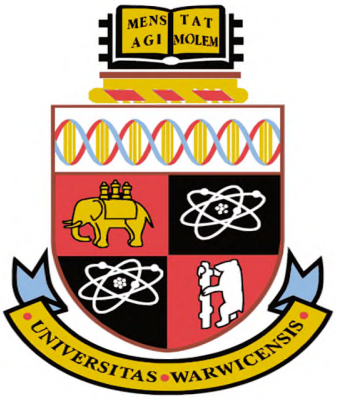
THE UNIVERSITY OF WARWICK



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Thesis: Biophysical characterisation and profile of HLA-specific antibodies in transplantation

Submitted to the University of Warwick

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God has a bigger plan for me than I have for myself!

Dedicated to

To my lovely wife Dr. Veena Daga and daughters Sneha, Hinal and Vibha and

To my respected parents

Mr. Omprakash Daga and Mrs. Godavari Daga.

Contents:

List of contents

List of Figures

List of Tables

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in Anaesthesia, she had always made time for me to do research and travel for conference and laboratory work in Oklahoma. My daughters, Sneha, Hinal and Vibha have been wonderful in this journey of project and I am enormously thankful for their sweet hugs and lovely smiles that were refreshing following a tiring day. I am thankful to my parents for inspiring me with their hard work and dedication in life.

Declaration

I declare that all the work presented in this thesis, except where specifically stated, and was original research performed by myself under the supervision of Dr. Daniel Mitchell, Professor Robert Higgins and Dr. Daniel Zehnder. None of this work has been previously submitted for any other degree. All sources have been acknowledged by means of references.

Dr. Sunil Daga

2nd January 2015

Abbreviations

AAV – ANCA associated vasculitis

ABOi – ABO incompatible

ACR – Acute cellular rejection

AHG – Anti-human globulin

AID – Activation-induced deaminase

AiT – Antibody incompatible transplantation

AMR – Antibody Mediated Rejection

ANA – Anti-nuclear antibody

ANCA – Anti-nuclear cytoplasmic antibody

APC – Antigen presenting cell

APRIL – A proliferation inducing ligand

ATG – Anti-thymocyte globulin

BAFF – B-cell activating factor

BCA – Bicinchoninic acid

BCR – B cell receptor

BSA – Bovine serum albumin

CD – Cluster of differentiation

CDC – Complement dependent cytotoxicity

CDRs – Complementarity determining regions

CHAPS - 3-[(3-cholamidopropyl) dimethylammonio-propane sulfonate

CKD – Chronic Kidney Disease

CREGs – Cross-reactive groups

cRF – Calculated reactive frequency

DEA – Diethyl amine

DFPP – Double-filtration plasmapheresis

DGF – Delayed graft function

DSA – Donor Specific Antibody

dsDNA – double stranded DNA

DTT – Dithiotheritol

EC_{50} – Effective concentration for 50% (of maximal response)

EDTA – Ethylene diaminetetra-acetic acid

eGFR – Estimated glomerular filtration rate

ELISA – Enzyme-linked immunosorbent assay

ESRD – End stage renal disease

Fab – Fragment for antibody binding

Fc – Fragment crystallisable

FFP – Fresh frozen plasma

FITC – Fluorecein isothiocyanate

F_R – Fractional binding

g – Glomerulitis

GBM – Glomerular basement membrane

HABA - 4'-hydroxyazobenzene-2-carboxylic acid

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HC- Heavy chain

HLA – Human Leukocyte Antigen

HLAi – HLA incompatible transplantation

HSP – Highly sensitized patients

Ig – Immunoglobulin

IgA – Immunoglobulin A

IgG – Immunoglobulin G

IgM – Immunoglobulin M

IgE – Immunoglobulin E

IgD – Immunoglobulin D

IHC – Immuno-histo-chemistry

IL2 – Interleukin-2

IL2R – Interleukin-2 receptor

IL-6 – Interleukin-6

LC – Light chain

LDS - Lithium dodecyl sulfate

MAb – Monoclonal antibody

MAC – Membrane attack complex

MES – 2-N morpholino ethanesulfonic acid

MFI – Mean fluorescence intensity

MHC – Major Histocompatibility Complex

Mm - Mismatch

MPO - Myeloperoxidase

MS – Mass spectroscopy

MWCO – Molecular weight cut-off

NDSA – Non-donor specific antibody

NHSBT – NHS Blood and Transplant

NHS-PEG - N-hydroxysuccinimide- polyethylene glycol

NLDKSS – National Living Donor Kidney Sharing Scheme

NSB – Non-specific binding

OD – Optical density

ODT – Organ Donor Transplant

PRA – Panel reactive antibody

PBS – Phosphate buffered saline

PE – Phycoerythrin

pI – isoelectric pH

PP – Plasmapheresis

PR3 – Proteinase-3

ptc – peri-tubular capiliaritis

RA – Renal association

RMF – Relative median fluorescence

Rpm – rotation per minute

Rmax – Maximal response

RU – response unit

SAB – Single antigen bead

SDS-PAGE – Sodium dodecyl sulfate – polyacrylamide gel eclectrophoresis

SEC – Size exclusion chromatography

SGF – Slow graft function

sHLA – Soluble HLA

SLE - Systemic lupus erythematosus

SPR – Surface Plasmon resonance

SPIRA – Solid phase radioimmunoassay

TCR – T-cell receptor

TG – Transplant Glomerulopathy

TLR – Toll like receptor

TMA – Thrombotic microangiopathy

UHB – University Hospital of Birmingham

UHCW – University Hospital of Coventry and Warwickshire

UNOS – United Network for Organ Sharing

USRDS – US renal data system

VH – Variable heavy-chain

VL – Variable light-chain

QOL – Quality of Life

Abstract

Following five decades of kidney transplantation, increasingly high risk immunological kidney transplantation (which previously was considered as sub-optimal) are carried out. The risk stratification with the current available assays have allowed safe transplantation in low risk non-sensitised patients and direct transplantation in high risk highly sensitised patients by removal of circulating donor specific antibodies (DSA) with reasonable outcomes. However, a large number of patients with chronic kidney disease and with low or intermediate antibody levels measured by current assay, the best way forward is uncertain resulting in denial of transplantation in some cases. Whilst in other cases, the solid phase Luminex assay may under or overestimate the risks of rejection and graft failure following direct kidney transplantation.

Currently only IgG-class of DSA is considered immunologically important and routinely measured in clinical laboratories. Other bio-physiological characteristics such as class, sub-class and binding kinetics of DSA may be more specific for risk stratification of immunological risks. In this thesis, we studied effect of de novo IgM class of HLA-specific antibodies on outcome of kidney transplantation and characterised binding kinetics and strength of HLA-specific antibodies.

De novo IgM or IgG HLA-specific responses alone were not associated with adverse outcomes following kidney transplantation. Presence of both IgM and IgG responses, however, was associated with poor graft function at 36 months. There was no temporal relationship of

antibody response and episodes of rejections. *De novo* Donor specific responses were less frequent compared to non-specific responses. A shorter follow-up and use of modern triple immunosuppressant therapy (Tacrolimus, Mycophenolate and Steroid) may explain this.

Binding kinetics measured by biosensor assay- surface plasmon resonance (SPR) on purified monoclonal HLA-specific antibodies showed binding kinetics and strength differed between HLA alleles despite same epitope and paratope interactions. There was a tendency towards higher affinity and faster association rate for HLA protein that was the initial immunizing antigen for the corresponding monoclonal HLA-specific antibodies. The dissociation constant (K_D) of human monoclonal HLA-specific antibodies range between 10^{-8} to 10^{-10} M. Thermodynamic analysis showed higher Gibbs free energy released for interactions with higher binding strength. The binding strength of mixed monoclonal HLA-specific antibodies is generally average of the strength of individual monoclonal HLA-specific antibodies.

Enriched polyclonal HLA-specific antibodies from clinical sample gave distinct binding response on bio-sensor based on SPR assay. Quantification of polyclonal HLA-specific antibodies using sandwich ELISA and SPR allowed quantitative measurement of binding kinetics and strengths. A range of binding strength was observed between patients and within same patient antibodies of different affinities was observed. Thus the antibodies could be grouped in four groups based on the strength of binding and this can serve as additional biomarker for risk stratifications.

Thus three important observations have been made during this thesis –

1) Presence of both IgM and IgG de novo HLA-specific responses was associated with poor graft outcomes
2) The difference in binding strength and binding energy may explain the difference of reactivity of antibodies despite same epitope and paratope interactions.
3) Polyclonal HLA-specific antibodies can be grouped in to four groups based on their binding strength and this can be used as an additional biomarker for risk stratification.

Further studies are required to establish clinical application of above observations.

1 Introduction

1.1 Introduction: Kidney transplantation – Success, challenges and strategies

Transplantation is the optimal treatment for end-organ failure. Apart from cost effectiveness, it has shown to improve QOL (Quality of Life) and life expectancy (Wolfe et al., 1999) (Figure 1-1). There have been significant improvements in reducing rates of acute rejection with the advent of new drugs and greater understanding of HLA matching procedures. The US Renal Data System (USRDS) data suggested acute rejection rate following kidney transplantation is now less than 10% (Collins et al., 2014) (Figure 1-2 (A)). However the improvement in the longer-term outcomes such as graft failure and return to dialysis has been slower comparatively (Figure 1-2 (B): USRDS 2013 data). UK data show similar slow progress in improving longer-term outcomes (Figure 1-3).

