weeks of thawing.

In addition, non-specific cleavage by IdeS was observed with bavituximab (Figure 4.13) but not with pooled human IgG (Figures 4.4 and 4.5). The only exception with IgG is the 2 h timepoint at pH 6.6 where additional bands at 40–45 kDa are present (Figure 4.4, panel C). The distinguishing feature of bavituximab compared to human IgG is the incorporation of

murine variable regions. However, the length of these variable regions (V_H : 120 amino acid residues/13.3 kDa; V_L : 108 residues/11.7 kDa) implies that any additional cleavage sites within the murine sequence would produce protein fragments too short to account for the 25–50 kDa banding seen here. Use of Western blotting using an anti-IgG antibody or Protein A as a probe would assist in the identification of these products.

It is encouraging that IdeS:IgG ratios as low as 1:1000 were as effective at reducing serum IgG binding to $Fc\gamma RIIIA$ (Figure 4.6) as the 1:600 ratio used in subsequent competition ELISAs (Figures 4.14 and 4.15). Future work would therefore focus on the use of a lower 1:1000 ratio, to test whether it is equally effective at reducing serum IgG competition in ELISA, and to identify the complicating factors present in the serum. Following this, a cellular ADCC assay using IdeS-resistant antibody and IdeS would confirm if increased receptor binding translated into increased killing of target cells.

Chapter 5

Exploring alternative phosphatidylserine-targeting methods

5.1 Creation of a TIM-4–IgG fusion protein

5.1.1 Strategy

PS is usually localised to the inner leaflet of the cellular membrane but is externalised under conditions of apoptosis, tumourigenesis or viral infection. It is therefore a marker to which potential treatments of these diseases can be targeted with high selectivity, leaving healthy cells untouched. This would ideally eliminate many of the side effects associated with current cancer treatments such as the standard chemotherapy regimes.

A chimeric monoclonal antibody, bavituximab, exploits this concept and is currently in clinical trials for multiple cancer indications [139]. The antibody binds PS indirectly via β 2-glycoprotein 1 (β 2-GP1) [115]. A potential disadvantage of β 2-GP1 dependence is its lack of selectivity as it binds cardiolipin, phosphatidylglycerol, and phosphatidic acid with similar affinities to PS [199].

TIM-4 is one member of a protein family that directly binds phosphatidylserine (PS). Human TIM-4 is composed of the ligand-binding IgV-like domain, a heavily O-glycosylated mucin domain, a transmembrane region and a short cytoplasmic tail which appears to lack the signalling function found in other family members [69]. Although structural characterisation was carried out only on the murine TIM proteins [158, 159], the human orthologs (hTIM) appear to be functionally equivalent. Assays to compare binding of hTIM-4 to phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylcholine (PC) and phosphatidylinositol (PI) demonstrated high selectivity for phosphatidylserine [102, 127].

We therefore designed a cancer- and virus-targeting agent composed of hTIM-4 fused to the Fc effector region of human IgG1. The hTIM-4 IgV domain would specifically direct this construct to PS-expressing cells; multiple immune effector mechanisms would thence be recruited via the Fc domain, particularly antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-mediated cytotoxicity (CDC). Unfortunately, the functionality of the fusion could not be evaluated due to problems expressing an intact protein.

5.1.2 Cloning and expression of initial fusion protein constructs

An initial set of constructs fused the C-terminus of hTIM-4 (IgV domain plus some or all of the mucin domain) with the N-terminus of hIgG1 Fc starting at the amino acid residue His224 (Eu numbering [60]). A schematic diagram of these constructs is illustrated in Figure 5.1. The pHLSec vectors carrying the constructs were transiently transfected into HEK 293T cells and the culture supernatant harvested after 5–7 days incubation. Proteins were purified from supernatant with nickel-affinity chromatography; final yield and purity was assessed using SDS-PAGE gel electrophoresis and Western blotting. Two TIM-4 constructs, containing some ('cryst') or all ('extracellular', 'EC') of the mucin domain to match the polypeptide lengths incorporated into the fusion protein, were expressed for comparison.

Expression of an intact protein was not observed for either of the fusion protein constructs (Figure 5.2). Additionally, integrity of protein expression varied between batches—for

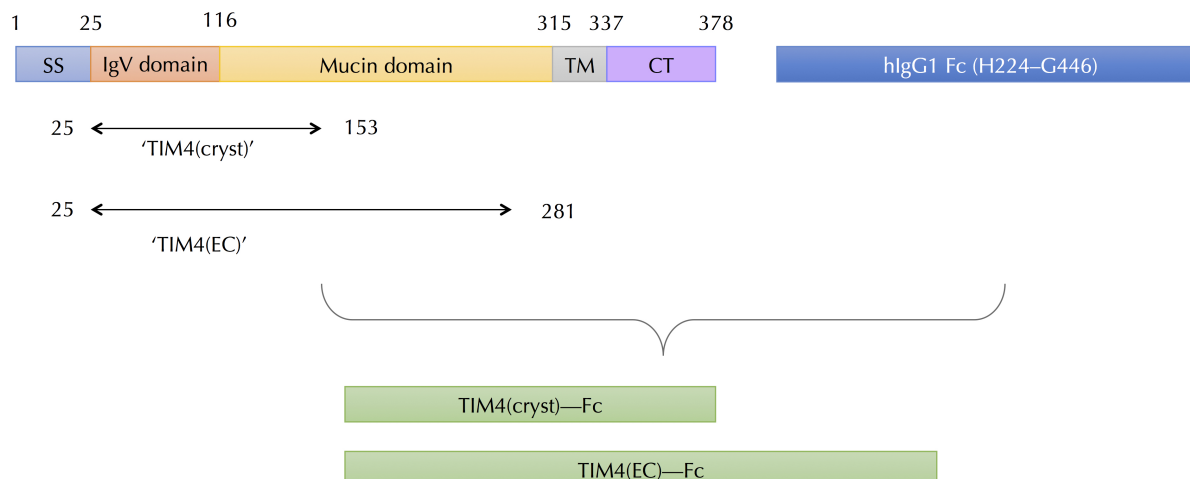


Figure 5.1: Schematic illustration of the amino acid sequences included in the first set of fusion protein constructs. SS, signal sequence; IgV, immunoglobulin V-like domain; TM, transmembrane domain; CT, C-terminal region; EC, extracellular.

example, a repeat production of TIM4(cryst)–Fc (Figure 5.3) yielded a greater proportion of TIM4(cryst)–Fc protein at the expected molecular weight (Figure 5.2). In the second instance, total protein yield was 2.2 mg/L culture supernatant.

5.1.3 Optimisation of domain boundaries

TIM-4

At the time this project was underway, no crystal structure for the human TIM proteins was available. Therefore, in order to optimise domain boundaries for expression, the human TIM-4 sequence (UniProt Q96H15) was compared to the sequences and crystal structures for the murine orthologue mTIM-4. The construct used to produce the structure of TIM-4 IgV in complex with phosphatidylserine (PDB 3BIB) incorporated residues 24–134 of the mTIM-4 protein. It can be seen from the structures, represented in Figure 1.4, that this protein sequence is likely to be the minimum necessary to produce a functional phosphatidylserine-binding domain. In particular, the FG loop (G being the N-terminal β -strand according to labelling

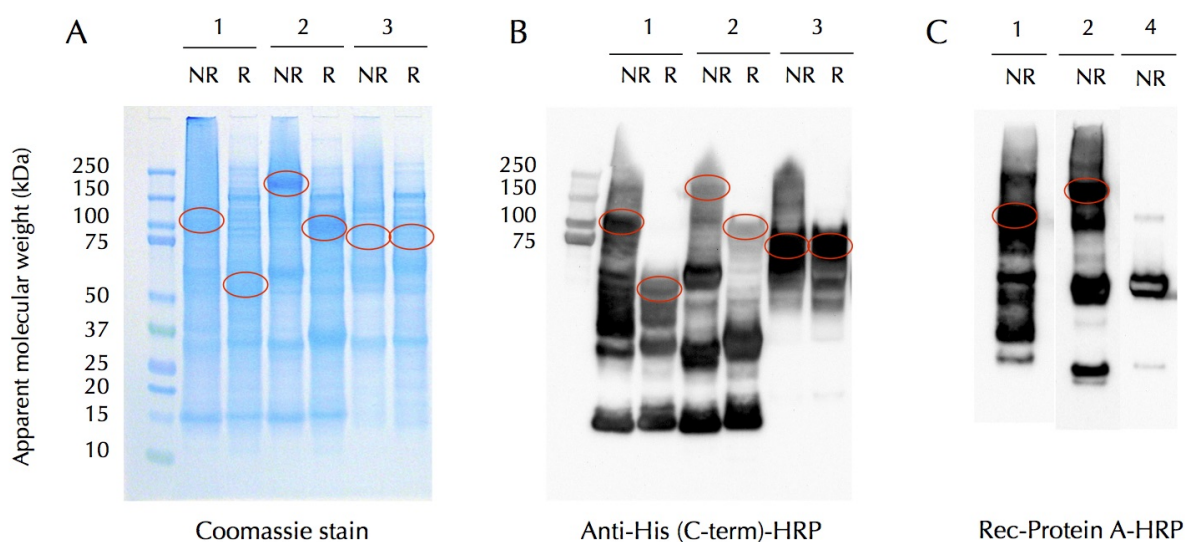


Figure 5.2: Gel electrophoretic and Western blot analysis of the first set of fusion proteins, purified by nickel-affinity chromatography as detailed in the Materials and Methods. In all panels, SDS-PAGE was first run using a 4–12% Bis-Tris gel then stain or antibody probe as indicated. ‘1’ refers to TIM4(cryst)–Fc, ‘2’ refers to TIM4(EC)–Fc, 3 to TIM4(EC) and ‘4’ to an IgG1 Fc control. NR: non-reduced sample; R: reduced sample. Red circles indicate the expected molecular weight of each protein if a monomer or a dimer is produced. **A**| Coomassie stain. **B**| Western blot with anti-His(C-terminal) HRP-conjugated antibody. **C**| Western blot with HRP-conjugated Protein A to detect fully-folded Fc domains.

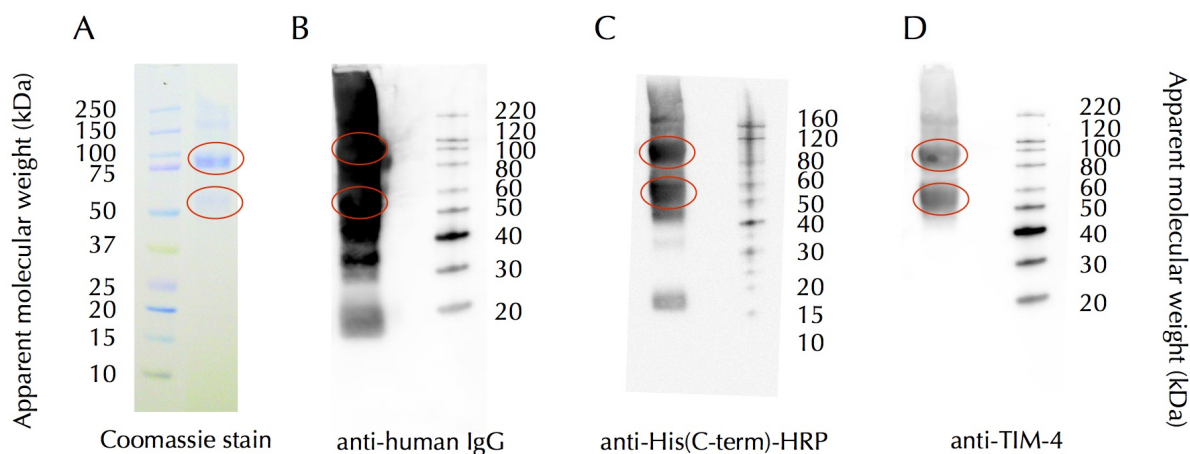


Figure 5.3: A repeat expression of TIM4(cryst)-Fc, purified by nickel-affinity chromatography, to illustrate variability in protein integrity. In all panels, SDS-PAGE was first run using a 4–12% Bis-Tris gel then stain or antibody probe as indicated. All samples are non-reduced. Red circles indicate the expected molecular weight if protein forms a monomer or a dimer. **A|** Coomassie stain. **B|** Western blot with rabbit anti-human IgG primary followed by anti-rabbit-HRP secondary. **C|** Western blot with anti-His(C-terminal) HRP-conjugated antibody. **D|** Western blot with rabbit anti-TIM-4 primary followed by anti-rabbit-HRP secondary.

by Santiago *et al* [158]) forms part of the PS-binding cavity. An alignment of the mTIM-4 and hTIM-4 protein sequences indicates that the hTIM-4 sequence corresponding to the crystallised murine equivalent consisted of residues 24–134 inclusive (Figure 1.5). This was therefore the sequence chosen for cloning of the second set of fusion constructs and was designated ‘hTIM4XII’.