Age at WL	Annual % Dead WL	RR long term	Days to Equal Risk	Days to Equal Survival	Lifetime* WL (yrs)	Extra Lifetime* Tx (yrs)
All	6.3	0.32	106	244	10	+10
0-19	2.2	0.33	3	5	26	+13
20-39	4.3	0.24	11	57	14	+17
40-59	6.5	0.33	94	255	11	+11
60-74	10.0	0.39	143	369	6	+5

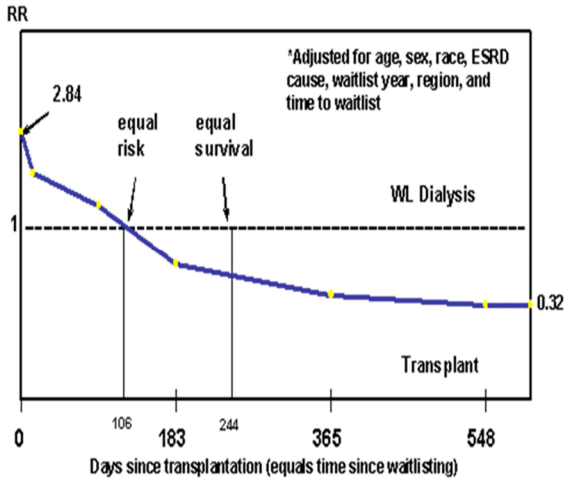


Figure 1-1: Adjusted relative risk of death following First Cadaveric Transplant. WL= waiting list, Tx= transplant

(Study group = 23,275 recipients & control group = 46,164 patient on dialysis on waiting list) Taken from ref (Wolfe et al., 1999)

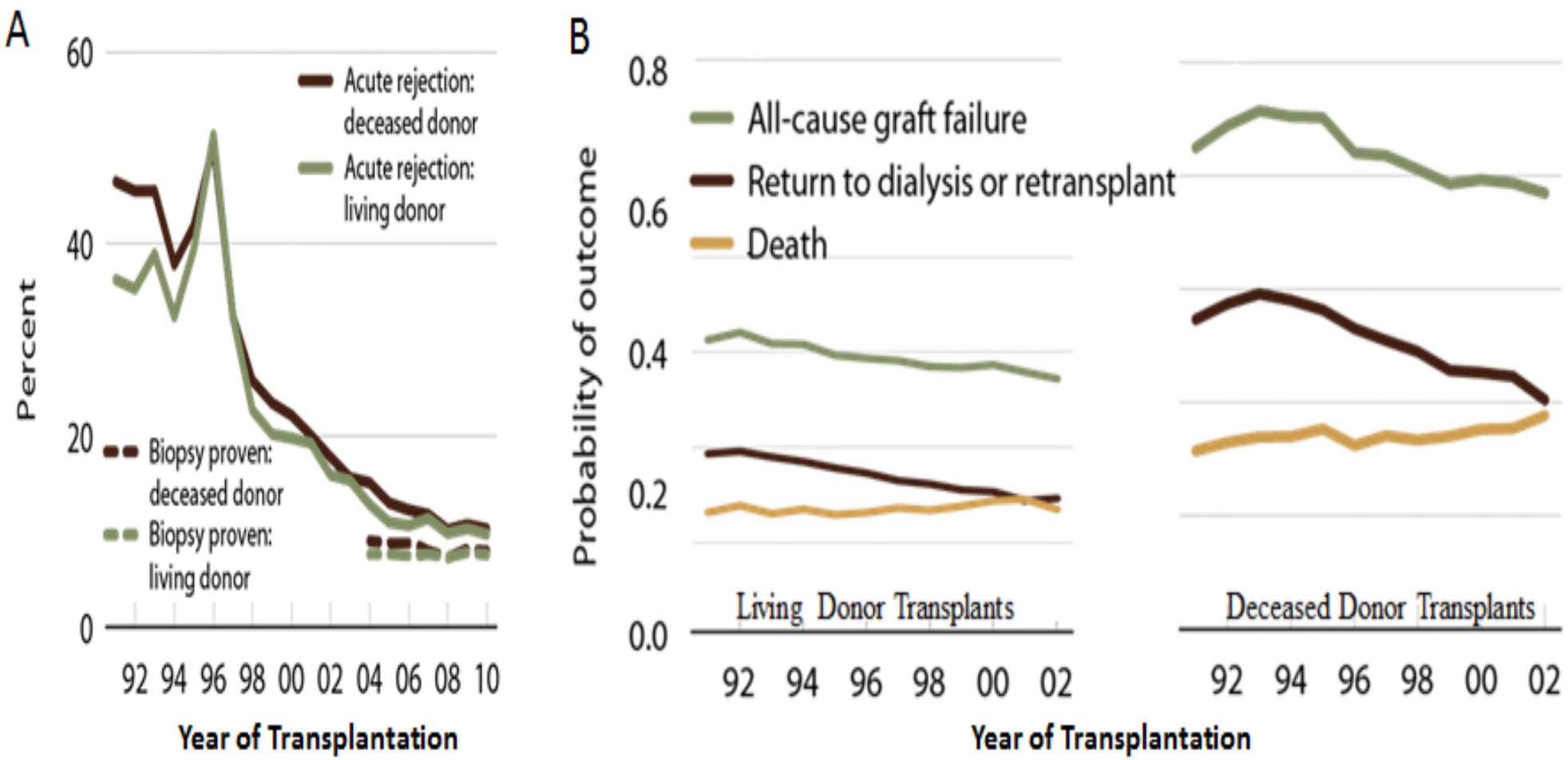


Figure 1-2: Outcome following first kidney transplantation

A – Acute rejection rate in the first year. B – Ten year outcomes following Living & Deceased donor Kidney Transplantation (Collins et al., 2014)

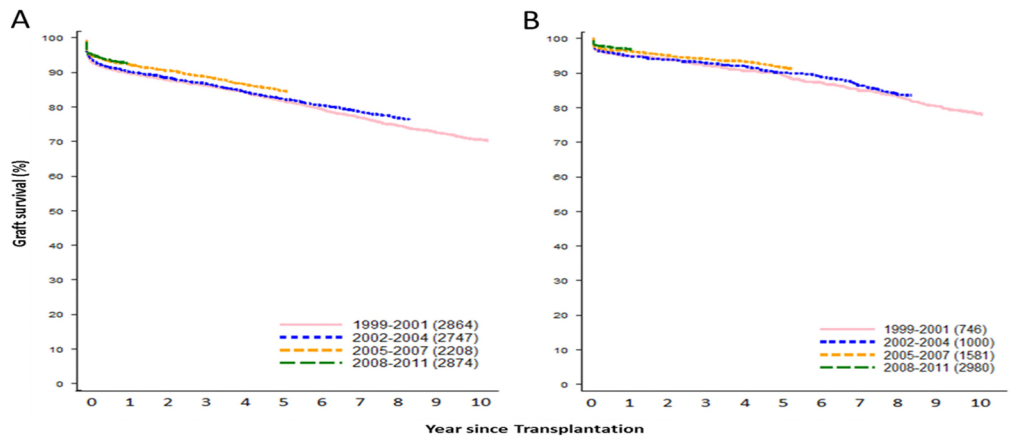


Figure 1-3 : Long-term Graft survival after first deceased [A] (DBD) and Living donor [B] kidney transplantation in UK (Modified from NHSBT (NHS Blood and Transplant) Transplant activity 2012-13)

A Longitudinal biopsy study has shown increasing chronic damage with time following kidney transplantation (Figure 1-4) (Nankivell et al., 2003). Graft failure could be due to many aetiologies either working alone or in combinations (Nankivell and Chapman, 2006). A significant number of these are thought to be mediated by antibodies directed against the donor kidney, particularly HLA-specific antibodies (Mao et al., 2007, Worthington et al., 2003, Lee et al., 2009, Terasaki and Cai, 2008).

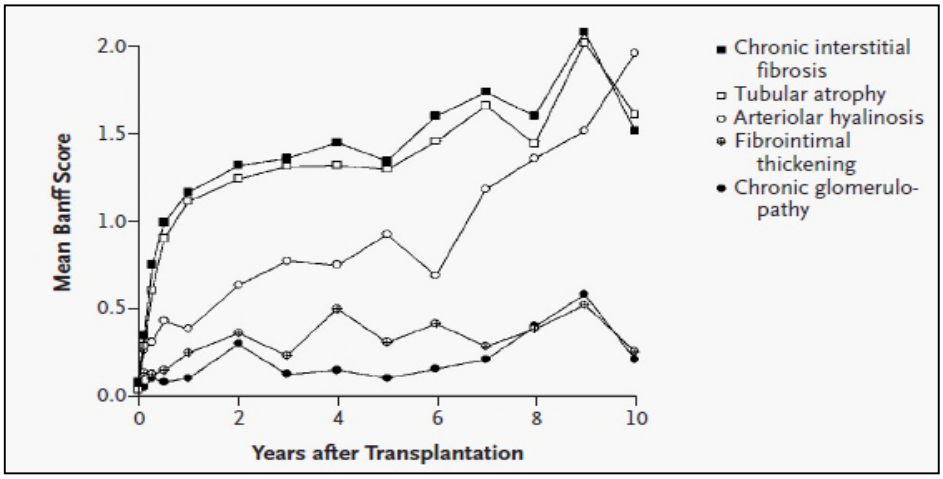


Figure 1-4 : Mean Banff score from serial protocol biopsy from kidney transplant recipients (Nankivell et al., 2003)

1.2 Humoral theory

The humoral immune mechanism can damage a transplanted organ via antibody formation against antigens on the tissue of graft. The most extensively studied are antibodies developed against HLA proteins (Terasaki, 2012). Many believed the major effector response to the transplanted organ was cell mediated and that the antibody response was a mere bystander effect. Over last few decades the understanding of the humoral response has improved due to availability of solid phase specific diagnostic assays. The majority of early work was done by Terasaki *et al*., and proposed mechanism of graft damage as seen in Figure 1-5 (Mao et al., 2007).

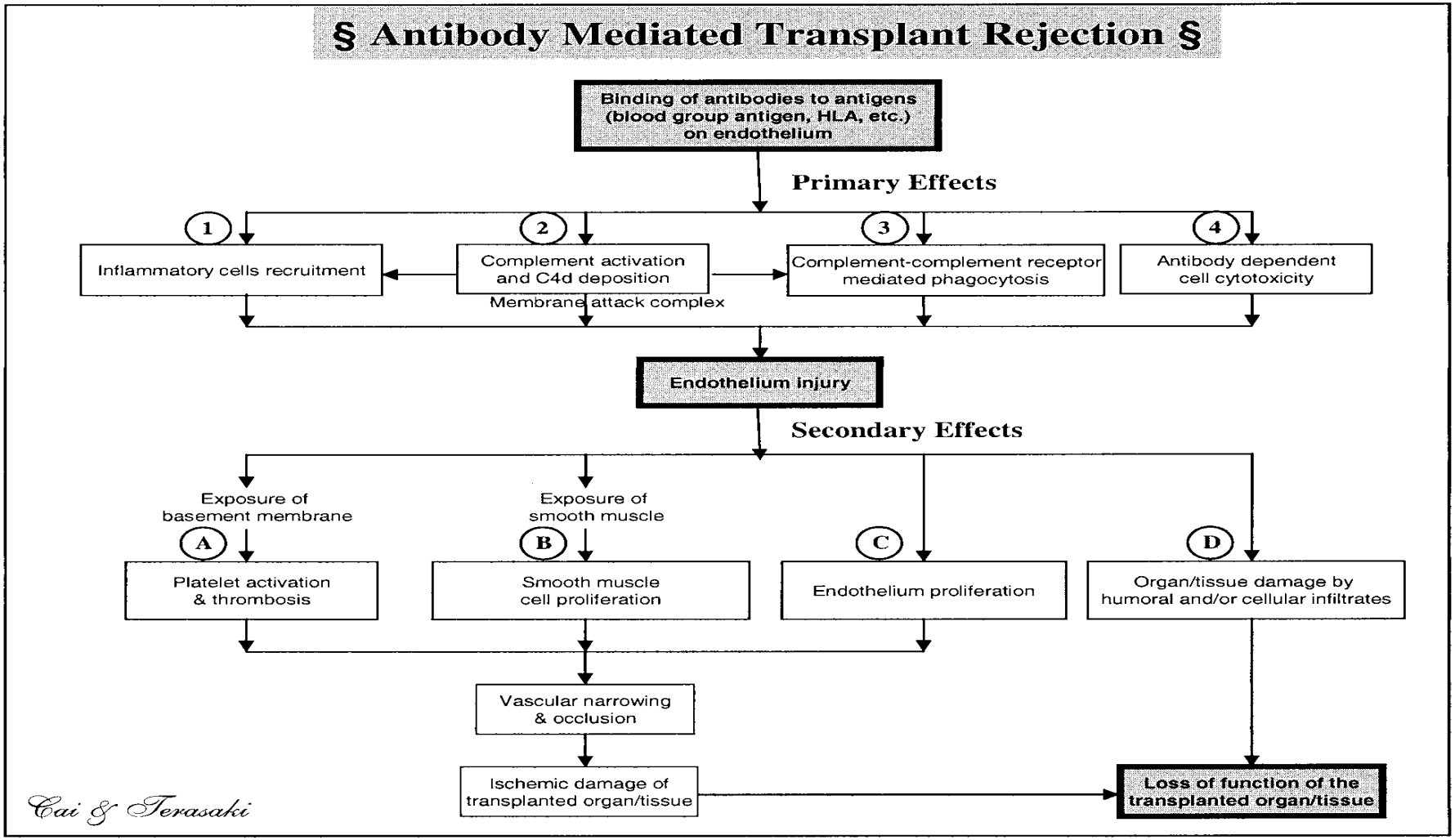


Figure 1-5 : Humoral immune response and damage to allograft (taken from (Cai and Terasaki, 2005)

1.2.1 *De novo* donor specific antibodies (*de novo* DSA):

Antibodies appearing after kidney transplantation are termed as *de novo*. Such transplant recipients did not have these antibodies historically or at the time of transplantation. Prevalence of *de novo* HLA is reported up to in 24 % in the first year post transplantation (Lachmann et al., 2006, Piazza et al., 2001, Terasaki and Ozawa, 2004). This is influenced by the type of baseline immunosuppression regimen (Lederer et al., 2005), sensitisation status, assays used to detect antibodies, time post-transplantation (Terasaki and Ozawa, 2004), compliance to drugs (Wiebe et al., 2012) and induction agents (Tinckam et al., 2004). These can be donor specific (DSA) or non-donor specific (NDSA) based on the presence or absence of corresponding HLA expressions on the donor kidney.

A prospective study of 315 transplant recipients followed over a median follow-up of 6.2(+/-2.9) years, showed that about 15% developed *de novo* DSA to HLA in a mean time of 4.6(+/-3.0) years post-transplantation. The median 10 year graft survival was 57% in cases that developed *de novo* DSA compared to 96% in cases who did not develop *de novo* DSAs (Wiebe et al., 2012). Screening for the development of *de novo* HLA-specific antibodies is recommended by BSHI/BTS (Howell et al., 2010), however this has been controversial (Cooper et al., 2011, Gill et al., 2010, Wiebe et al., 2012).

NDSA are observed frequently following transplantation. Some of these appear together with DSA, and are detected on assays due to shared epitopes on different alleles, or many may appear without any detectable DSAs (thus not due to shared epitopes). Explanations for the latter are not clear and could be due to a re-call response from past sensitisation or non-

specific immune up-regulation following transplantation/ infections (Briggs et al., 2009). It has also been suggested that some of these could be natural antibodies (such as observed in a study of un-transfused males (Morales-Buenrostro et al., 2008)).

1.2.2 Prevention of HLA-specific antibody development post transplantation

Various strategies are proposed to reduce the development of *de novo* DSA post-transplantation. This includes an immunosuppressant regimen that involves Mycophenolate Mofetil (Lederer et al., 2005), and epitope matching (Duquesnoy, 2014a) and increased adherence to the immunosuppressant regimen (Wiebe et al., 2012). It is also postulated that certain HLA-antigens are more immunogenic than others and assays that could assess immunogenicity are under development. Studying electrostatic potential over the epitope surface has been put forward as a promising tool to quantify epitope immunogenicity (Mallon et al., 2014, Kosmoliaptsis et al., 2011). Another important observation is that transplantation across CREG (cross reactive group) of HLA-antigen reduces the risk of an alloresponse (McKenna and Takemoto, 2000). The underlying mechanism is epitope matching between donor and recipient HLA antigens. Epitope consists of antigen determinant amino acid residue that differs between HLA alleles. However, epitopes can be shared between different HLA alleles and as a result presence of antibody against specific epitope gives positive signal for many HLA-specificities defined at antigen levels by current assays. Epitope matching can be done using computer algorithm (Duquesnoy, 2014a). Section 1.7.1. discusses more on concept of epitope. Current UNOS (United Network for Organ Sharing) proposals in the USA will allow better matched kidneys to be transplanted in younger recipients. This will allow the kidney to last longer with less risk of sensitisation (Reese and Caplan, 2011).

1.2.3 Pre-formed HLA-specific antibodies and highly sensitised recipient

Many patients with CKD (Chronic kidney disease) have pre-formed HLA-specific antibodies present whilst on the transplant waiting list. These antibodies develop in response to a sensitising event where there has been previous exogenous HLA-antigen exposure; such as pregnancy (Masson et al., 2013), blood product transfusion (Leffell et al., 2014), and/or previous organ transplantation (Clatworthy et al., 2010). The presence of these antibodies is measured as the breadth of reactivity against the last 10,000 donor HLA types on a national database and expressed as the calculated reactive frequency (cRF) (see Table 1-1). The higher the cRF, the more difficult it is to obtain a compatible transplant offer.

cRF	HD	PD	Pre-emptive	Total
<10%	51%	63%	71%	57%
10 to 85 %	19%	23%	21%	21%
>85%	30%	14%	8%	23%

Table 1-1 : Proportion of sensitised patients in UK (HD – Haemodialysis, PD – Peritoneal dialysis, cRF – calculated reactive frequency) taken from Renal Registry annual report 2013

Patients with cRF > 85% are defined as Highly Sensitised Patients (HSP) and they have to wait longer before a transplant offer. Some 43% of cases on the waiting list are sensitised and 23% are highly sensitised (Table 1-1) (Pruthi et al., 2013). Transplantation in the presence of high levels of pre-formed HLA-specific antibodies can result in immediate hyper acute rejection (Patel and Terasaki, 1969) and poor transplant outcomes (Patel et al., 1971). Various strategies are used to increase the chances of transplantation in such groups; 1) Prioritisation of highly sensitised patients, 2) Modified unacceptable antigen listing, 3) Kidney sharing

schemes and 4) Direct transplantation in the presence of donor specific antibodies. The first two approaches are for cases that are on the national deceased donor waiting list.

1.2.3.1 *Prioritisation of highly sensitised patient*

1.2.3.1.1 UK kidney allocation scheme

In the UK, unacceptable antigens are listed with ODT (Organ Donor Transplantation) and when a kidney becomes available it is allocated based on the national allocation scheme. In 2006, the kidney allocation scheme, which was largely based on HLA matching, was changed with a view to reduce variations in waiting times (Higgins et al., 1997) (see Table 1-2). Prior to the 2006 scheme, 15 % highly sensitised patients were on waiting lists for 5 or more years; after implementing the new scheme it was reduced to 10%. However, subsequently this remained static; hence in 2011 Level 3 HLA mismatch (0-DR and 2-B or 1-DR and 0/1-B) kidneys were allowed for cases with cRF > 85%. But that only had minor improvement due to the large residual pool. Recently, the scheme has changed to allow Level 2 HLA mismatch (0-DR and 1/0-B) for cases with cRF > 85%. (KAG – Kidney advisor report UK, November 2013; See Appendix 1) The effect of new changes will need to be assessed after its implementation in 2014.

Tier scheme	Points
Tier A **000 mismatched children (DR homozygous or HSP)** Tier B **000 mismatched children (all others)** **Within tiers A and B: patients are prioritised by waiting time only** Tier C **000 mismatched adults (DR homozygous or HSP)** Tier D **000 mismatched adults & favourably mismatched children** Tier E **All other eligible patients** **Within tiers C to E: patients are prioritised by point score**	**Waiting time** points: 1 point for each day on list **HLA match & age** points combined: max 3,500 **Age** difference points: –0.5*(donor-recipient age diff)2 Location points: 900 same centre, 750 local area **HLA homozygous** points: HLA-B 100, HLA-DR 500 **Blood group** points: –1000 for B patients when donor is O

Table 1-2 : UK National organ allocation scheme 2006

1.2.3.1.2 Euro-Transplant Acceptable Mismatch Program

The Euro-Transplant Acceptable Mismatch Program uses a computer algorithm (HLA match maker) to identify HLA-antigens that are likely to give a negative cross match. Acceptable antigens are defined as ones which the patient has never formed antibodies against (Claas et al., 2004). When such a donor becomes available the kidney is shipped to the transplanting centre, and wet cross match is performed. This strategy has allowed 65% of highly sensitive patients on waiting lists to receive transplants within 2 years. However not all cases could be transplanted using this strategy and the authors have suggested the role of desensitisation in selected cases (Doxiadis and Claas, 2009).

Thus, although the strategy used in the UK and Euro-Transplant program has improved the chances of a transplantation offer in highly sensitised cases, there are still a large number of cases that need to wait longer; and thus we require additional approaches.

1.2.3.2 *Modified unacceptable HLA-specificities listing*

In the UK, unacceptable HLA specificities are reported to ODT (Organ Donor Transplant). Different profiles of unacceptable antigens can be registered for the deceased donor waiting list. Individual clinicians go through the list of HLA-specificities in highly sensitised patients, and if the levels are low, or historic positive and current negative, or deemed false positive, these are considered negative and listed them as modified unacceptable HLA-antigens.

1.2.3.2.1 Historic positive but current negative HLA-specificities

Some HLA-specific antibodies can disappear following the initial response and these specificities are removed from unacceptable list. This is supported by a study using AHG-CDC (Anti-human globulin augmented complement dependent cytotoxicity) assay that showed no difference in graft function or rejection between groups of patients with historic positive and historic negative AHG-CDC (Baron et al., 2002). However other studies have given contrary conclusions (Avlonitis et al., 2000); 10 out of the 14 cases lost their graft despite negative pre-transplant flow cross match and five year survival was 43%. Similarly, another study suggested higher peak historic Mean Fluorescence intensity (MFI) (Luminex assay) levels predicted antibody mediated rejection better than current DSA levels (Lefaucheur et al., 2010). Ideally such cases should have assessment for likelihood of memory response by studying memory B cell (Eisen, 2014). A pragmatic approach is however taken in such cases with augmented immunosuppressive treatment followed by careful monitoring.