IgG Fc

The IgG Fc boundaries were altered to incorporate more of the hinge region with the hope that this might facilitate expression. Asp221 (Eu numbering [60]) was chosen to form the N-terminus of the sequence used, corresponding to the N-terminus of the Fc sequence in the pFUSE-hIgG1-Fc expression vectors (Section 2.1.2). This sequence is referred to as ‘hIgGII’ when used in the fusion constructs in this chapter.

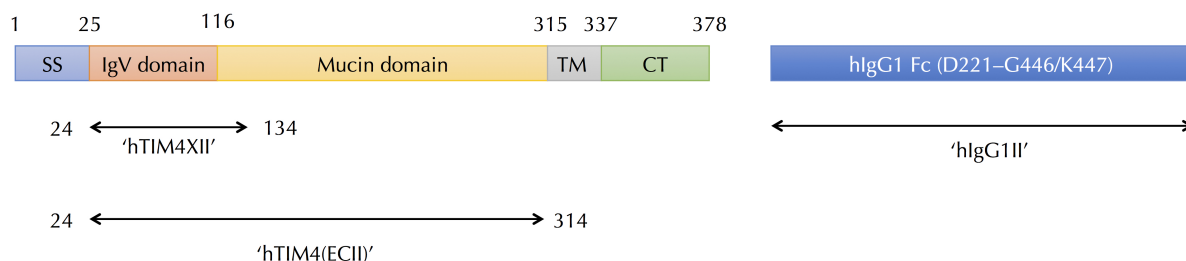


Figure 5.4: Schematic illustration of the amino acid sequences included in the second set of fusion protein constructs. SS, signal sequence; IgV, immunoglobulin V-like domain; TM, transmembrane domain; CT, C-terminal region; EC, extracellular.

5.1.4 Expression of constructs with altered domain boundaries

A second set of constructs was created according to the parameters defined in Section 5.1.3, as illustrated in Figure 5.4. This second set of constructs was subsequently expressed using the same procedure as previously (Section 5.1.2) and assessed with gel electrophoresis and Western blotting using a variety of antibodies to different regions of the fusion protein (Figure 5.6).

5.1.5 Size exclusion purification

Each of hTIM4XII-hlgG1II, hlgG1II-hTIM4XII and TIM4(cryst)-Fc proteins underwent a further gel filtration step but sufficient UV absorbance for protein retrieval was observed for TIM-4(cryst)-Fc only (Figure 5.7). Peak 2 from the chromatogram of TIM4(cryst)-Fc appears to indicate the presence of the desired protein at the correct molecular weight as indicated by gel electrophoresis and Western blotting (Figure 5.8). However, the majority of the protein retrieved from the size exclusion step consisted of aggregates (peak 1); the TIM-4(cryst)-Fc therefore consisted of <20% protein retrieved. The final yield from 600 mL culture supernatant was <0.01 mg.

Yields for all protein constructs described here were therefore too low to pursue functional assays.

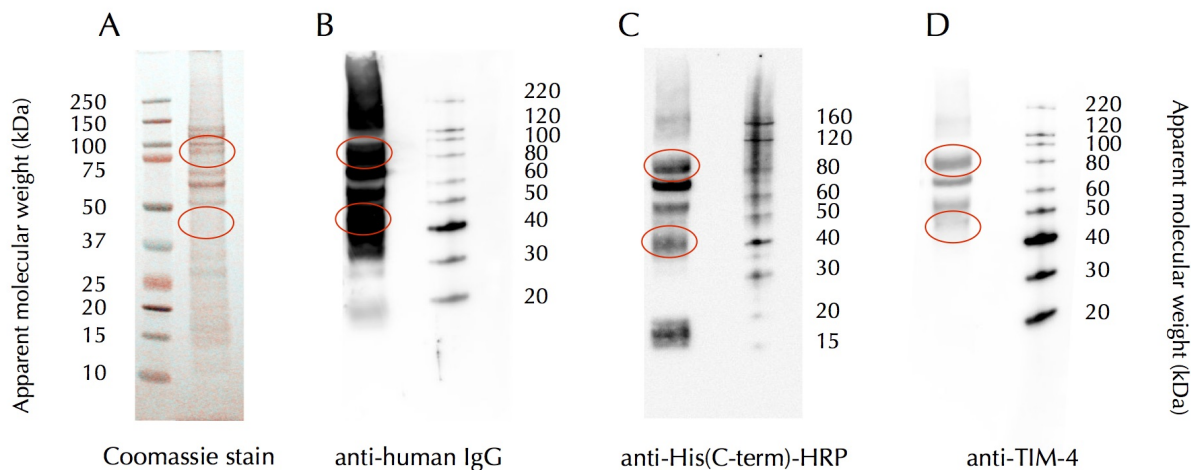


Figure 5.5: Expression of hTIM4XII-hIgG1II. In all panels, SDS-PAGE was first run using a non-reducing 4–12% Bis-Tris gel then stain or antibody probe as indicated. All samples are non-reduced. Red circles indicate the expected molecular weights if protein forms a monomer or a dimer. **A|** Coomassie stain. **B|** Western blot with rabbit anti-human IgG primary followed by anti-rabbit-HRP secondary. **C|** Western blot with anti-His(C-terminal) HRP-conjugated antibody. **D|** Western blot with rabbit anti-TIM-4 primary followed by anti-rabbit-HRP secondary.

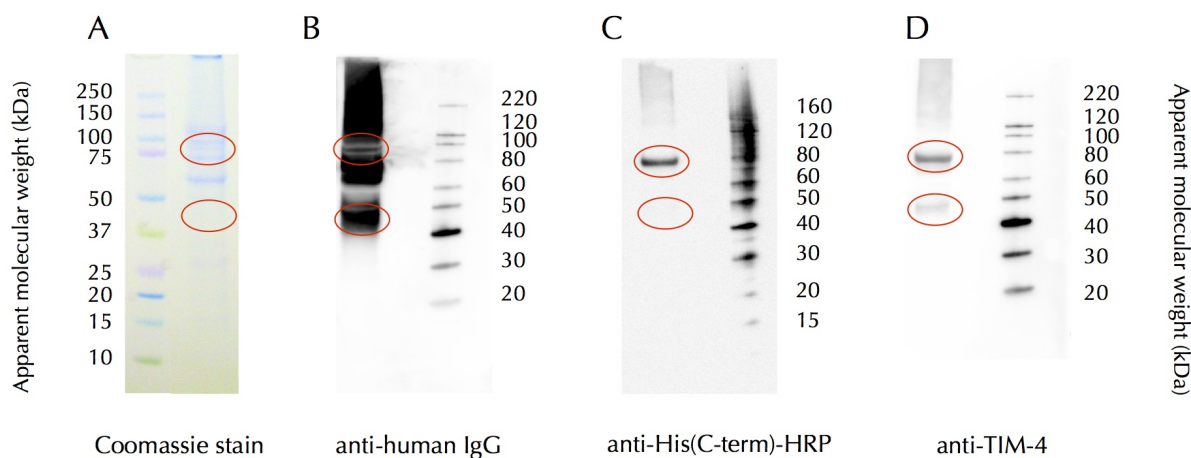


Figure 5.6: Expression of hIgG1II-hTIM4XII. In all panels, SDS-PAGE was first run using a non-reducing 4–12% Bis-Tris gel then stain or antibody probe as indicated. All samples are non-reduced. Red circles indicate the expected molecular weights if protein forms a monomer or a dimer. **A|** Coomassie stain. **B|** Western blot with rabbit anti-human IgG primary followed by anti-rabbit-HRP secondary. **C|** Western blot with anti-His(C-terminal) HRP-conjugated antibody. **D|** Western blot with rabbit anti-TIM-4 primary followed by anti-rabbit-HRP secondary.

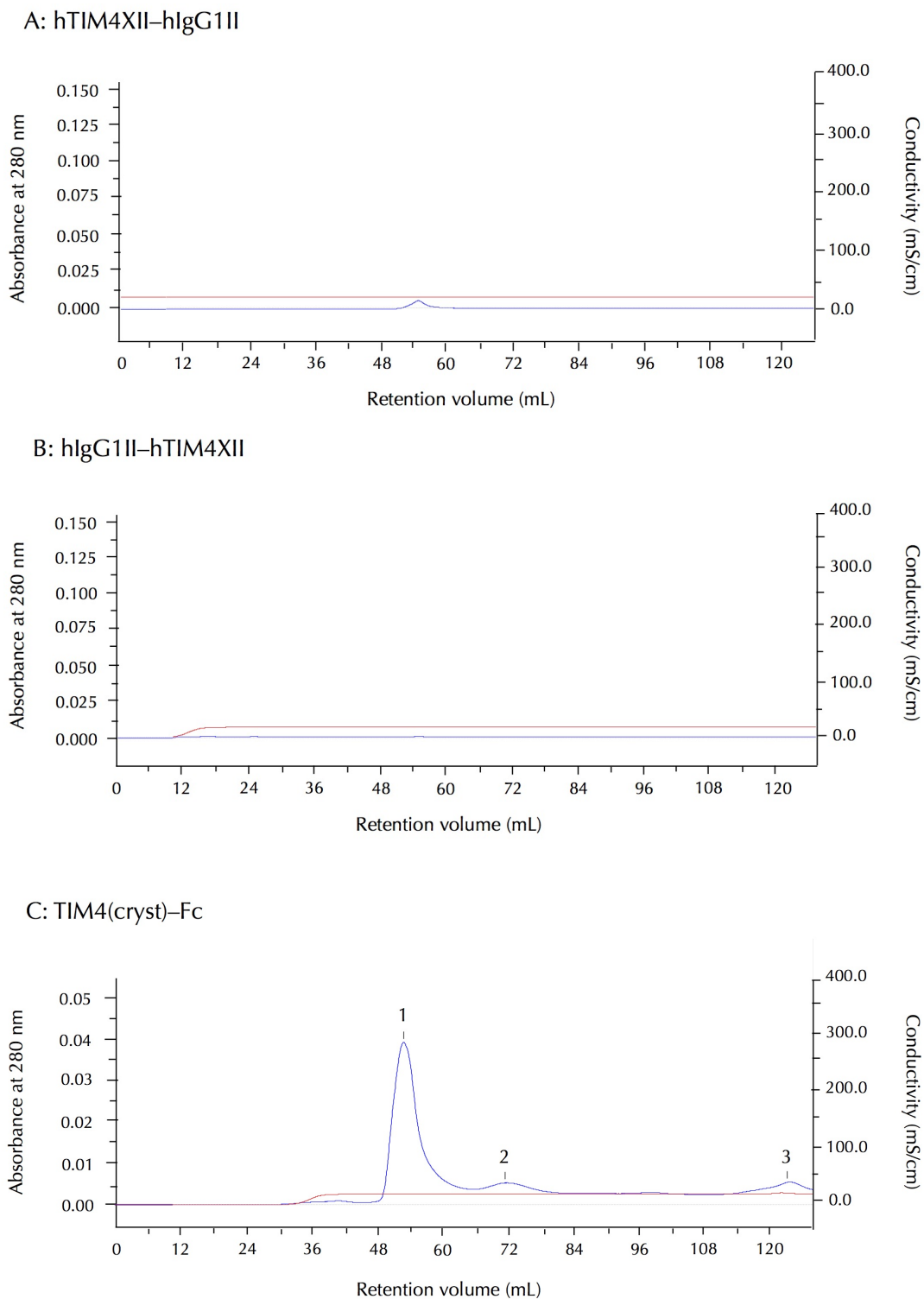


Figure 5.7: Size exclusion purification of fusion proteins using a Superdex 200 pg 16/600 column. Blue trace, UV absorbance at 280 nm; red trace, conductivity. Injected masses were as follows: **A**| hTIM4XII-hIgG1I, 0.57 mg; **B**| hIgG1II-hTIM4XII, 0.90 mg; **C**| TIM4(cryst)-Fc, 0.91 mg. Peak fractions were collected for TIM4(cryst)-Fc as indicated by numbers 1, 2 and 3.

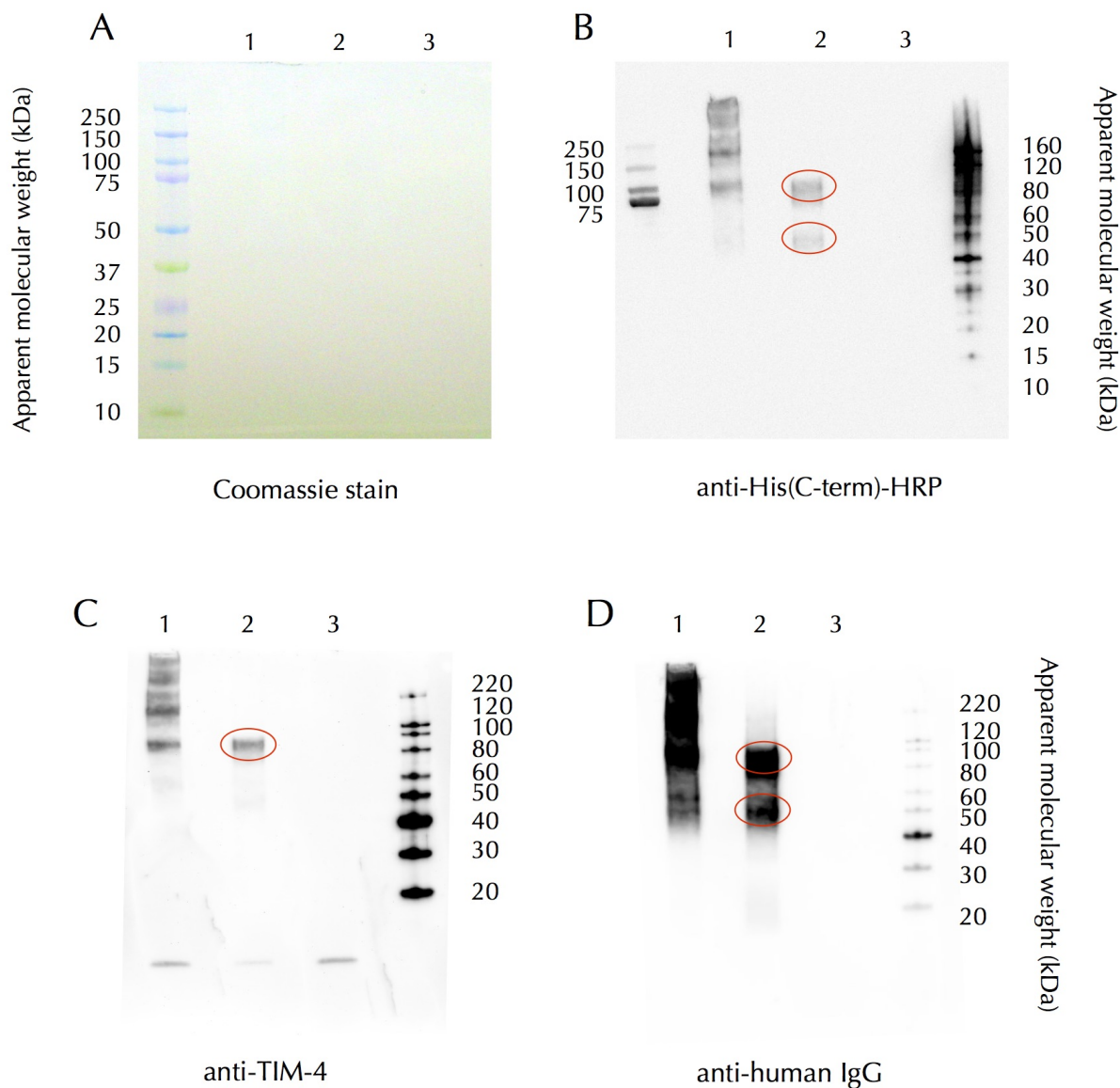


Figure 5.8: SDS-PAGE and Western blot analysis of pooled fractions following gel filtration purification of TIM-4(cryst)-Fc. Samples were run on a non-reducing 4–12% Bis-Tris gel and either stained with Coomassie Blue (panel **A**) or transferred to a PVDF membrane and probed with antibodies as indicated (panels **B–D**). Lane numbers correspond to labelled peaks from the UV absorbance trace of TIM4(cryst)-Fc (Figure 5.7, panel C).

5.2 Investigation of phosphatidylserine exposure on Semliki Forest Virus

5.2.1 Strategy

The general involvement of phosphatidylserine (PS) in viral infection, for most if not all virus families, is now well-established (Section 1.5). However, its importance at the various stages of the viral life cycle continues to be elucidated. There is conflicting evidence regarding the exposure of PS on the surface of virions — for example, Callahan *et al* (2003) [33] and Moody *et al* (2010) [129] both investigated the exposure of PS on HIV-1 virions. The former group used Annexin V-coated beads in a pull-down assay to show that virions carried exposed PS; the latter group tested binding of various anti-phospholipid antibodies using surface plasmon resonance (SPR) and found that only one antibody, PGN632, bound. PGN632 also showed the greatest response when binding to immobilised PS liposomes using the same method. Furthermore, the level of PS exposure and accessibility may be dependent on the structure of the protein coat, which in turn is determined by the particular virus species. The binding of PS may also be affected by size of the PS receptor used, the epitope it recognises and the detection technique.

We decided to explore the role of different PS-binding proteins in a model alphavirus, Semliki Forest Virus (SFV). Being one of the simplest icosahedral enveloped RNA viruses, SFV has been structurally well-characterised [117, 194, 202] and has proved tractable for both laboratory culture and cryo-electron microscopy (cryo-EM). The aim was to investigate the protein features that determined binding to virion-exposed PS, and to use cryo-electron microscopy to probe the structure of the resulting complex more closely.

Binding to SFV was tested using ELISA with three anti-PS proteins as the analytes: TIM-1, TIM-4 and bavituximab. TIM-1 and TIM-4 are members of the same protein family and have a similar domain structure, including the IgV domain which is responsible for binding PS (Section 1.4.1). In this instance, the human variants for both proteins were used. Bavituximab is a chimeric antibody with mouse variable regions and human constant regions. It has been

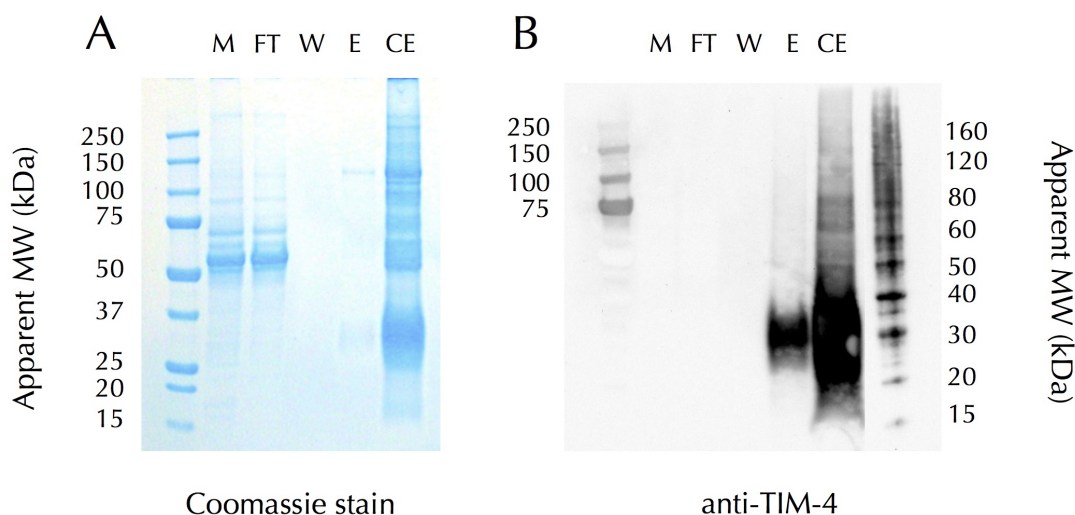


Figure 5.9: Trial expression of TIM-4 in HEK-293T cells followed by nickel-affinity purification. Samples from purification stages were analysed by non-reducing SDS-PAGE using 4-12% Bis-Tris gels, followed by either **A**| Coomassie stain or **B**| Western blot with rabbit anti-TIM-4 primary and anti-rabbit-HRP secondary. M, media; FT, flow-through (unbound); W, wash; E, eluate; CE, concentrated eluate; MW, molecular weight.

shown to bind PS on the surface of virus-infected cells and virions and has also demonstrated anti-viral effects *in vivo* (see Table 1.2 for a detailed summary).

5.2.2 Cloning and expression of PS-binding proteins

The TIM-4 protein used in this section contained amino acid residues 24–153 inclusive, thereby incorporating the IgV domain and a portion of the mucin domain. An initial transfection of HEK 293T cells showed successful expression of TIM-4, as confirmed with an anti-TIM-4 antibody (Figure 5.9). For subsequent protein production, TIM-4 was purified by nickel-affinity followed by size exclusion (Figure 5.10). The TIM-1 protein contained the IgV domain only (residues 22–129) and was kindly expressed and purified from *E. coli* by Xiangxi Wang (Division of Structural Biology, University of Oxford).

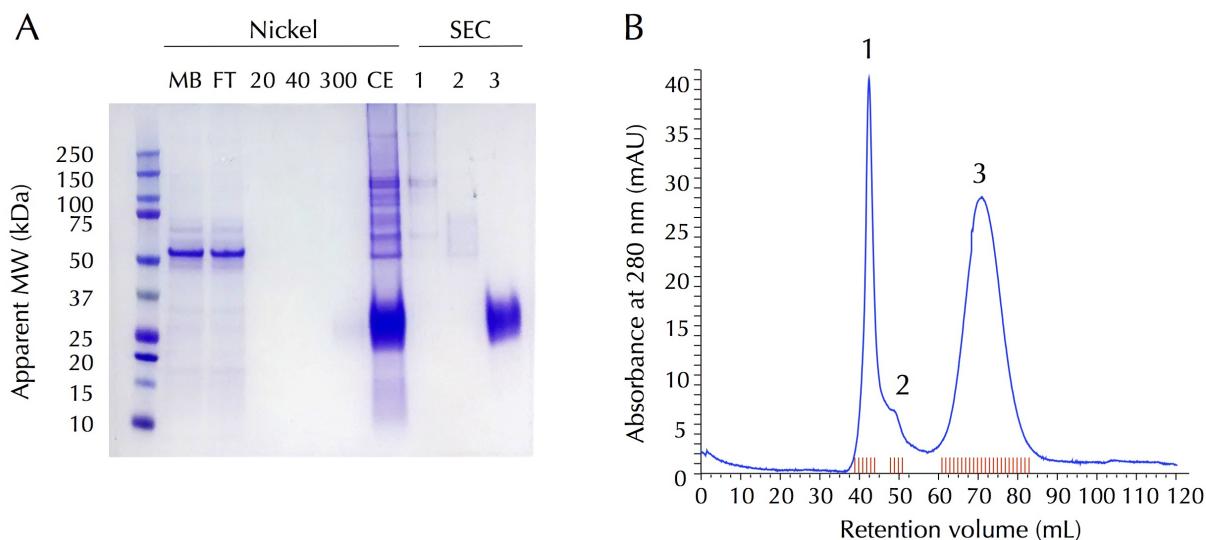


Figure 5.10: Expression and purification of TIM-4 containing the IgV domain and a portion of the mucin domain (residues 24–153). **A** SDS-PAGE analysis of TIM-4 fractions following nickel-affinity purification ('Nickel') and size exclusion ('SEC'). MB, media + buffer; FT, flow-through; 20/40/300, washes/elutions with imidazole concentrations in mM. The 300 mM imidazole fraction was concentrated ('CE') before gel filtration. 1/2/3 refer to peaks corresponding to pooled and concentrated fractions following gel filtration (panel B). Molecular weight (MW) markers are given on the left-hand side. **B** Gel filtration chromatogram of TIM-4 using a Superdex 75 pg 16/600 column. Fractions from the labelled peaks were pooled and concentrated. Blue trace indicates UV absorbance at 280 nm; red vertical lines indicate the start and end of fraction collection.

Full length bavituximab was also expressed in HEK 293T cells. Initial purifications were carried out using nickel affinity chromatography (Section 2.6.1), the results of which can be seen in Figure 4.7. Expression of the full length antibody was confirmed by SDS-PAGE and Western blotting using Protein A-HRP (Figure 4.7). The integrity of the PS-binding domain was confirmed by colleagues using SKBr3 cells treated with etoposide to induce PS exposure [186]. Both the positive control Annexin V (Section 1.4.4) and bavituximab selectively bound the etoposide-treated cells, facilitating adenoviral infection via an Fc γ RIIIA-containing adaptor protein. For the ELISA work presented in this chapter, bavituximab was expressed in HEK 293T or 293F cells and purified using Protein A (Section 4.4).

5.2.3 Binding of anti-PS proteins to SFV by ELISA

An ELISA was conducted, with all incubations at room temperature, to test binding of immobilised SFV to TIM-1, TIM-4 and bavituximab. Binding was detected only for TIM-1 (Figure 5.11). This was surprising given the similarity of the TIM-1 and TIM-4 PS-binding domains (Section 1.4.1). An absence of binding might be partly due to close packing of the viral protein coat; an increase in binding temperature to 37°C might consequently cause structural rearrangement of the protein coat to expose the previously hidden PS epitope. This phenomenon has been demonstrated previously for antibody binding to dengue [68, 209] and West Nile Virus (WNV) [58]. Accordingly, the ELISA was repeated using TIM-4 and bavituximab with blocking and incubation steps at 37°C (Figure 5.12). No binding was detected, despite increasing the maximum concentration of TIM-4 tested (for which protein quantities were limited by low expression yields) from 8 μ M to 20 μ M.