1.2.3.2.2 Low level (MFI) HLA-specific antibodies

Lack of standardisation, coupled with variability of assay protocols used across the world, has led to different H&I Units having different cut-off points for positivity of MFI levels; which ranges from 300 to 2000 MFI values. Each Unit sets up a cut-off threshold based on response from the negative sera using their protocols. So it is difficult to compare results of transplantation between various Centres. It is generally believed that transplantation across low level MFI (<2000) carries low immunological risks.

Transplanting across low level antibodies need careful approach, as studies have shown poor outcome in such cases (Gupta et al., 2008). We have reported that certain HLA-specificities provoke more vigorous response than others and that this depends on the sensitisation event that was responsible for synthesis of DSA in first instance (Higgins et al., 2014b). Thus history of sensitisation events is important; this is of particular significance if the donor is the husband or child for a female transplant recipients, as even a low level of DSA is important (see Figure 1-6) (Higgins et al., 2014b).

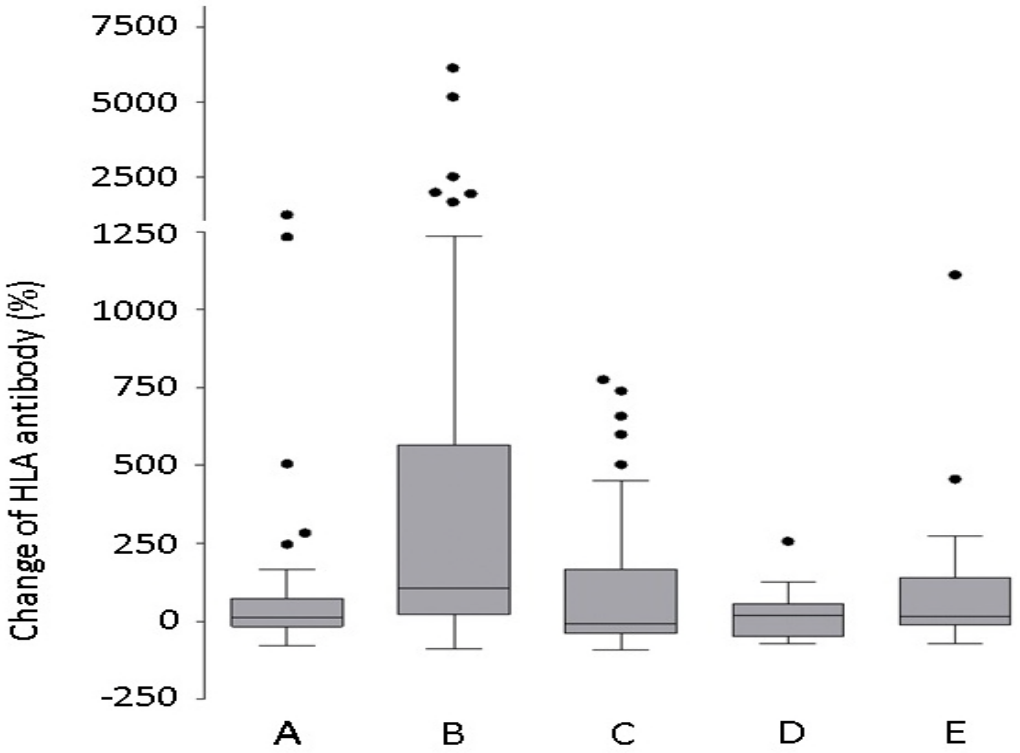


Figure 1-6 : Percentage changes in HLA-specific antibody levels (from pre-treatment levels) according to mode of original sensitisation event (Higgins et al., 2014b)

Box plot showing median, 25%, 75% and inter-quartile range; A = no known sensitising event; B = pregnancy; C = transfusion; D = transplant, repeat antigen mismatch; E = transplant, repeat epitope mismatch ($p < 0.0001$ (Independent samples Kruskal Wallis test))

1.2.3.3 *National living donor kidney sharing scheme (NLDKSS)*

This is a collective scheme whereby the donated kidneys are shared across UK. This includes paired/pooled donation (PPD) and altruistic donor chains (ADC). It was first proposed in 1986 (Ross et al., 1997) and later developed in Korea, Netherlands and USA (Kaplan et al., 2005, Gentry et al., 2011, Montgomery et al., 2011a). A paired donation can take place between two antibody incompatible pairs, where by the respective donors can donate kidney to the opposite recipients such that the immunological risk is lower and the transplantation is antibody compatible (Figure 1-7, A). More pairs can be added in to this and the best possible donation is identified in the pool for every recipient and this is called pooled donation (Figure 1-7, B). The pooled donation can be facilitated further by participation of an altruistic (non-directed) donor, whereby an additional kidney improves matching in the pool and one kidney at the end goes to patient waiting on national waiting list (Figure 1-7, C). Thus by kidney sharing scheme the highly sensitised patients with a living donor could have a compatible kidney transplantation with lower immunological risks.

National kidney sharing scheme was introduced in UK (see Figure 1-7 and 1-8). However up to 50% of incompatible pairs fails to find a suitable match (Montgomery, 2010). This is due to the presence of larger numbers of blood group ‘O’ patients compared to fewer donor and cases with cRF over 95%. This limitation could be partly overcome by allowing compatible pairs (Gentry et al., 2007), altruistic donor (Johnson et al., 2008) and direct transplantation with antibody removal treatment for selected cases (Montgomery et al., 2011a). See Appendix – B (Kidney Advisory Report December 2014)

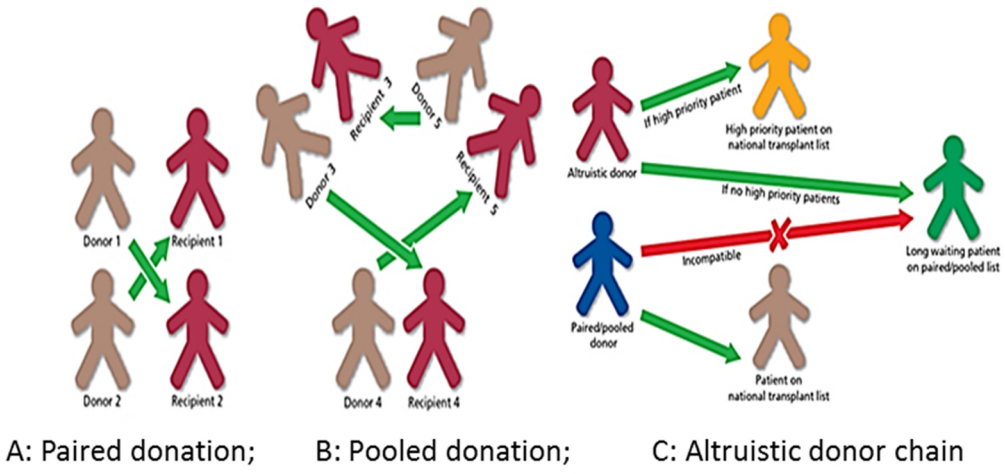


Figure 1-7 : Kidney sharing scheme (adapted from NHSBT website)

A- Paired exchange between pair-1 and pair-2; B – kidney exchanged in three way swap (pooled donation) and C – Altruistic donor donating to highly priority patient (Yellow colour) and in absence of highly priority patient donates kidney to long waiting patients on paired or pooled donation (Green) and then the corresponding recipient (blue colour) donates the kidney to patient on national waiting list (brown colour)

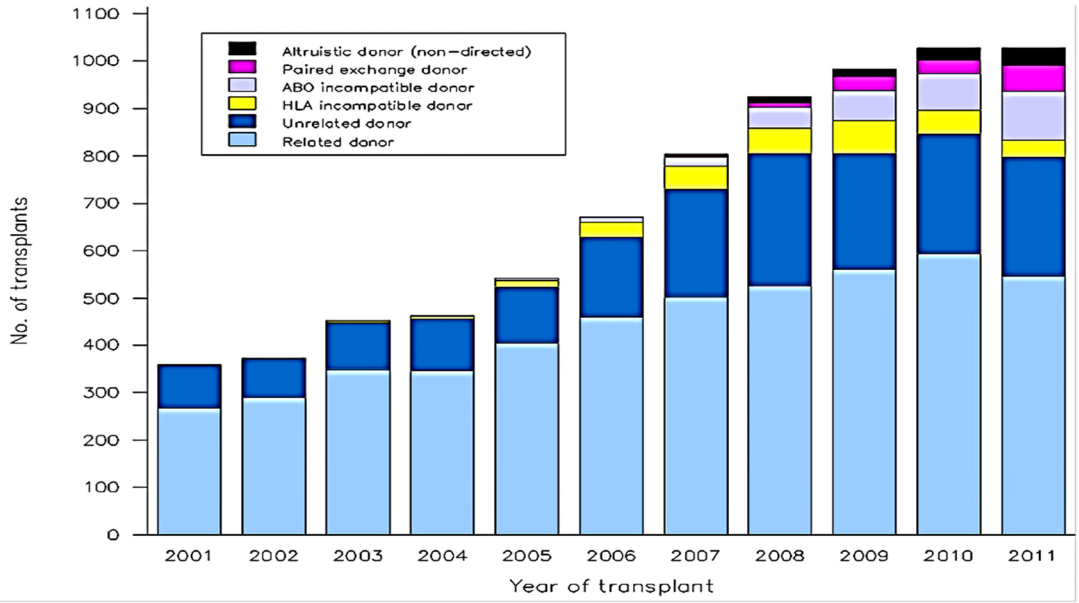


Figure 1-8 : Profile of Living donor kidney transplantation in UK (adapted from UKT report)

1.2.3.4 *Direct transplantation with donor specific antibody removal*

In highly sensitised patients, Antibody removal combined with other conditioning therapies such as IVIg and Rituximab can render cross match negative (Genberg et al., 2008; Gloor & Stegall, 2010; Higgins et al., 2010; Higgins et al., 2011b; Higgins et al., 2011c; Montgomery et al., 2011b; Stegall et al., 2006), thus allowing kidney transplantation. (See Figure 1-9 for UK activity). Following HLA-incompatible kidney transplantation, the dynamics of HLA-specific antibodies over tine are monitored carefully (Higgins et al., 2009b) and treatment adapted accordingly. Transplanting across higher levels of DSA (complement dependent cytotoxicity (CDC) positive) is associated with poor graft survival compared to standard compatible kidney transplantation.