5.2.4 Discussion

The binding of TIM-1 to SFV by ELISA provides evidence that PS is exposed on the virion following our purification and assay conditions. There are several possible reasons for the binding differences between TIM-1, TIM-4 and bavituximab.

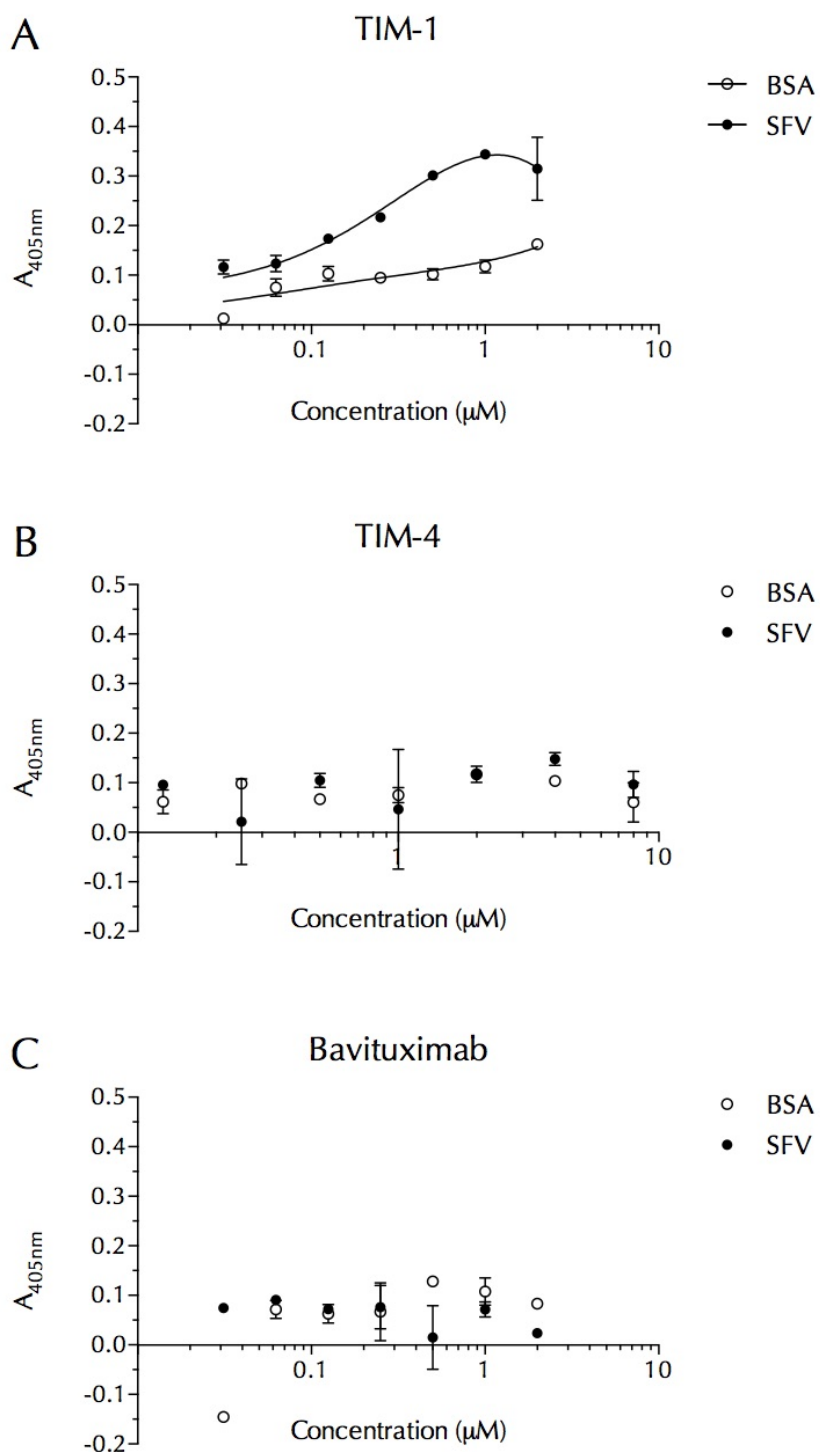


Figure 5.11: ELISA probing binding of TIM-1 (panel A), TIM-4 (panel B) and bavituximab (panel C) to Semliki Forest Virus (SFV), at room temperature. SFV was plated overnight at 4°C, blocked at room temperature then incubated at room temperature with a dilution series of the appropriate analyte. Bovine serum albumin (BSA) was used in place of SFV as a negative control. Values are plotted as mean $\pm$ standard error of the mean (SEM) for duplicate measurements.

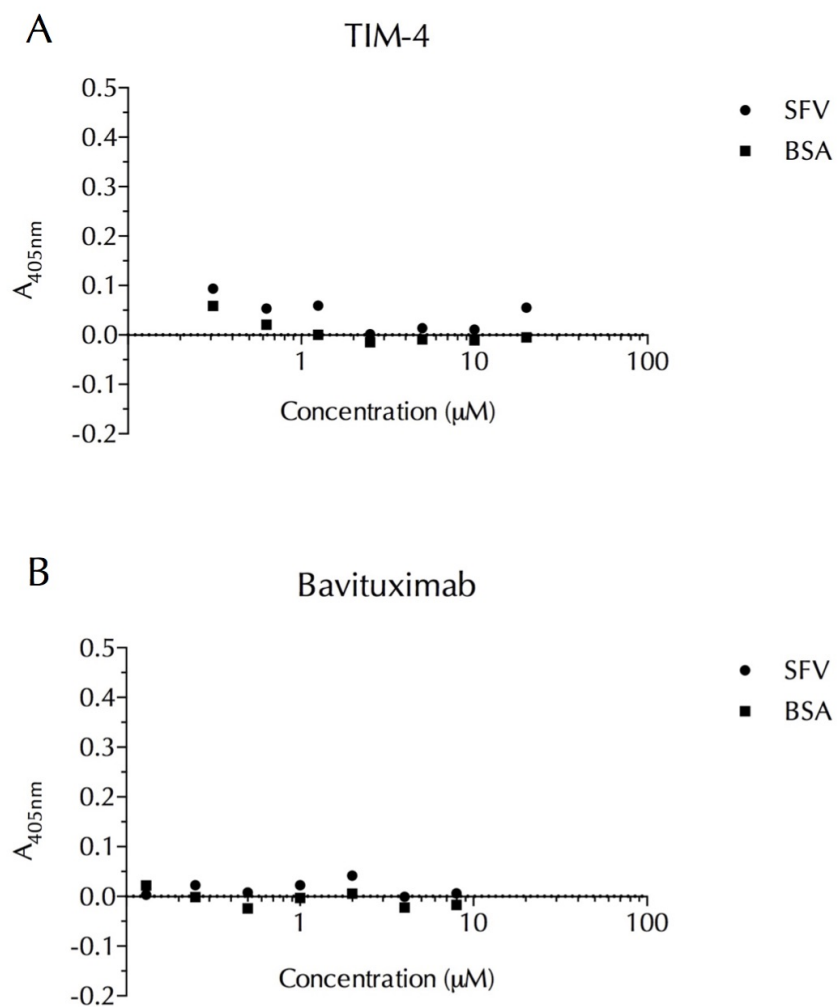


Figure 5.12: ELISA probing binding of TIM-4 (panel A) and bavituximab (panel B) to SFV, at 37°C. SFV was plated overnight at 4°C, blocked at 37°C then incubated at 37°C with a dilution series of the appropriate analyte. BSA was used in place of SFV as a negative control. Values are plotted as mean $\pm$ SEM for duplicate measurements.

Firstly, TIM-1 and TIM-4 are of different sizes. TIM-1 has an apparent molecular weight of 12 kDa, as it contains only the IgV binding domain and was expressed in *E. coli*. It is therefore much smaller than the 30 kDa TIM-4 construct used here (Figure 5.10), which incorporates some of the mucin domain in addition to the IgV. As TIM-4 was expressed in mammalian cells, it is likely that the mucin domains is heavily O-glycosylated [169]. It is possible that the ‘holes’ in the viral protein coat may be of a size to allow the smaller and more compact TIM-1 to access PS while preventing the bulkier TIM-4 from binding. Planned future work is to express a shorter construct of TIM-4 containing residues 24–134 and to assay binding at both room temperature and 37°C.

Regarding bavituximab, a similar logic could apply. Bavituximab itself has an apparent MW of 165 kDa when expressed in HEK 293T cells (Section 4.4) but unlike the TIM-1 and TIM-4 proteins it does not bind PS directly but via β 2-GP1. β 2-GP1 is a 50 kDa protein and thus larger than TIM-4; if steric hindrance prevented TIM-4 from accessing PS, this is likely to apply to β 2-GP1 also.

However, an alternative explanation also exists. β 2-GP1 is not specific to PS but also binds other negatively-charged phospholipids with similar affinities — namely phosphatidylinositol, phosphatidylglycerol, phosphatidic acid and cardiolipin [199, 76]. The exception to this series is phosphatidylcholine, for which the negative charge on the phosphate is countered by the positive charge on the choline head group (Section 1.2, Figure 1.1). This suggests that binding is mainly dependent on electrostatic interactions and can be disrupted by cations. In accordance with this, a study by Willems *et al* (1996) [199] found that increasing the ionic strength of the buffer reduced the affinity of β 2-GP1 for membranes containing 20 mol% PS, particularly with the addition of divalent calcium ions. For example, increasing the CaCl_2 concentration from 1 mM to 3 mM, while maintaining NaCl at 120 mM, caused an increase in the K_D from 0.63 μM to 3.9 μM .

The buffer used in the preceding ELISAs contained 100 mM NaCl and 5 mM CaCl_2 . These specific conditions were not tested by Willems and his colleagues but the relatively high

calcium concentration may therefore have contributed to the lack of binding observed. Interestingly, serum calcium concentration is 10 mg/dL [119], equivalent to 2.5 mM, of which 40% is bound to protein. This equates to a free calcium concentration in the blood of 1.5 mM. Further work would include a repeat of the ELISA using bavituximab in a buffer containing calcium at this concentration to reflect physiological conditions as closely as possible.

A third hypothesis is drawn from the findings of Yuan *et al* (2015) [208], whose paper became available following the submission of this thesis. In addition to publishing the first crystal structures of hTIM-1 and hTIM-4, the authors demonstrated binding of the Ebola glycoprotein (GP) to hTIM-1, but not to hTIM-3 or hTIM-4. Affinity of hTIM-1 for Ebola GP was greater than its affinity for PS. The binding site for Ebola GP was contiguous with that for PS binding; key structural differences in hTIM-1 that may contribute to selectivity for this particular family member are a shorter BC loop and a putative GP-binding groove containing positively-charged arginines [208]. hTIM-1 has also been found to interact directly with Ebolavirus Niemann-Pick C1 (NPC1) to promote membrane fusion. Given this evidence, it seems feasible that the binding seen in Figure 5.11 with hTIM-1 only is due to an interaction of hTIM-1 with the SFV glycoprotein rather than with PS.

TIM-1 is being taken forward for cryo-EM studies in collaboration with colleagues at the Division of Structural Biology, University of Oxford, in the hope that this would further elucidate the nature of its interaction with the SFV virion.

Chapter 6

Perspectives

Therapeutic antibodies have several advantages over conventional small molecule drugs, not least their target specificity and their ability to recruit immune effector functions [35]. Phosphatidylserine (PS) is a novel target that is exposed on a wide variety of tumour cell types, virus-infected cells and virions (Sections 1.5 to 1.6). It therefore forms a prime candidate for both anti-viral and anti-cancer mAbs and is being commercially exploited in the form of bavituximab, a therapeutic mAb shown to have *in vivo* efficacy against virus infections and cancer (Section 1.7).

Bavituximab relies predominantly on ADCC, and therefore on Fc γ R binding, for its anti-tumour effect [171]. A limitation common to ADCC-dependent antibodies is competition with high serum IgG concentrations *in vivo* [143], which reduces mAb binding and decreases therapeutic potency. One aim of this thesis was to reduce serum IgG concentrations and improve mAb binding with the use of an IgG-cleaving enzyme, IdeS. The strict specificity of IdeS for IgG [191] and the low affinity of its F(ab')₂ and Fc products for Fc γ R [26] render it highly suitable for the selective removal of IgG. Despite being of bacterial origin, it was well tolerated during a recent Phase I clinical trial and effectively cleaved almost all serum IgG within hours of administration [200]. While its lead indication is transplant rejection, work

presented here explored its use in combination with therapeutic antibodies, using bavituximab as an example.