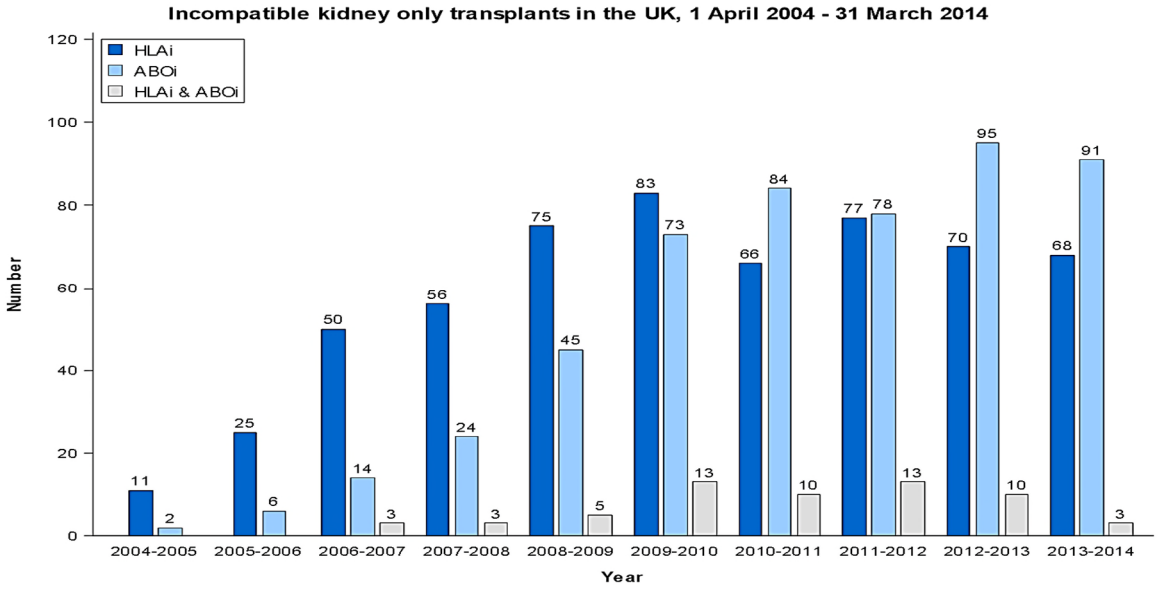


Figure 1-9 : Antibody incompatible kidney transplantation in UK

(Taken from NHSBT Organ & Transplantation report 2013-14)

Data from our Centre showed that graft survival is dependent on the level of antibodies (see Figure 1-10), and that cases with CDC negative have similar 10-year graft survival as standard kidney transplantation. Similarly, a multicentre study form US showed poor graft and patient survival associated with higher levels of antibodies (Lentine et al., 2014, Orandi et al., 2014) (see Figure 1-11). Hence Kidney Advisory Group (see Appendix B) recommended direct transplant in pairs with positive CDC should be considered only in exceptional circumstances. Despite of high clinical and immunological risk, direct transplantation with antibody removal in highly sensitised patients confers survival benefit when compared to patient staying on dialysis (Montgomery et al., 2011b) (Figure 1-12).

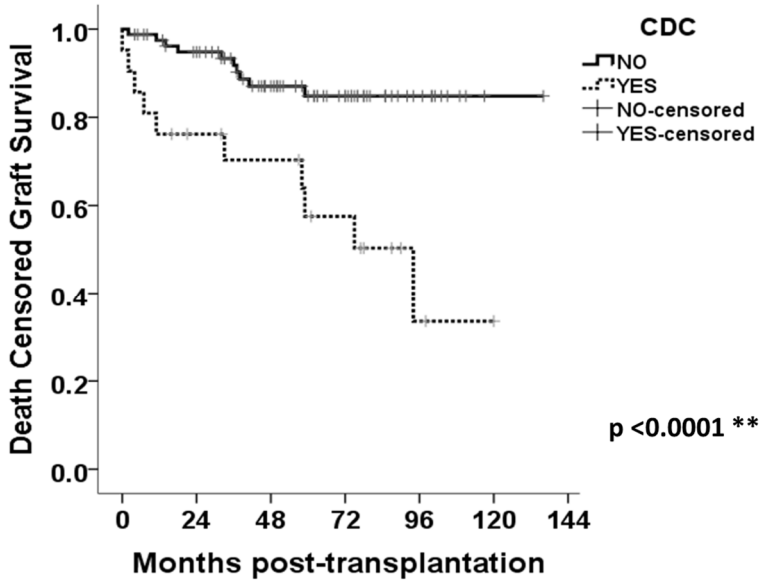


Figure 1-10: Death censored graft failure of HLA-incompatible kidney transplantation stratified according to the reactivity of the donor antibodies (Higgins et al., 2014a)

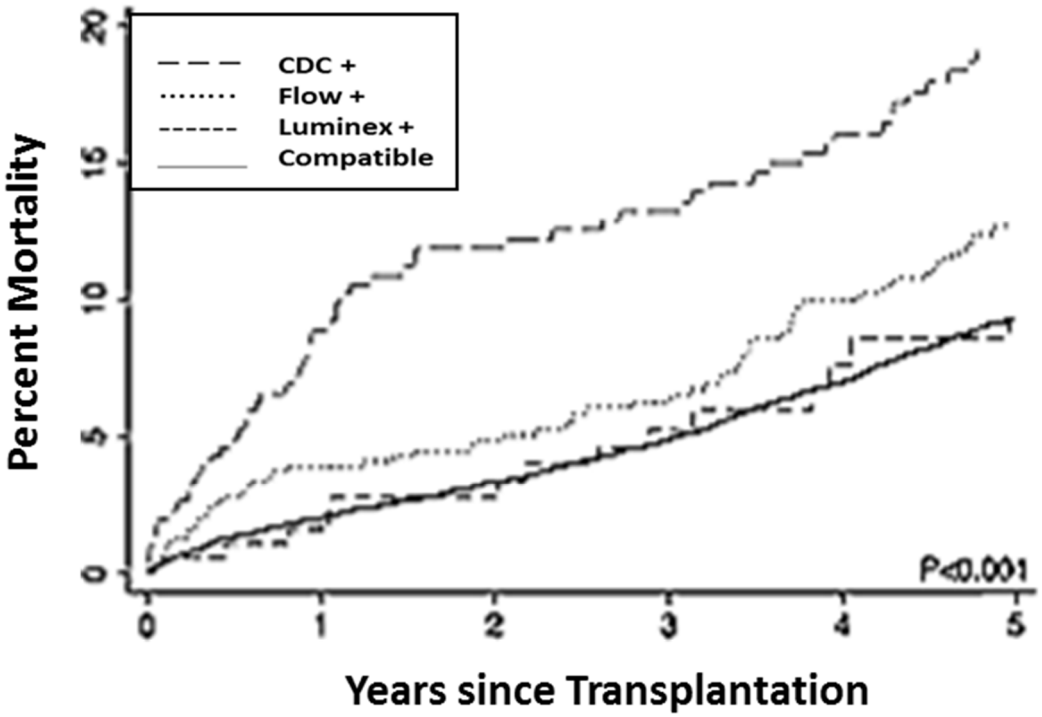


Figure 1-11 : Survival curve showing patient mortality based on antibody strength. (Modified from ref (Orandi et al., 2014))

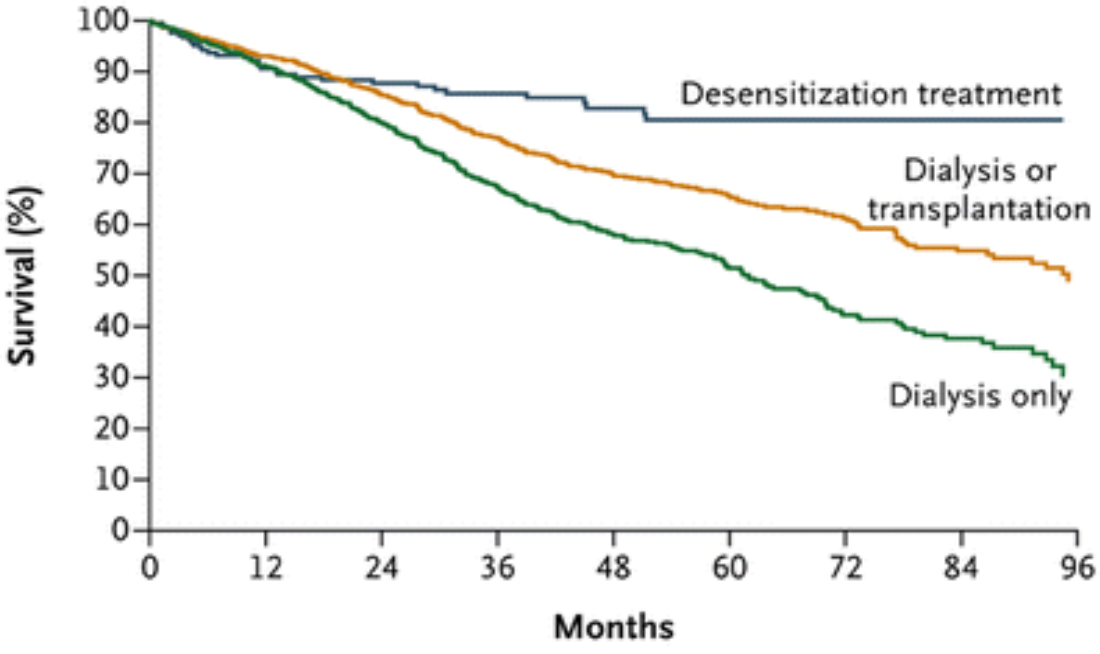


Figure 1-12: Patient survival comparing patients who underwent antibody incompatible kidney transplantation with patients who stayed on dialysis (p < 0.001) (Montgomery et al., 2011b)

ABO-incompatible kidney transplantation (ABOi) is considered a lesser immunological risk compared to HLA-incompatible kidney transplantation and single centre data show that the outcome from ABOi is similar to compatible kidney transplantation (Higgins et al., 2011a, Tyden et al., 2007, Takahashi and Saito, 2006, Ichimaru and Takahara, 2008, Tanabe et al., 2013, Toma et al., 2001). Recent multi-centre European studies showed similar results of graft survival at three years compared to standard transplantation (Opelz G[1], 2014). A US multi-centre study showed day 8 as higher risk for acute antibody mediated rejection with graft failure (Montgomery et al., 2012) (see Figure 1-12).