Although IdeS is rapidly cleared from the bloodstream, which would allow administration of therapeutic mAb within the few days following IdeS treatment, we designed an IdeS-resistant therapeutic mAb to ensure maximal efficacy and to widen the potential therapeutic window. IdeS is known to cleave in the hinge region of IgG but is thought to have an additional interface on the IgG C γ 2 or C γ 3 domains [191]. The design of an optimal IdeS-resistant antibody therefore requires detailed knowledge of the amino acid residues of IgG Fc involved in IdeS binding. This was the subject of study in Chapter 3: systematic mutagenesis of residues identified Pro232–Gly237 (the hinge cleavage region), Ser267 and Pro329–Ile332 as possible residues involved in the interaction, although only Ile332 appears to have a significant role. This was consistent with previously published studies of IdeS-resistant antibodies [101] but did not identify any additional important residues. The key conclusion from this chapter was the confirmation of the presence of an exosite in the Fc region of IgG: IdeS bound an IgG Fc construct lacking the hinge, with only slightly reduced affinity compared to the wild type Fc containing the hinge.

Future work would include testing the panel of mutants produced for Fc γ RI, Fc γ RIIA/B/C, and Fc γ RIIIB receptors. It is anticipated that mutations in the hinge region would reduce affinity to all Fc γ Rs, in accordance with previous findings [166]. Further mutations in the C γ 2 region are likely to show differing affinity for receptors—for example, the mutation S298A reduced binding to Fc γ RII but improved binding to Fc γ RIIA. Comprehensive knowledge of these properties would enable targeting of therapeutic antibodies, such as bavituximab, to the desired receptors to elicit an enhanced response.

The creation of an IdeS-resistant bavituximab incorporated the mutations previously identified by Kinder *et al.*: E233P, L234V, L235A, G236X, S239D and I332E. These mutations successfully conferred IdeS-resistance under physiological conditions, as determined by *in vitro* assay. Bavituximab binding to Fc γ RIIA was dramatically reduced in the presence of competing IgG

but completely restored upon pre-treatment of the IgG with IdeS. Pre-treatment of whole serum with IdeS also produced some improvement in bavituximab binding; however, the effect was less significant and appears to be modified by additional, as yet unidentified, serum factors. These serum factors could be interacting with the IdeS and/or the Fc γ RIIIA. Importantly, bavituximab binding to Fc γ RIIIA should be tested in the presence of serum depleted of IgG to confirm that the complicating factors are not IgG fragments. IdeS is currently thought to cleave only IgG and is not known to interact with any other serum components; to investigate this, a pull-down assay could be used with the inactive IdeS C94A mutant as bait, to identify any additional serum proteins with which IdeS may interact. Interestingly, there is evidence that IdeS binds to Fc γ RIIIB to inhibit the release of reactive oxygen species from polymorphonuclear leukocytes [108, 173]. However, in the presence of '0% serum + IdeS', bavituximab binding to Fc γ RIIIA was similar to binding in PBS alone ('0% serum') (Figure 4.15), suggesting that IdeS binding to Fc γ RIIIA was not the complicating factor in this experiment. Lastly, it would be worth exploring the effects of plasma compared to serum: the clotting process that produces serum from plasma involves the activation of serine proteases [51], which may be affecting the activity of IdeS.

The receptor refocusing approach using IdeS therefore holds promise; further work is required to determine whether the improved binding translates into clinical benefit using *in vivo* studies. If this is shown to be the case, IdeS could potentially be used in conjunction with EndoS, another *S. pyogenes*-derived enzyme that renders IgG inactive by cleaving the N297 oligosaccharide [44]. EndoS was previously shown to increase the binding of a monoclonal antibody to Fc γ RIIIA in the presence of 20% serum [12]. Consecutive use of these two enzymes would increase the potential timespan in which to administer a therapeutic antibody with reduced competition from serum IgG. IdeS completely cleaves the serum IgG pool within 6 hours and the majority is cleared from the bloodstream within 24 hours [200]. The serum IgG levels begin to rise within 3-4 days; IdeS therefore provides a window of only ~2 days in which a non-IdeS-resistant therapeutic antibody can be administered, and 3 days if an IdeS-resistant therapeutic antibody is used. EndoS, at 108 kDa [44], is above the kidney filtration cut-off of

70 kDa [114]; it would therefore have a longer half life and provide a longer timespan in which serum IgG levels would be reduced.

A third line of investigation pursued in this thesis was the creation of alternatives to bavituximab that bypass the requirement for β 2-GP1. TIM-4 is a cell surface receptor that directly binds PS [69] and was therefore chosen for the creation of IgG1 Fc fusion proteins; the incorporation of the Fc domain was intended to confer the ability to recruit immune effector functions, comparable to a therapeutic mAb. Unfortunately, all such constructs designed so far exhibited poor and fragmented expression. Personal communication with Dr Gerardo Kaplan, who leads a research group working with this family of proteins, indicated that this might be due to the lack of incorporation of an intron splice site between the TIM protein and the Fc domain, and this remains a potential line of enquiry.

TIM-4, the related protein TIM-1 and bavituximab were used to explore viral PS exposure with Semliki Forest Virus (SFV) as a model. The aim was to determine the density of PS exposure and its accessibility by different PS receptors, as an initial step in resolving some conflicts in the literature. Unexpectedly, out of these three PS-targeting proteins only TIM-1 bound to SFV. As explained in Section 5.2.4, the differences in TIM-1, TIM-4 and bavituximab binding may be due to three possible factors: glycoprotein-mediated spatial restriction of PS accessibility to TIM-4 and bavituximab/ β 2-GP1; the presence of calcium in the binding buffer inhibiting the binding of β 2-GP1; or selective binding of SFV glycoproteins to hTIM-1 due to structural differences between TIM family members. Although this study remains in preliminary stages, cryo-EM data from the TIM-1–SFV complex would provide insight to inform future work. This study could be expanded by including other virus families, such as the *Filoviridae* and *Retroviridae*, for which the Ebolaviruses and HIV viruses respectively are already the subject of much investigation (Table 1.2).

Current *in vivo* evidence appears to support the targeting of PS in viral infections, given the success of bavituximab in animal models of Pichinde virus, Punta Toro virus, and mouse cytomegalovirus [47, 171]. There seems to be a consensus that PS and PS receptors (PSRs)

enhance viral entry, via the engagement of host cell PSRs either by virion-incorporated PS or by viral proteins (Section 1.5). However, the importance of this pathway is variable depending on the virus species and the host cell type, and the full process from PS engagement to virion internalisation is not fully elucidated. Furthermore, in the case of HIV-1 the PSR TIM-1 was found to enhance virus entry but inhibit its release, producing a net decrease in viral production from the host cell line. The addition of PS or PSR blockers may therefore have the undesirable effect of increasing virus titres. This suggests that the targeting of PS in viral infections should be evaluated on a case-by-case basis with regard to both the virus species and the targeting agent used.

Targeting phosphatidylserine in cancer indications has shown clinical success, also with the use of bavituximab which is currently in Phase III trials for non-small cell lung cancer. The specific localisation of anti-cancer antibodies to tumour blood vessels [147] is promising evidence of tumour specificity and may reduce angiogenesis, a main driver of tumour growth. However, comparison of cancer cell lines *in vitro* showed a wide variation in the extent of phosphatidylserine exposure; the efficacy of PS-targeting agents as anti-cancer drugs may therefore vary significantly depending on cancer type. A disadvantage of using antibodies is their limited tissue penetration [37], as confirmed with the imaging studies using bavituximab [147]; it may also be that PS is not expressed uniformly across the heterogeneous cell types present in the tumour and therefore cell killing will be incomplete. These limitations could be overcome by engineering bispecific antibodies, or bispecific antibody fragments, to simultaneously target PS plus a tumour type-specific antigen.

In summary, the data presented in this thesis demonstrate the potential utility of IdeS in a receptor refocusing approach to enhance the efficacy of therapeutic antibodies. Complementary biophysical approaches are needed to fully identify the binding interface with IgG, but there is strong evidence for the presence of a secondary binding site in addition to the hinge region. As demonstrated by the success of bavituximab to date, PS may be a valuable ligand for both anti-cancer and anti-viral antibodies; further elucidation of its role in viral life

cycles and clinical anti-cancer efficacy is required to assist the design of optimal PS-targeting agents.

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Appendix A

Expression vectors

A.1 DNA sequences

pHLSec

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 CAAAGTGACGCAGTTGCCGGCCGGGTCGCGCAGGGCGAACTCCCGCCCCCACGGCT

GCTCGCCGATCTCGGTCATGGCCGGCCCCGGAGGCGTCCCGGAAGTTCGTGGACACGA
CCTCCGACCACTCGGCGTACAGCTCGTCCAGGCCGCGCACCCACACCCAGGCCAGGG
TGTTGTCCGGCACCACCTGGTCCTGGACCGCGCTGATGAACAGGGTCACGTCGTCCCG
GACCACACCGGCGAAGTCGTCTCCACGAAGTCCCGGGAGAACCCGAGCCGGTTCGG
TCCAGAACTCGACCGCTCCGGCGACGTCGCGCGCGGTGAGCACCGGAACGGCACTG
GTCAACTTGGCCATGATGGCTCCTCTGTCAAGGAGAGGAAAGAGAAGAAGGTTAGTA
CAATTGCTATAGTGAGTTGTATTATACTATGCAGATATACTATGCCAATGATTAATTGT
CAAAGTAGGGCTGCAGGGTTCATAGTGCCACTTTTCTGCACTGCCCCATCTCCTGCC
CACCCTTTCCCAGGCATAGACAGTCAGTGAAGTACCAAAGTACAGGAGGGGAGAAGG
CAGAAGCTTGAGACAGACCCGCGGGACCGCCGAAGTGCAGGGGGACGTGGCTAGGG
CGGCTTCTTTTATGGTGCGCCGGCCCTCGGAGGCAGGGCGCTCGGGGAGGCCTAGCG
GCCAATCTGCGGTGGCAGGAGGCGGGGCCGAAGGCCGTGCCTGACCAATCCGGAGC
ACATAGGAGTCTCAGCCCCCGCCCCAAAGCAAGGGGAAGTCACGCGCCTGTAGCG
CCAGCGTGTTGTGAAATGGGGGCTTGGGGGGGTGGGGGCCCTGACTAGTCAAAACAA
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TCCACGCCCATTGATGTACTGCCAAAACCGCATCATCATGGTAATAGCGATGACTAA
TACGTAGATGTACTGCCAAGTAGGAAAGTCCCATAAGGTCATGTACTGGGCATAATG
CCAGGCGGGCCATTTACCGTCATTGACGTCAATAGGGGGCGTACTTGGCATATGATA
CACTTGATGTACTGCCAAGTGGGCAGTTTACCGTAAATACTCCACCCATTGACGTCAA
TGGAAGTCCCTATTGGCGTTACTATGGGAACATACGTCATTATTGACGTCAATGGGC
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CAGGTAAATTAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAA
AGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCTGACGAGCATCACAAAA
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TGTTTTTTTGTGTGAATCGTAACATAACGCTCTCCATCAAAACAAACGAAACAA
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TATCGAA

A.2 Vector maps

Please see the following pages.

Created with SnapGene®

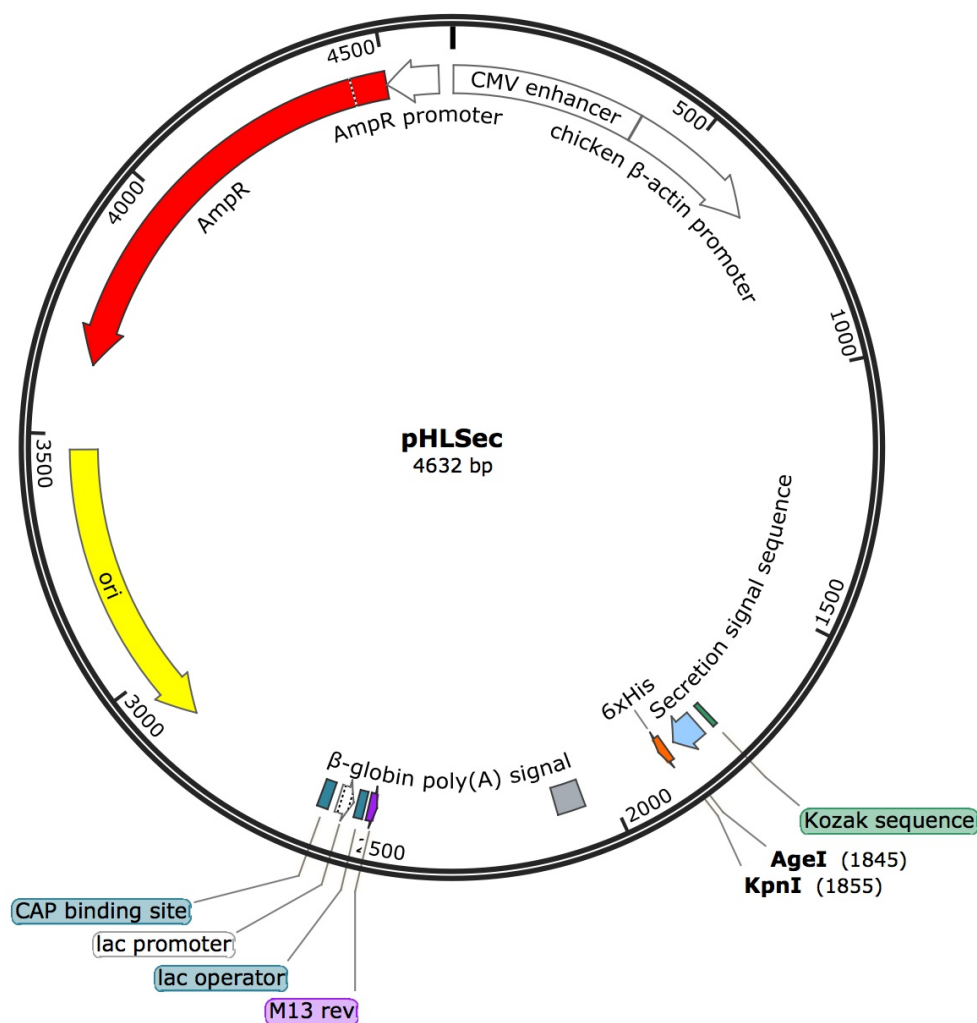


Figure A.1: pHLSec vector map. AgeI and KpnI are unique restriction sites in the multiple cloning region.