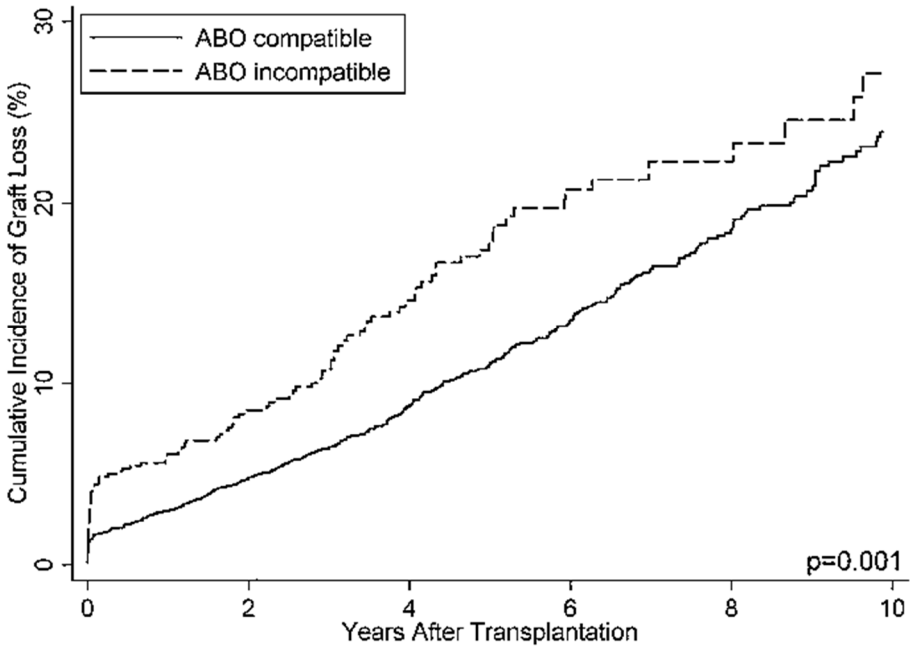


Figure 1-13: Outcome of ABO-incompatible kidney transplantation(Montgomery et al., 2012)

1.3 Alloresponse following kidney transplantation

Following organ transplantation, the donor HLA-proteins are presented to recipient T cells by antigen presenting cells through three distinct pathways (see Figure 1-14). On activation of recipient T cell, they can drive T cell proliferation and B cell activation.

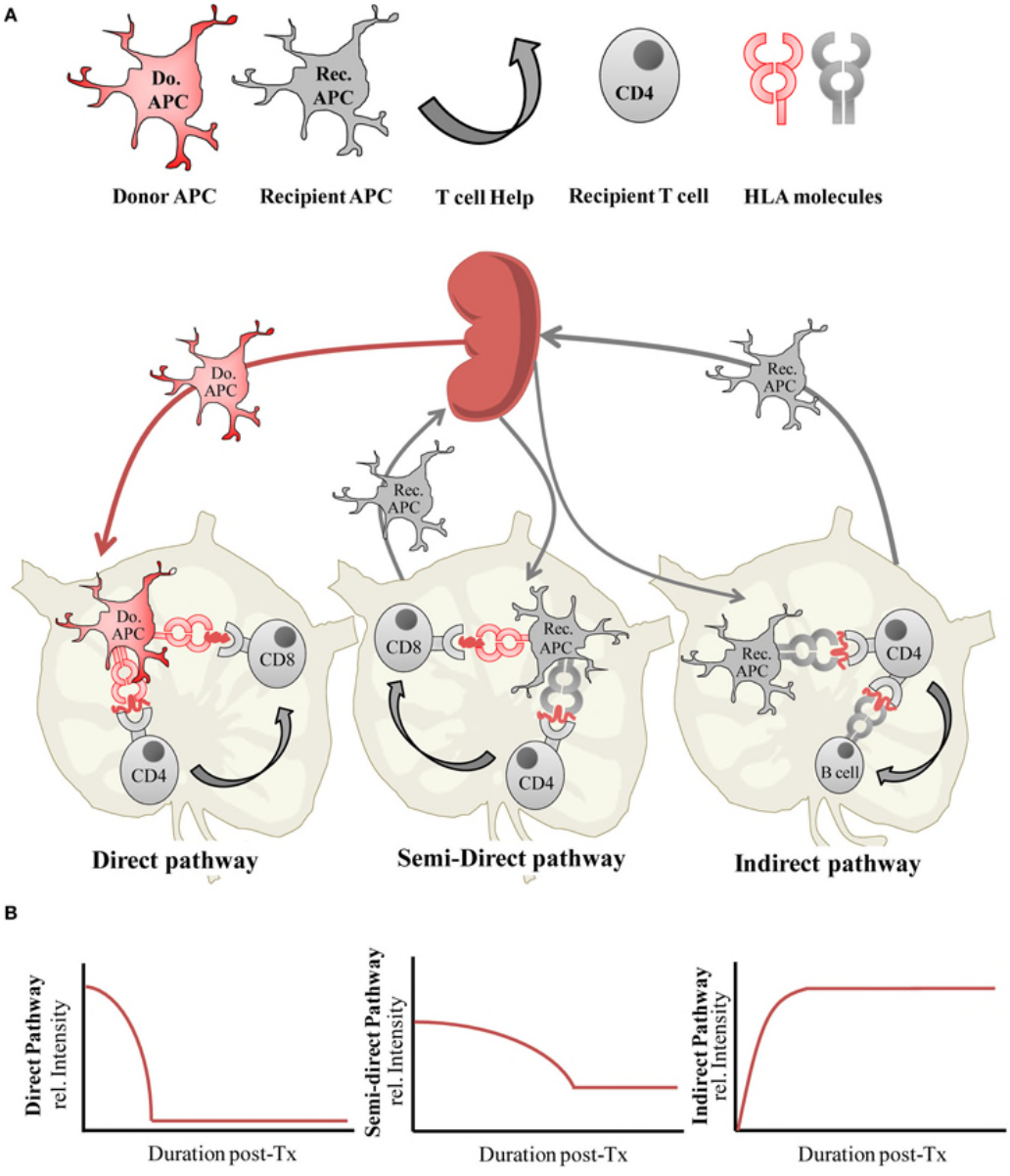


Figure 1-14 : Pathways for allorecognition following transplantation (taken from ref (Sagoo et al., 2012)) (A – illustration of pathways and B – time-line of contribution from each pathways)

1.3.1 Direct pathway

As seen in the Figure 1-14, a donor antigen presenting cell (APC) presents donor MHC to recipients T-cells. This alloimmune response is unique to organ transplantation and lasts as long as the donor APC survives. The donor APC expresses MHC +/- peptides for both recipient CD4 and CD8 T cells, and thus CD4 T cells provide ***direct help*** for cytotoxic CD8 T cell-mediated alloimmunity. This pathway does not provide help for antibody production as demonstrated in a murine adoptive-transfer mode (Taylor et al., 2007). This pathway is intense in the early post-transplant period and is responsible for acute cellular rejection observed in first three months post-transplantation.

1.3.2 Indirect pathway

As seen in the Figure 1-14, recipient APC MHC molecules present processed donor MHC class II peptides to the recipient CD4 T cell leading to T cell activation and proliferation. This pathway is similar to normal physiological responses to a foreign protein antigen and is important for humoral immunity. The CD4 T cell help is delivered directly to the B cells, resulting in alloantibody production (Steele et al., 1996).

Here CD4 T cell help is delivered ***indirectly*** to the CD8 T cell via the APC which acts as an intermediary cell expressing MHC class I and presents antigen to the CD8 T cell (not shown in the Figure 1-14).

1.3.3 Semi-direct pathway

As seen in the Figure 1-14, donor class I MHC molecules can be presented on recipient APC and donor peptide is presented through recipients' class II MHC molecules. Thus both CD4 and CD8 T cell can interact with recipient APC and ***direct*** CD4 T cell help is provided to the cytotoxic CD8 T cells (Figure 1-14).

Indirect and semi-direct pathways are responsible for continuing allorecognition long term and responsible for rejection (both acute and chronic).

1.3.4 Direct allorecognition by pre-existing donor specific antibodies

Pre-existing donor specific antibodies circulating in the recipient's circulation can recognise the donor HLA molecules on endothelial cells and thus cause direct allorecognition. Following binding of the antibodies, the complement system is activated which leads to activation of T cells, neutrohils and macrophages. (Figure 1-25)

1.3.5 B cell activation and proliferation of B cells and Plasma cells

B cell activation and alloantibody production occurs via the indirect allorecognition pathway described above. The Indirect pathway involves direct B cell and T cell interactions (cognate interaction). Cognate interaction is important for immunoglobulin class-switching and affinity maturation. Additionally, B cell can be activated by direct interaction with class I MHC on donor APCs similar to CD8 T-cell in direct pathway described above. This is a non-cognate interaction and the immune response is characterised by an IgM only response (Steele et al., 1996).

As seen in the Figure 1-15, following allorecognition and activation, the B cells migrate to the T/B border in secondary lymphoid organs and interact with CD4 helper T cells. This cognate interaction is augmented via the costimulatory interactions between the molecules CD40 and CD40L and is followed by local cytokine release (IL4) inducing clonal expansion and differentiation. Cells entering germinal centres undergo somatic hypermutation and class switch recombination. These further differentiate in to memory B cells and plasma cells. Ectopic formation of germinal centre in transplant graft has been demonstrated (Thaunat et al., 2010). Some activated B cells remain in extra-follicular / marginal zones and produce poly-specific low affinity antibodies (IgM, IgG and IgA) (Baumgarth, 2011, Griffin et al., 2011). The B cells These T-independent pathways of B cell activation are faster than T-dependent which can take up to five to seven day in response to microbial exposure. These B cells differentiate into short lived plasma cells or IgM+ memory B cells.

Long lasting plasma cells are high affinity variants and migrate to bone marrow, where they form a niche and become fully differentiated and continue to produce antibodies of high affinity (Clatworthy, 2011). As seen in Figure 1-16, plasma cells reside in a cellular niche that provides a microenvironment favorable for the survival of these long lived cells (Tangye, 2011). Various mediators take part in the activation and survival of plasma cells, particularly IL6, BAFF (B-cell activating factor) and APRIL (a proliferation inducing ligand) (Tangye, 2011, Clatworthy et al., 2010). FcγRIIB controls bone marrow plasma cell apoptosis (Xiang et al., 2007). Long lived plasma cells do not express receptors for their cognate antigen, and give rise to constitutive production of low levels of high affinity antibodies (Manz et al., 1998, Manz et al., 2005). These circulating donor specific antibodies can be removed via an assortment of plasma treatments to allow direct transplant in highly sensitised patients.

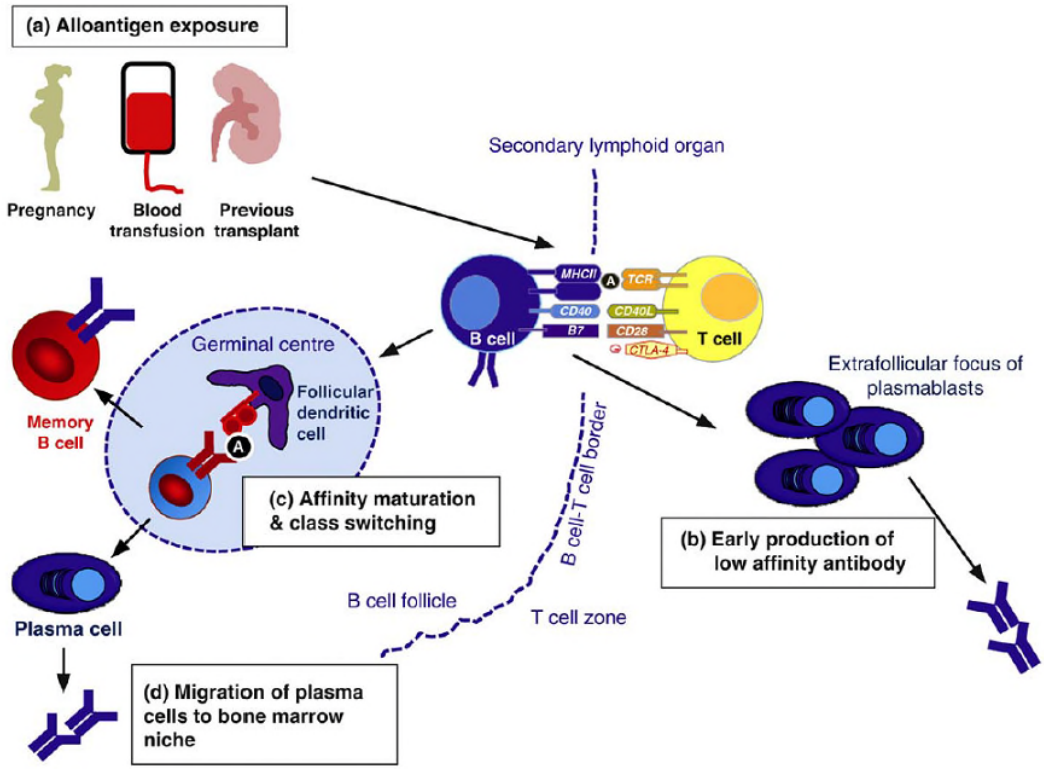


Figure 1-15: Alloantibody production (taken from (Clatworthy et al., 2010))

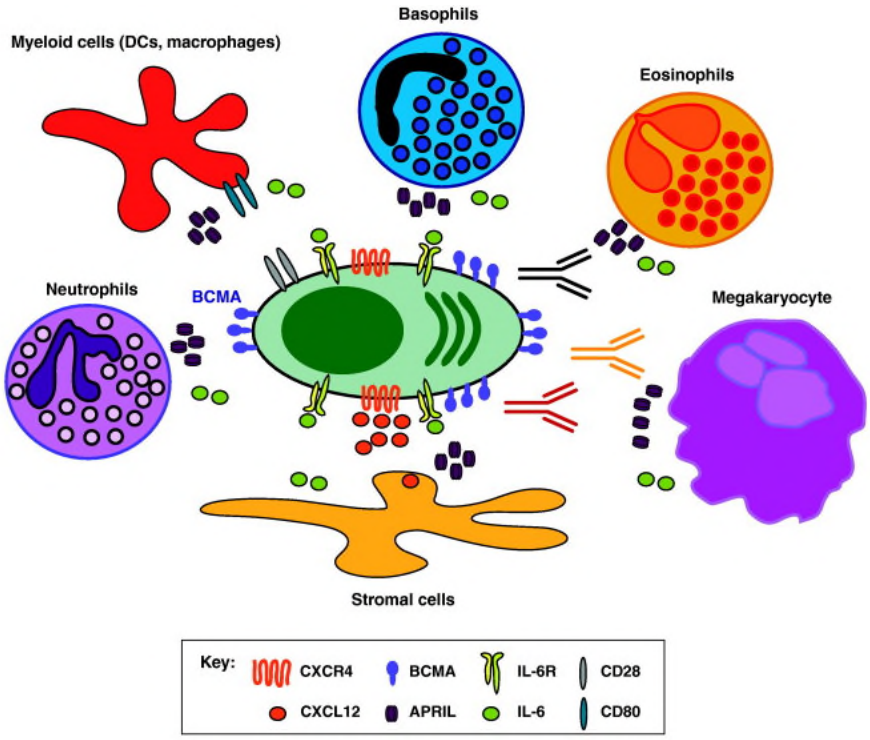


Figure 1-16 : Survival factors for long lived plasma cell in bone marrow (taken from (Tangye, 2011))

Memory B cells expresses antigen receptors and thus require antigens to mount a re-call response. They rapidly expand and develop into short-lived plasma cell under cognate control of memory helper T cells. This is characterised by increased concentrations of high affinity IgG antibodies and can result in rejection in sensitised patients undergoing direct transplantation. The antigen-driven reactivated memory B cells can return to germinal centres for further maturation.

In mice, experiments have shown that the development of high avidity memory B cells occurs in two stages. In the first stage following primary sensitisation, the IgG-bearing memory cells do not require help from T cells. But in the second stage, there is production of high avidity memory B cells due to interaction with T cell in presence of antigenic stimulation. IgM+

Memory B cells, generated in a T-independent manner through innate responses (e.g. Toll like receptor 9) are described (Weller et al., 2001) and they are thought to have role in fighting infections (Capolunghi et al., 2013). The response regenerated by IgM+ memory B cells is usually short lived (Rabant et al., 2013). These processes are not well delineated following organ transplantation, and it is unclear which cells contribute to the antibody response.

1.3.5.1 *Response in highly sensitised patients*

As discussed above, the humoral response is different for *de novo* alloantibody production compared to a re-call response following a direct transplantation in highly sensitised patients. In summary, the baseline antibody levels are due to long lived plasma cells residing in bone marrow – antibodies which can be removed by depletion therapy (Higgins et al., 2010) but the levels usually rise following direct transplantation (Higgins et al., 2009b). The rise is usually after 4-7 days post transplantation and this could be due to saturation of adsorption of the alloantibodies to the graft or soluble HLA molecules or increases in antibody synthesis following secondary memory B cells responses which are T cell dependent

The high affinity baseline antibodies from long-lived plasma cells can cause direct allorecognition and bind to the endothelial cells on the kidney graft causing complement activation with subsequent tissue damage and rejection. This may not respond to the anti-T cell treatment typically given to transplant patients, as the rejection process is ultimately driven by long-lived plasma cells that do not typically respond to standard immunosuppressive medications. However, the memory response is T cell dependant and can potentially be ameliorated by anti-T cell treatment. The antibodies generated following allorecognition in direct transplants within highly sensitised patients are polyclonal with varied physio-chemical properties such as sub-class of IgG and target binding affinity. This may partly explain the difference in humoral response following allorecognition. Thus treatment of antibody mediated rejection needs the above consideration and a combination of therapies may need to be used.

1.3.6 Antibody (Immunoglobulin)

An antibody (also called immunoglobulin) molecule structure primarily consists of two heavy (H) and two light (L) chains. Disulphide (covalent) bond holds these chains together and form a '**Y**' shape structured molecule. The antibody molecule is further divided in to variable (V) and constant (C) regions. The N-terminal end (Fab) of heavy and light chains has variable amino acid sequences and serves as the antigen binding site. The constant portion (Fc) serves as the binding site for effector mechanisms such as Fc receptors and the complement system (Figure 1-17). Based on variations in heavy chains (HC), the immunoglobulin molecule can be further classified in to five classes or isotypes (Table 1-3). The light chains (LC) consist of two types: kappa (κ) and lambda (λ).

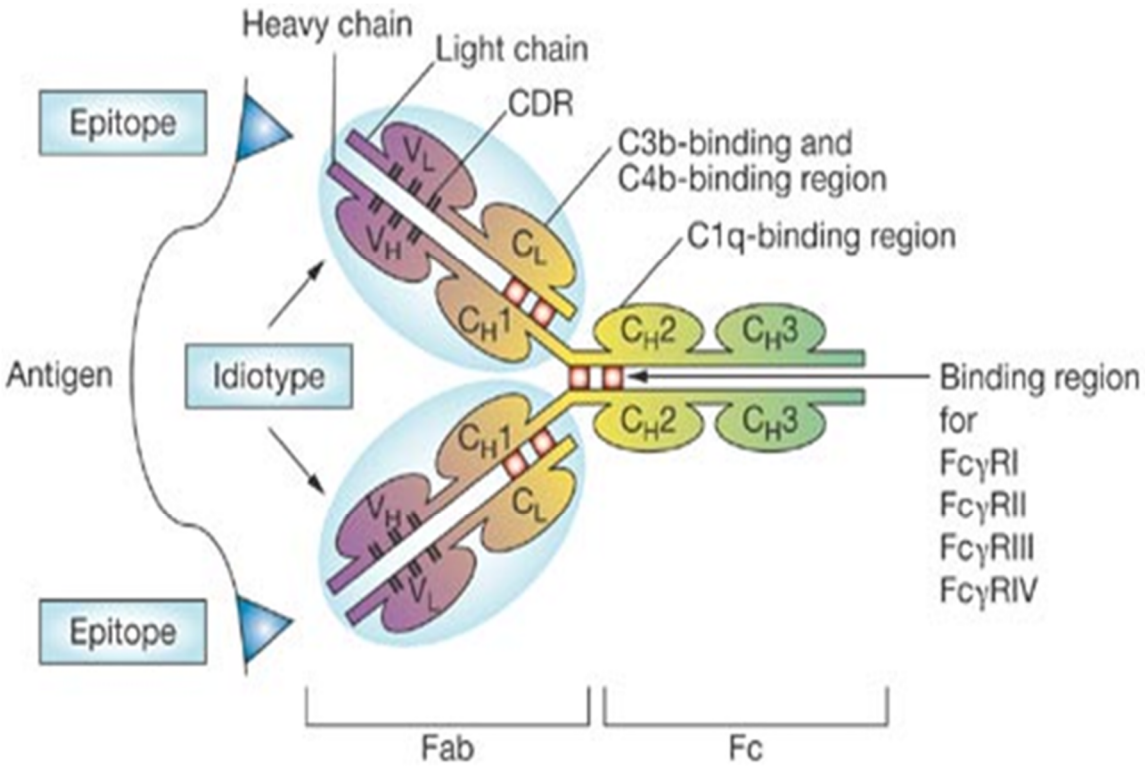


Figure 1-17: Structure of antibody (immunoglobulin) molecule (Bayry et al., 2007)

Class	IgA	IgD	IgE	IgG	IgM
Heavy chain	α1,α2	Δ	E	γ1, γ2, γ3, γ4	μ
Molecular weight (D)	160,000	184,000	188,000	146,000	970,000
Serum concentration (mg/ml)	0.5-3	0.03	0.00005	0.5-9	1.5
Half life (days)	6	3	2	7-21	10
intravascular distribution	42%	75%	50%	45%	80%

Table 1-3: Classes of antibody

1.3.6.1 *Antibody binding to the antigen*

The antigen binding site (paratope) is located in the region of variable heavy chain (VH) and variable light chain (VL) domains (Figure 1-18(A)). The antigen-binding site consists of amino acid residues of six hyper variable loops (L1-3 are the LC CDR loops shown in the order of their location in the amino acid sequence; H1-3 are the HC CDR loops) and these interacts with epitope region on the antigen. The binding interaction consists of specific binding between the paratope and epitope and non-specific binding to the surrounding amino acid residues. The overall surface interacting determines stability of binding.