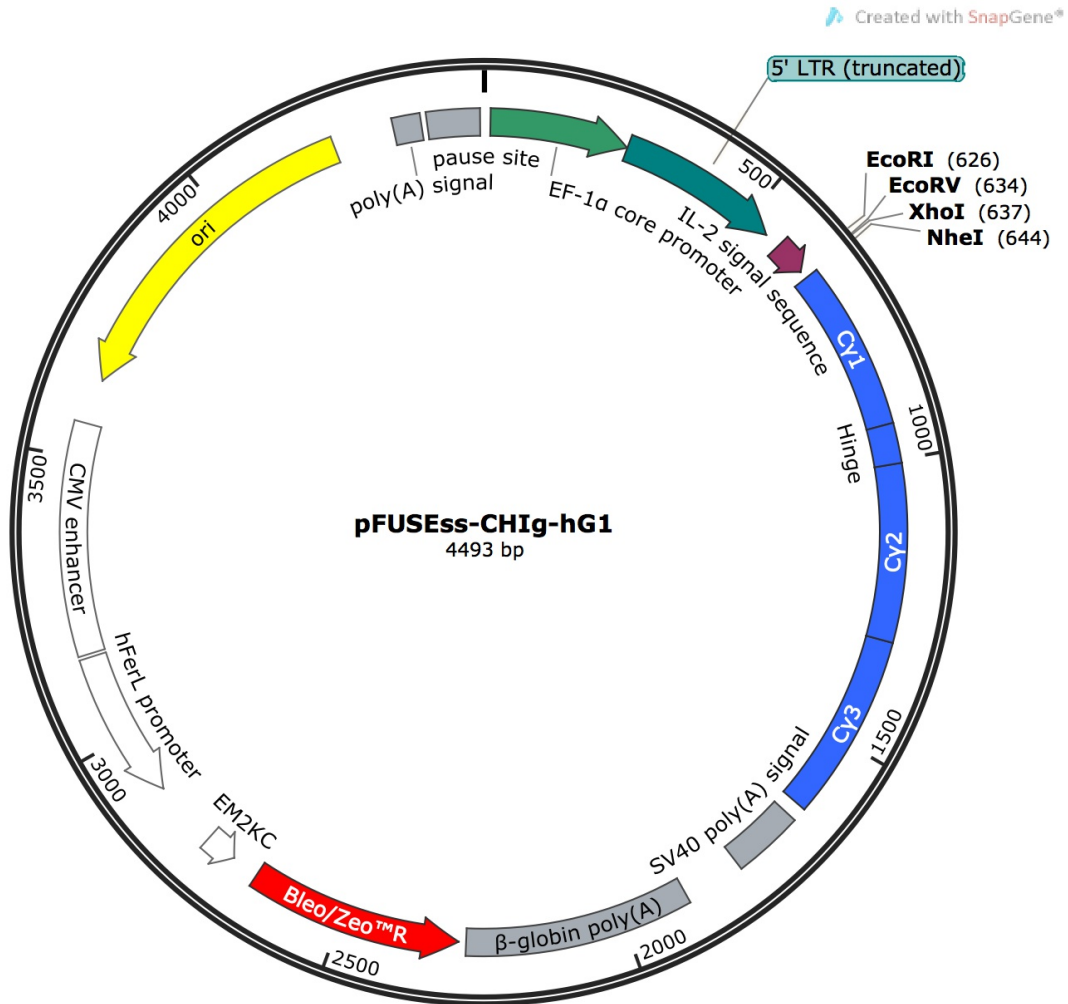


Figure A.2: pFUSEss-CHIg-hG1 vector map. EcoRI, EcoRV, XhoI and NheI are unique restriction sites in the multiple cloning region.

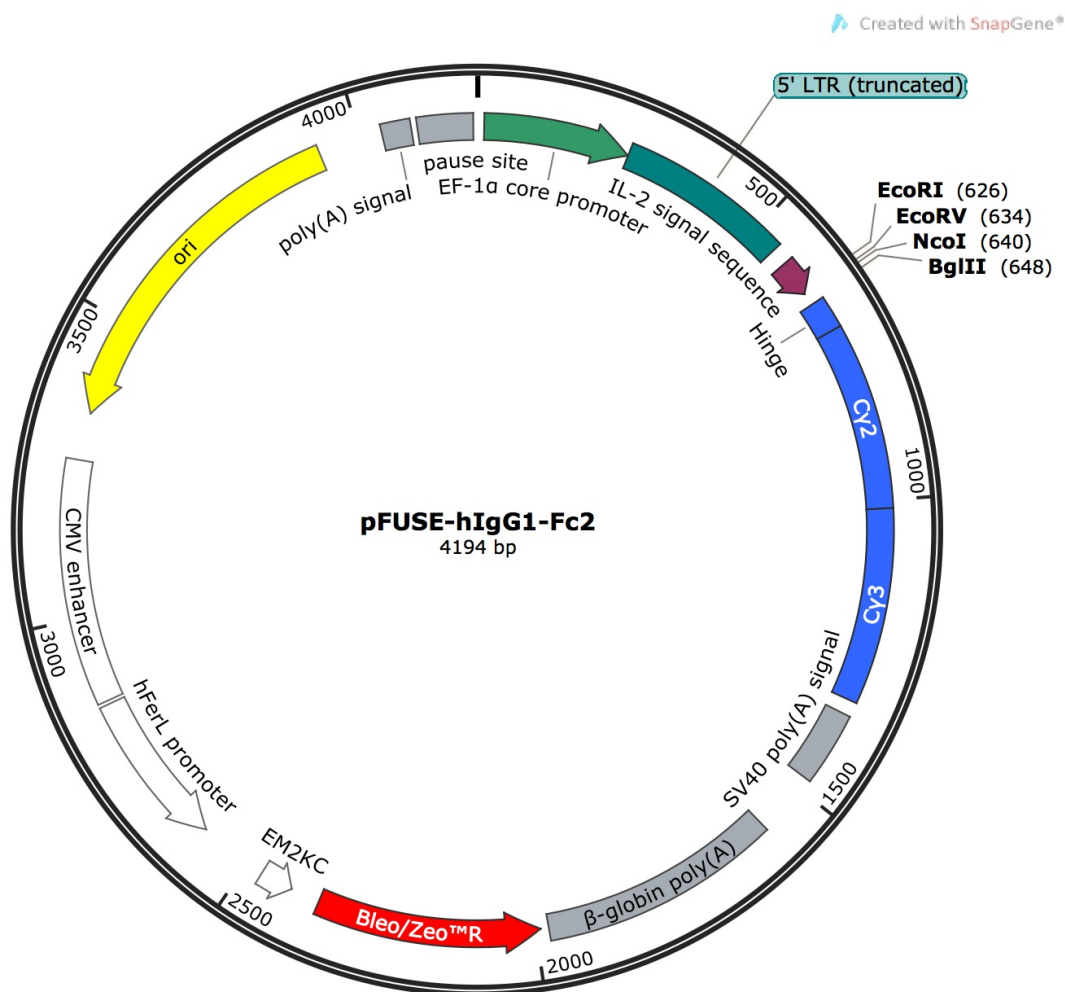


Figure A.3: pFUSE-hIgG1-Fc2 vector map. EcoRI, EcoRV, XhoI and BglII are unique restriction sites in the multiple cloning region.

Appendix B

Sequences of recombinantly expressed proteins used in this thesis

B.1 Bavituximab

Mouse variable regions are given in blue.

Protein

Heavy chain

EVQLQQSGPELEKPGASVKLSCKASGYSFTGYNMNWVKQSHGKSLEWIGHIDPYYGDTSY
NQKFRGKATLTVDKSSSTAYMQLKSLTSEDSAVYYCVKGGYYGHWYFDVWGAGTTVTVS
SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSG
LYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSV
FLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYR
VVSVELTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQ
VSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVF
SCSVMHEALHNHYTQKSLSLSPGK

Light chain

DIQMTQSPSSLSASLGERVSLTCRASQDIGSSLNWLQQGPDGTIKRLIYATSSLD SGVPKRFSG

SRSGSDYSLTISSLESEDFVDYYCLQYVSSPPTFGAGTKLELKRADAAPSVFIFPPSDEQLKSG
TASVVCLNNFYYPREAKVQWKVDNALQSGNSQESVTEQDSKDYSLSTLTLSKADYEKH
KVYACEVTHQGLSSPVTKSFNRGEC

DNA

The DNA sequences given here are those produced by GeneArt™ (Invitrogen) following back translation and codon optimisation for human expression.

Heavy chain

GAAGTGCAGCTGCAGCAGTCTGGCCCCGAGCTGGAAAAACCTGGCGCCTCCGTGAA
GCTGAGCTGCAAGGCCAGCGGCTACAGCTTCACCGGCTACAACATGAACTGGGTCAA
GCAGAGCCACGGCAAGAGCCTGGAATGGATCGGCCACATCGACCCCTACTACGGCG
ACACCAGCTACAACCAGAAGTTCGGGGCAAGGCCACCCTGACCGTGGACAAGTCT
AGCAGCACCGCCTACATGCAGCTGAAGTCCCTGACCAGCGAGGACAGCGCCGTGTA
CTACTGTGTGAAGGGCGGCTACTATGGCCACTGGTACTTCGACGTGTGGGGAGCCGG
CACCACCGTGACAGTGTCTAGCGCCTCTACCAAGGGCCCCAGCGTGTTCCCTCTGGCC
CCTAGCAGCAAGAGCACATCTGGCGGAACAGCCGCCCTGGGCTGCCTCGTGAAGGA
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CTGTGCCCAGCAGCAGCCTGGGCACCCAGACCTACATCTGCAACGTGAACCACAAGC
CCAGCAACACCAAGGTGGACAAGAAGGTGGAACCCAAGAGCTGCGACAAGACCCA
CACCTGTCCCCCTTGTCCTGCCCCTGAACTGCTGGGCGGACCTTCCGTGTTCCCTGTTCC
CCCCAAAGCCCAAGGATACCCTGATGATCAGCCGGACCCCCGAAGTGACCTGCGTGG
TGGTGGATGTGTCCACGAGGACCCTGAAGTGAAGTTCAATTGGTACGTGGACGGCG
TGGAAGTGCACAACGCCAAGACCAAGCCCAGAGAGGAACAGTACAACAGCACCTAC
CGGGTGGTGTCCGTGCTGACAGTGCTGCACCAGGACTGGCTGAACGGCAAAGAGTAC
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GGCCAAGGGCCAGCCCCGCGAACCCCAGGTGTACACACTGCCTCCCAGCAGGGACG
AGCTGACCAAGAACCAGGTGTCCCTGACATGCCTCGTGAAAGGCTTCTACCCCTCCG
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CCCCCTGTGCTGGACAGCGACGGCTCATTCTTCCTGTACAGCAAGCTGACTGTGGATA
AGAGCCGGTGGCAGCAGGGCAACGTGTTTCAGCTGCTCCGTGATGCACGAGGCCCTGC
ACAACCACTACACCCAGAAAAGCCTGTCCCTGAGCCCCGGCAAG

Light chain

GACATCCAGATGACCCAGAGCCCCAGCAGCCTGTCTGCCTCTCTGGGCGAGAGAGTG

TCCCTGACCTGTAGAGCCAGCCAGGACATCGGCAGCTCCCTGAACTGGCTGCAGCAG
GGCCCAGACGGCACCATCAAGAGACTGATCTACGCCACCTCCAGCCTGGACAGCGG
CGTGCCCAAGAGATTCAGCGGCAGCAGAAGCGGCAGCGACTACAGCCTGACCATCT
CTAGCCTGGAAAGCGAGGACTTCGTGGACTACTACTGCCTGCAGTACGTGTCCAGCC
CCCCACATTTGGAGCCGGCACCAAGCTGGAAGTGAAGAGAGCCGACGCCGCTCCC
AGCGTGTTTCATCTTCCCACCTAGCGACGAGCAGCTGAAGTCCGGCACAGCCTCTGTCTG
TGTGCCTGCTGAACAACCTTCTACCCCCGCGAGGCCAAGGTGCAGTGAAGGTGGACA
ATGCCCTGCAGAGCGGCAACAGCCAGGAAAGCGTGACCGAGCAGGACAGCAAGGA
CTCCACCTACTCCCTGAGCAGCACCTGACCCTGAGCAAGGCCGACTACGAGAAGCA
CAAGGTGTACGCCTGCGAAGTGACCCACCAGGGCCTGTCTAGCCCCGTGACCAAGAG
CTTCAACCGGGGCGAGTGT

B.2 TIM-4

Protein

ETVVTEVLGHRVTLPCLYSSWSHNSNSMCWGKDQCPYSGCKEALIRTDGMRVTSRKSAY
RLQGTIPRGDVSLTILNPSESDSGVYCCRIEVPGWFNVDVKINVRLNLQRASTTTHTRTATTTTR
RTTTTS

DNA

GAGACTGTTGTGACGGAGGTTTTGGGTCACCGGGTGACTTTGCCCTGTCTGTACTCAT
CCTGGTCTCACAACAGCAACAGCATGTGCTGGGGGAAAGACCAGTGCCCCTACTCCG
GTTGCAAGGAGGCGCTCATCCGCACTGATGGAATGAGGGTGACCTCAAGAAAGTCA
GCAAAATATAGACTTCAGGGGACTATCCCGAGAGGTGATGTCTCCTTGACCATCTTA
AACCCAGTGAAAGTGACAGCGGTGTGTACTGCTGCCGCATAGAAGTGCCTGGCTGG
TTCAACGATGTAAAGATAAACGTGCGCCTGAATCTACAGAGAGCCTCAACAACCACG
CACAGAACAGCAACCACCACCACACGCAGAACAAACAACAAGC

B.3 TIM4(EC)

Protein

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RTTTTSPTTTTRQMTTTPAALPTTVVTTPDLTTGTPLQMTTIAVFTTANTCLSLTPSTLPEEAT
GLLTPEPSKEGPILTAESETVLPDSWSSAESTSADTVLLTSKESKVWDL PSTSHVSMWKTS
D
SVSSPQPG

DNA

GAGACTGTTGTGACGGAGGTTTTGGGTCACCGGGTGACTTTGCCCTGTCTGTACTCAT
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GCAAAATATAGACTTCAGGGGACTATCCCGAGAGGTGATGTCTCCTTGACCATCTTA
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TTCAACGATGTAAAGATAAACGTGCGCCTGAATCTACAGAGAGCCTCAACAACACG
CACAGAACAGCAACCACCACCACACGCAGAACAAACAACAAGCCCCACCACCA
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CCCAGTGATTCTGGAGTAGTGCTGAGTCTACTTCTGCTGACACTGTCCTGCTGACATC
CAAAGAGTCCAAAGTTTGGGATCTCCCATCAACATCCACGTGTCAATGTGGAAAAC
GAGTGATTCTGTGTCTTCTCCTCAGCCTGGAGGTACC

B.4 TIM4(cryst)–Fc

The TIM-4 sequence is given in green.

Protein

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RTTTTSHTCPPCPAPPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYV
DGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKA
KGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLD
DGSFFLYSKLTVDKSRWQQGNVFCSTMHEALHNHYTQKSLSLSPG

DNA

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B.5 TIM4(EC)–Fc

The TIM-4 sequence is given in green.

Protein

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 SVSSPQPGHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNW
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DNA

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B.6 hTIM4XII-hIgG1II

The TIM-4 sequence is given in green.

Protein

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 VEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAK
 GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLD
 SDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG

DNA

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 GCCTCCCGTGCTGGACTCCGACGGCTCCTTCTCCTCTACAGCAAGCTCACCGTGGAC
 AAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCGTGATGCATGAGGCTCTG
 CACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCGGGT

B.7 hIgG1II–hTIM4XII

The TIM-4 sequence is given in green.

Protein

DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDG
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 QPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDG
 SFFLYSKLTVDKSRWQQGNVFSVSMHEALHNHYTQKSLSLSPGKSETVVTEVLGHRVTLP
 CLYSSWSHNSNSMCWGKDQCPYSGCKEALIRTDGMRVTSRKS AKYRLQGTIPRGDVSLTIL
 NPSESDSGVYCCRIEVPWFNDVKINVRNLQRASTTHRTATTTTRRTTTTSPTTTRQMTT
 TPAALPTTVVTTPLDITGTPQMTHIAVFTTANTCLSLTPSTLPEEATGLLTPEPSKEGPILTA
 ESETVLPDSWSSVESTSADTVLLTSKESKVWDLPTSHVSMWKTSDSVSSPQPGASDTAVPE
 QNKTTKTGQMDGIPMSMKNEMPISQ

DNA

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 AACGAGTGATTCTGTGTCTTCTCCTCAGCCTGGAGCATCTGATACAGCAGTTCCTGAG
 CAGAACAAAACAACAAAACAGGACAGATGGATGGAATACCCATGTCAATGAAGA
 ATGAAATGCCCATCTCCCAA

B.8 Fc γ RIIIA

Protein

GMRTEDLPKAVVFLEPQWYRVLEKDSVTLKCQGAYSPEDNSTQWFHNESLISSQASSYFID
 AATVDDSGEYRCQTNLSTLSDPVQLEVHIGWLLLQAPRWVFKEEDPIHLRCHSWKNTALH
 KVTYLQNGKGRKYFHHNSDFYIPKATLKDSGSYFCRGLFGSKNVSETVNITITQGLAVSTIS
 SFFPPGYQ

DNA

GGCATGCGGACTGAAGATCTCCCAAAGGCTGTGGTGTTCCTGGAGCCTCAATGGTAC
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 GACAATTCCACACAGTGGTTTCACAATGAGAGCCTCATCTCAAGCCAGGCCTCGAGC
 TACTTCATTGACGCTGCCACAGTTGACGACAGTGGAGAGTACAGGTGCCAGACAAAC
 CTCTCCACCCTCAGTGACCCGGTGCAGCTAGAAGTCCATATCGGCTGGCTGTTGCTCC
 AGGCCCCCTCGGTGGGTGTTCAAGGAGGAAGACCCTATTCACCTGAGGTGTCACAGCT
 GGAAGAACACTGCTCTGCATAAGGTCACATATTTACAGAATGGCAAAGGCAGGAAG
 TATTTTCATCATAATTCTGACTTCTACATTCCAAAAGCCCACTCAAAGACAGCGGCT
 CCTACTTCTGCAGGGGGCTTGTGTTGGGAGTAAAAATGTGTCTTCAGAGACTGTGAACAT
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 AA

B.9 IdeS, M1 serotype, wild type**Protein**

MIRYSEVTPYHVTSVWTKGVTPPANFTQGEDVFHAPYVANQGWYDITKTFNGKDDLCCG
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 EYFKEKAFPYLSTKHLGVFPDHVIDMFINGYRLSLTNHGPTPVKEGSKDPRGGIFDAVFTRG
 DQSKLLTSRHDFKEKNLKEISDLIKKELTEGKALGLSHTYANVRINHVINLWGADFDNSNGN
 LKAIVTDSDSNASIGMKKYFVGVSAGKVAISAKEIKEDNIGAQVLGLFTLSTGQDSWNQ
 TNLE

DNA

ATGATCCGTTATAGCGAAGTTACCCCGTATCATGTTACCAGCGTTTGGACCAAAGGTG
 TTACCCCTCCGGCAAATTTTACCCAGGGTGAAGATGTTTTTCATGCACCGTATGTTGC
 AAATCAGGGCTGGTATGATATCACCAAAACCTTTAATGGCAAAGATGATCTGCTGTG
 CGGTGCAGCAACCGCAGGTAATATGCTGCATTGGTGGTTTGATCAGAATAAAGATCA
 GATCAAACGCTATCTGGAAGAACATCCGGAAAAACAGAAAATCAATTTTAACGGCG

AGCAGATGTTTCGATGTGAAAGAAGCAATTGACACCAAAAACCATCAGCTGGATAGC
AAACTGTTTCGAGTACTTTAAAGAAAAAGCCTTCCCGTATCTGAGCACCAAACATCTG
GGTGTGTTTCCGGATCATGTTATCGATATGTTTATTAACGGTTATCGTCTGAGCCTGAC
CAATCATGGTCCGACACCGGTTAAAGAAGGTAGCAAAGATCCGCGTGGTGGTATTTT
TGATGCAGTTTTTACCCGTGGTGATCAGAGCAAACCTGCTGACCAGCCGTCATGATTTT
AAAGAGAAAAACCTGAAAGAGATCAGCGACCTGATCAAAAAAGAACTGACCGAAG
GTAAAGCACTGGGTCTGAGCCATACCTATGCAAATGTTTCGTATTAACCATGTGATTAA
TCTGTGGGGTGCAGATTTTGATAGCAATGGTAATCTGAAAGCCATCTATGTTACCGAT
AGCGATAGTAATGCAAGCATCGGGATGAAAAAATACTTTGTGGGTGTTAATAGCGCA
GGCAAAGTTGCAATTAGCGCGAAAGAAATCAAAGAGGATAATATTGGCGCACAGGT
TCTGGGTCTGTTTACCCTGAGCACCGGTCAGGATAGCTGGAATCAGACCAATCTCGAG

Appendix C

Calculation of Fc glycan masses

Retention times, peak areas, and percentage peak areas were calculated by Empower Pro software (Waters). Unassigned peaks were removed from the calculation; total peak area for each Fc construct therefore does not amount to 100% in every case. Mass values for individual glycan structures were calculated using <http://web.expasy.org/glycanmass/>, using underivatised monosaccharide residues and average mass values (Table C.1).

Table C.1: Masses of individual glycan structures as calculated by ExPASy.

Glycan	Mass (Da)
A2	1317
A2F	1463
A2BF	1667
A2G1F 6-arm	1626
A2G1F 3-arm	1625
A2BG1F	1829
A2G2	1642
A2G2F	1788
A2G2BF	1991
A2G2BFS1	2282

Table C.2: Glycan mass calculations for IgG1 Fc constructs used for analysis of IdeS binding.

Construct	Retention time (mins)	Area (% total)	Assignment	Mass (Da)	Mass x Area%
Fc WT	12.7	2.2	A2	1317	2858
	14.4	36.1	A2F	1463	52886
	15.8	4.9	A2BF	1667	8149
	17.8	25.5	A2G1F 6-arm	1626	41385
	18.3	10.0	A2G1F 3-arm	1625	16197
	18.7	2.7	A2BG1F	1829	4883
	19.6	2.9	A2G2	1642	4810
	22.0	12.7	A2G2F	1788	22721
	23.9	1.3	A2G2BFS1	2282	2853
	Sum total	98.2	N/A	N/A	156742
Weighted average:					1596
Fc S239-K447	12.5	1.3	A2	1317	1739
	14.2	22.6	A2F	1463	33072
	15.5	4.3	A2BF	1667	7233
	17.5	12.1	A2G1F 6-arm	1626	19620
	18.1	4.8	A2G1F 3-arm	1625	7814
	18.5	8.1	A2BG1F	1829	14849
	19.9	1.1	A2G2	1642	1756
	21.7	27.8	A2G2F	1788	49607
	22.2	7.0	A2G2BF	1991	13996
	24.8	3.4	A2G2BFS1	2282	7805
	Sum total	92.5	N/A	N/A	157491
Weighted average:					1702
T225A	12.2	3.7	A2	1317	4808
	13.9	43.2	A2F	1463	63217
	15.4	3.2	A2BF	1667	5250
	17.2	21.5	A2G1F 6-arm	1626	34916
	17.8	9.1	A2G1F 3-arm	1625	14751
	21.4	6.4	A2G2F	1787	11435
	Sum total	87.0	N/A	N/A	134377
Weighted average:					1545

Table C.2 continued: Calculated glycan masses

Construct	Retention time (mins)	Area (% total)	Assignment	Mass (Da)	Mass x Area%
C226A	12.2	3.6	A2	1317	4782
	13.9	46.2	A2F	1463	67593
	15.4	4.3	A2BF	1667	7100
	17.2	21.1	A2G1F 6-arm	1626	34314
	17.8	8.0	A2G1F 3-arm	1625	13062
	21.4	6.0	A2G2F	1787	10684
	Sum total	89.2	N/A	N/A	137534
Weighted average:					1542
P228A	12.2	4.4	A2	1317	5783
	13.9	41.9	A2F	1463	61300
	15.4	4.2	A2BF	1667	7000
	17.2	19.2	A2G1F 6-arm	1626	31128
	17.8	7.5	A2G1F 3-arm	1625	12217
	21.4	7.3	A2G2F	1787	13025
	Sum total	84.4	N/A	N/A	130453
Weighted average:					1545
C229A	12.2	3.9	A2	1317	5124
	13.9	41.0	A2F	1463	60042
	15.4	4.1	A2BF	1667	6883
	17.2	20.5	A2G1F 6-arm	1626	33372
	17.8	7.9	A2G1F 3-arm	1625	12753
	21.4	8.0	A2G2F	1787	14257
	Sum total	85.4	N/A	N/A	132431
Weighted average:					1551
P230A	12.2	4.3	A2	1317	5677
	14.0	47.1	A2F	1463	68851
	15.4	3.3	A2BF	1667	5500
	17.3	19.3	A2G1F 6-arm	1626	31356
	17.8	8.1	A2G1F 3-arm	1625	13078
	21.5	5.0	A2G2F	1787	8880

Table C.2 continued: Calculated glycan masses

Construct	Retention time (mins)	Area (% total)	Assignment	Mass (Da)	Mass x Area%
	Sum total	87.0	N/A	N/A	133342
				Weighted average:	1533
A231T	12.7	1.4	A2	1317	1844
	14.4	50.5	A2F	1463	73958
	15.8	6.8	A2BF	1667	11399
	17.8	20.7	A2G1F 6-arm	1626	33681
	18.3	9.2	A2G1F 3-arm	1625	14898
	18.7	3.0	A2BG1F	1829	5431
	22.0	7.1	A2G2F	1788	12728
	25.2	1.2	A2G2BFS1	2282	2807
	Sum total	100.0	N/A	N/A	156746
				Weighted average:	1568
P232A	12.2	5.1	A2	1317	6678
	13.9	42.9	A2F	1463	62778
	15.4	3.4	A2BF	1667	5650
	17.3	18.9	A2G1F 6-arm	1626	30706
	17.8	7.5	A2G1F 3-arm	1625	12249
	21.4	7.4	A2G2F	1787	13275
	Sum total	85.2	N/A	N/A	131336
				Weighted average:	1541
E233A	12.7	1.0	A2	1317	1357
	14.4	37.5	A2F	1463	54862
	15.8	4.7	A2BF	1667	7900
	17.8	26.2	A2G1F 6-arm	1626	42637
	18.3	11.4	A2G1F 3-arm	1625	18488
	18.6	5.2	A2BG1F	1829	9564
	22.0	13.9	A2G2F	1788	24848
	Sum total	100.0	N/A	N/A	159655
				Weighted average:	1597
L234A	12.7	1.0	A2	1317	1344

Table C.2 continued: Calculated glycan masses

Construct	Retention time (mins)	Area (% total)	Assignment	Mass (Da)	Mass x Area%
	14.5	32.8	A2F	1463	47998
	15.8	4.5	A2BF	1667	7500
	17.8	27.4	A2G1F 6-arm	1626	44474
	18.4	10.3	A2G1F 3-arm	1625	16782
	18.7	4.0	A2BG1F	1829	7223
	22.1	18.2	A2G2F	1788	32499
	25.3	1.9	A2G2BFS1	2282	4245
	Sum total	100.0	N/A	N/A	162065
	Weighted average:				1621
L235A	12.8	4.1	A2	1317	5414
	14.5	43.8	A2F	1463	64110
	15.9	6.9	A2BF	1667	11533
	17.9	21.5	A2G1F 6-arm	1626	34916
	18.4	6.6	A2G1F 3-arm	1625	10657
	18.7	2.2	A2BG1F	1829	4005
	19.7	4.6	A2G2	1642	7584
	22.1	6.3	A2G2F	1788	11316
	Sum total	96.0	N/A	N/A	149534
	Weighted average:				1557
G236A	12.9	3.4	A2	1317	4479
	14.6	38.2	A2F	1463	55901
	16.0	5.7	A2BF	1667	9533
	17.9	25.2	A2G1F 6-arm	1626	41012
	18.5	8.2	A2G1F 3-arm	1625	13289
	18.7	2.7	A2BG1F	1829	4992
	19.8	3.4	A2G2	1642	5598
	22.2	11.1	A2G2F	1788	19754
	Sum total	97.9	N/A	N/A	154556
	Weighted average:				1578
G237A	12.9	5.2	A2	1317	6889
	14.6	46.0	A2F	1463	67315

Table C.2 continued: Calculated glycan masses

Construct	Retention time (mins)	Area (% total)	Assignment	Mass (Da)	Mass x Area%
	15.9	6.7	A2BF	1667	11083
	17.9	20.2	A2G1F 6-arm	1626	32835
	18.5	7.7	A2G1F 3-arm	1625	12542
	18.8	2.0	A2BG1F	1829	3694
	19.8	3.6	A2G2	1642	5877
	22.2	6.3	A2G2F	1788	11298
	Sum total	97.7	N/A	N/A	151532
	Weighted average:				1551
P238A	12.9	0.9	A2	1317	1186
	14.6	26.3	A2F	1463	38545
	16.0	6.6	A2BF	1667	10966
	18.0	18.1	A2G1F 6-arm	1626	29373
	18.6	6.9	A2G1F 3-arm	1625	11193
	18.9	9.9	A2BG1F	1829	18122
	22.3	17.8	A2G2F	1788	31838
	22.6	2.1	A2G2BF	1991	4141
	25.5	3.1	A2G2BFS1	2282	6983
	Sum total	91.6	N/A	N/A	152348
	Weighted average:				1662
S267A	12.6	1.6	A2	1317	2121
	14.3	35.1	A2F	1463	51393
	15.7	5.2	A2BF	1667	8583
	17.7	20.6	A2G1F 6-arm	1626	33404
	18.3	6.7	A2G1F 3-arm	1625	10869
	18.6	6.4	A2BG1F	1829	11722
	21.9	17.7	A2G2F	1788	31606
	25.1	2.0	A2G2BFS1	2282	4587
	Sum total	95.2	N/A	N/A	154284
	Weighted average:				1620
H268A	12.6	2.0	A2	1317	2608
	14.3	32.8	A2F	1463	47984

Table C.2 continued: Calculated glycan masses

Construct	Retention time (mins)	Area (% total)	Assignment	Mass (Da)	Mass x Area%
	15.6	5.4	A2BF	1667	9066
	17.7	20.8	A2G1F 6-arm	1626	33794
	18.2	6.8	A2G1F 3-arm	1625	11080
	18.6	6.2	A2BG1F	1829	11393
	21.9	19.4	A2G2F	1788	34752
	22.2	2.2	A2G2F	1788	3986
	25.1	1.6	A2G2BFS1	2282	3629
	Sum total	97.3	N/A	N/A	158292
	Weighted average:				1627
	P271A	12.5	0.9	A2	1317
14.2		38.1	A2F	1463	55783
15.6		6.6	A2BF	1667	10916
17.6		22.0	A2G1F 6-arm	1626	35712
18.2		9.7	A2G1F 3-arm	1625	15726
18.5		5.5	A2BG1F	1829	9985
21.8		11.4	A2G2F	1788	20343
25.1		1.9	A2G2BFS1	2282	4313
Sum total		95.9	N/A	N/A	153952
Weighted average:				1605	
K274A	12.5	1.1	A2	1317	1475
	14.2	50.7	A2F	1463	74251
	15.5	5.5	A2BF	1667	9166
	17.6	20.5	A2G1F 6-arm	1626	33339
	18.1	9.1	A2G1F 3-arm	1625	14833
	18.5	2.7	A2BG1F	1829	4956
	21.8	6.0	A2G2F	1788	10708
	Sum total	95.7	N/A	N/A	148728
Weighted average:				1554	
N276A	12.5	0.7	A2	1317	909
	14.2	26.1	A2F	1463	38165
	15.6	5.4	A2BF	1667	9016

Table C.2 continued: Calculated glycan masses

Construct	Retention time (mins)	Area (% total)	Assignment	Mass (Da)	Mass x Area%
	17.6	20.8	A2G1F 6-arm	1626	33827
	18.1	7.4	A2G1F 3-arm	1625	12006
	18.5	8.6	A2BG1F	1829	15763
	21.8	18.1	A2G2F	1788	32285
	22.2	1.9	A2G2BF	1991	3763
	25.0	3.3	A2G2BFS1	2282	7440
	Sum total	92.2	N/A	N/A	153173
	Weighted average:				1661
T289A	14.3	20.8	A2F	1463	30453
	15.4	2.5	A2BF	1667	4166
	17.1	27.7	A2G1F 6-arm	1626	45010
	17.5	11.2	A2G1F 3-arm	1625	18163
	17.8	5.8	A2BG1F	1829	10661
	20.1	25.2	A2G2F	1788	45067
	20.5	1.5	A2G2BF	1991	2986
	Sum total	73.9	N/A	N/A	126054
	Weighted average:				1706
K290A	14.3	28.8	A2F	1463	42204
	15.5	2.4	A2BF	1667	4033
	17.1	29.1	A2G1F 6-arm	1626	47286
	17.5	9.6	A2G1F 3-arm	1625	15661
	17.8	4.0	A2BG1F	1829	7333
	20.1	18.3	A2G2F	1788	32750
	20.5	0.9	A2G2BF	1991	1871
	Sum total	64.4	N/A	N/A	108935
	Weighted average:				1691
P291A	14.3	32.5	A2F	1463	47501
	15.5	3.1	A2BF	1667	5183
	17.1	26.4	A2G1F 6-arm	1626	42865
	17.5	11.0	A2G1F 3-arm	1625	17822
	17.9	4.6	A2BG1F	1829	8412

Table C.2 continued: Calculated glycan masses

Construct	Retention time (mins)	Area (% total)	Assignment	Mass (Da)	Mass x Area%
	20.2	19.5	A2G2F	1788	34913
	Sum total	97.0	N/A	N/A	156695
	Weighted average:				1615
E293A	14.3	23.7	A2F	1463	34638
	15.4	8.6	A2BF	1667	14282
	17.1	14.1	A2G1F 6-arm	1626	22903
	17.5	11.5	A2G1F 3-arm	1625	18618
	17.8	10.3	A2BG1F	1829	18836
	20.2	18.2	A2G2F	1788	32553
	Sum total	86.3	N/A	N/A	141830
				Weighted average:	1644
E294A	14.3	42.9	A2F	1463	62764
	15.4	5.4	A2BF	1667	9016
	17.1	18.6	A2G1F 6-arm	1626	30202
	17.5	13.1	A2G1F 3-arm	1625	21234
	17.8	3.7	A2BG1F	1829	6785
	20.2	11.5	A2G2F	1788	20558
	Sum total	95.2	N/A	N/A	150558
				Weighted average:	1582
P329A	12.5	1.2	A2	1317	1567
	14.1	26.7	A2F	1463	39013
	15.5	2.1	A2BF	1667	3433
	17.4	30.4	A2G1F 6-arm	1626	49480
	18.0	12.1	A2G1F 3-arm	1625	19576
	18.4	3.1	A2BG1F	1829	5578
	21.7	19.4	A2G2F	1788	34627
	Sum total	94.8	N/A	N/A	153275
				Weighted average:	1616
P331A	12.4	1.0	A2	1317	1344
	14.1	28.4	A2F	1463	41589

Table C.2 continued: Calculated glycan masses

Construct	Retention time (mins)	Area (% total)	Assignment	Mass (Da)	Mass x Area%
	15.4	3.1	A2BF	1667	5200
	17.5	28.5	A2G1F 6-arm	1626	46246
	18.0	10.2	A2G1F 3-arm	1625	16636
	18.4	4.0	A2BG1F	1829	7260
	21.7	20.5	A2G2F	1788	36683
	Sum total	95.7	N/A	N/A	154956
	Weighted average:				1619
I332A	12.5	0.9	A2	1317	1212
	14.1	20.3	A2F	1463	29677
	15.5	2.1	A2BF	1667	3416
	17.5	29.6	A2G1F 6-arm	1626	48066
	18.0	10.4	A2G1F 3-arm	1625	16847
	18.4	5.3	A2BG1F	1829	9601
	21.7	25.7	A2G2F	1788	45960
	Sum total	94.2	N/A	N/A	154780
	Weighted average:				1644
E333A	12.5	0.8	A2	1317	1067
	14.1	22.9	A2F	1463	33570
	15.5	2.6	A2BF	1667	4300
	17.5	31.5	A2G1F 6-arm	1626	51252
	18.1	12.3	A2G1F 3-arm	1625	19999
	18.4	4.4	A2BG1F	1829	7973
	21.7	22.0	A2G2F	1788	39310
	Sum total	96.5	N/A	N/A	157471
	Weighted average:				1631