As antibody has more than one binding site (e.g. IgG = 2; IgM = 10) the overall binding strength is a result of binding interaction at different site and this is called avidity. Affinity is used to describe the binding strength at one specific interaction. Avidity will be affected by antigen density whilst affinity is unaffected by antigen density.

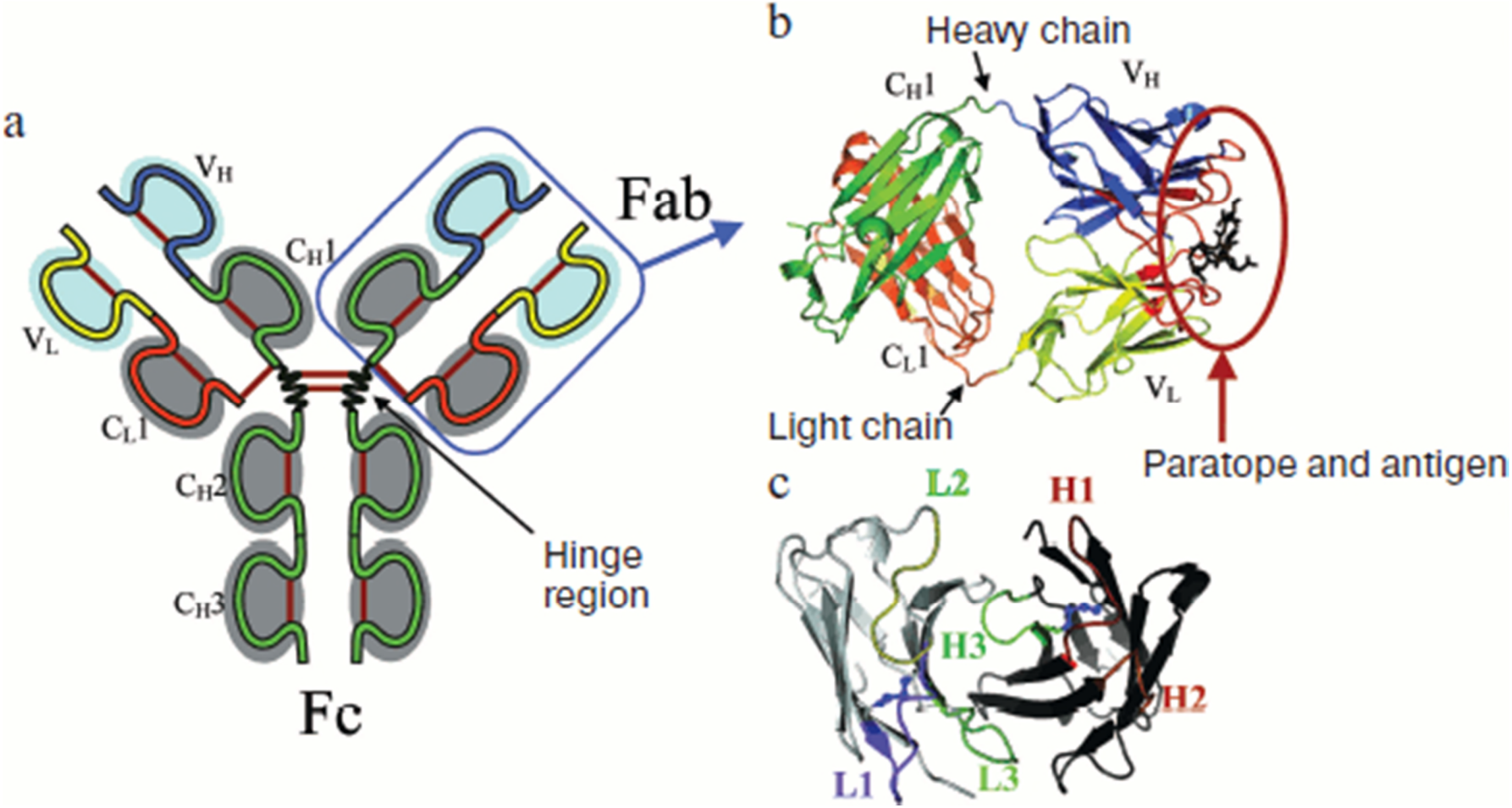


Figure 1-18: Epitope – Paratope interactions (adapted from (Altshuler et al., 2010))

A – IgG molecule; B – interaction of antigen and variable portion (V_H and V_L) of antibody shown in red oval area. Within the highlighted area, the CDR loops are marked in red colour; C – Shows the CDR loops in color and labelled L1-3 and H1-3 on light and heavy chain respectively.

1.3.6.2 *Class switching*

The IgM class can be produced by short lived plasma cells following direct B-cell interaction with antigen, and this can happen independent of T cell help, or it could be produced by an activated B cell with T cell help in a germinal centre. IgM has a pentameric structure. On account of this clustering of Fc domains, it has strong complement activating properties, and higher avidity when compared to the other immunoglobulins. Class-switching from IgM to IgG or IgA generally requires T cell help and happens in germinal centres; but it can happen to a lesser degree in extra-follicular regions due to innate system interactions. Within IgG, the subclasses switch with IgG3 appearing first followed by IgG1-2-4.

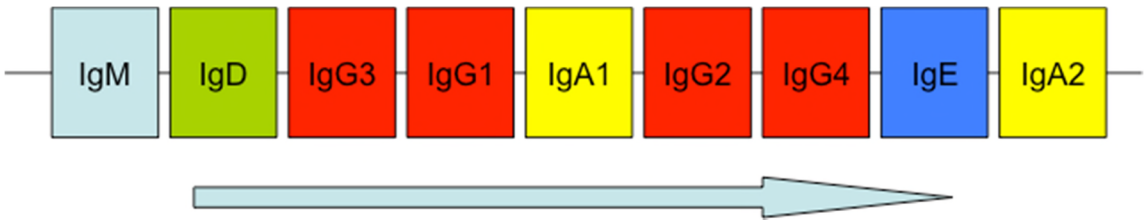


Figure 1-19: Sequence of class switching of immunoglobulin molecule

The molecular mechanisms that underlie this class switching have been described. Briefly, the sequence of class switch is summarised in Figure 1-19. Hereby, the VDJ exon that encodes the heavy chain V (variable) domain recombines with a downstream C (constant) region gene segment and the intervening DNA is deleted (Figure 1-20).

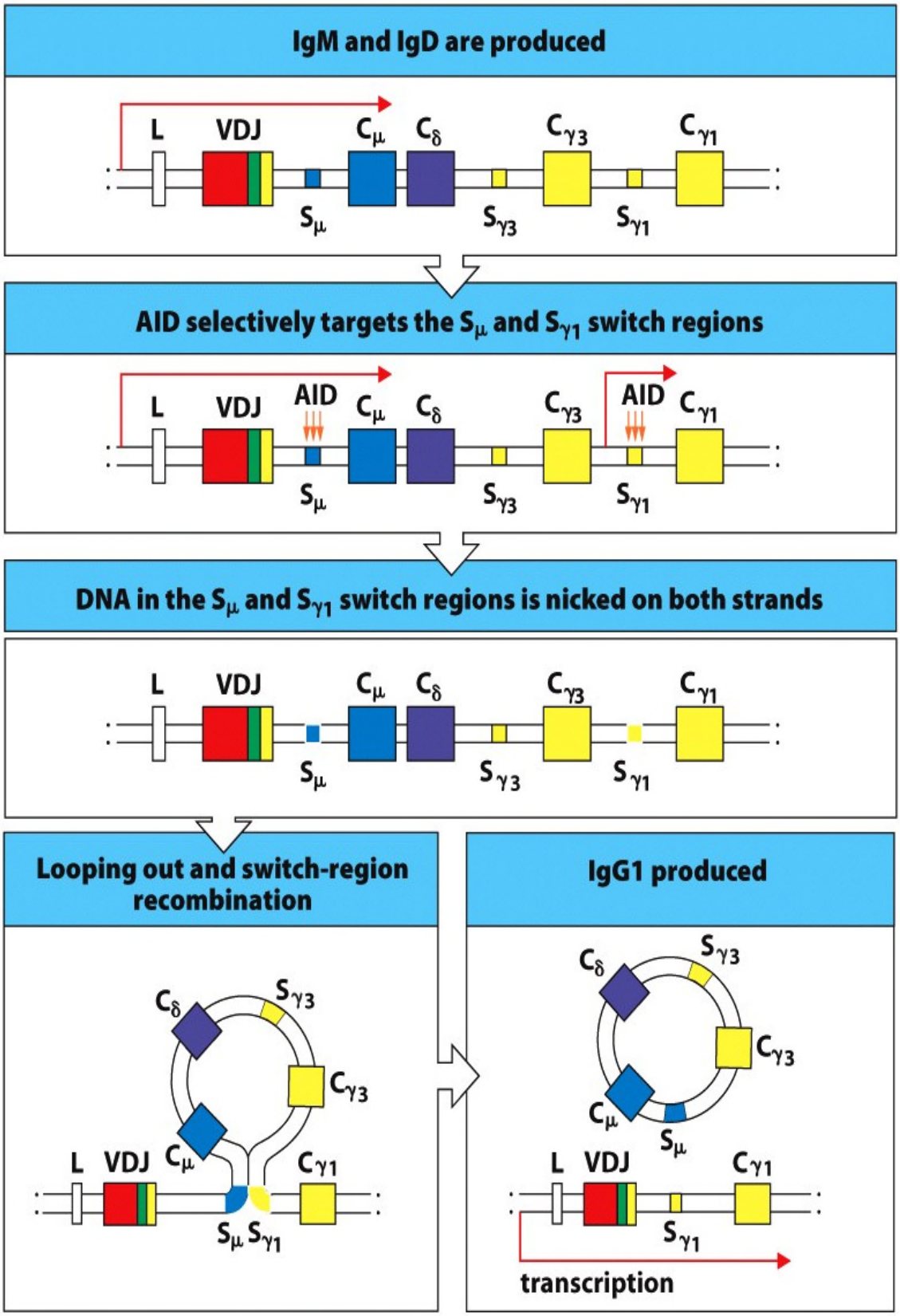


Figure 1-20: Class switch recombination (taken from The Immune system, 3ed ©Garland Science 2009)

IgG subclasses differ in hinge region and amino acid residues within the constant domain of the antibody structure (Figure 1-21). These structural differences give them functional diversity, with IgG3 being the strongest complement activator followed by IgG1, and then IgG2. IgG4 is believed to be a non-complement activating immunoglobulin. They also differ in antigenic interactions; with IgG2 preferring carbohydrate antigen and IgG1 & IgG3 preferring protein antigen. IgG4 has a unique property whereby it can exchange N-terminal Fab arms and behave as a bispecific molecule interacting with two different antigens (Schuurman et al., 1999). Isotype pattern varies according to the mode of B cell activation (Esser & Radbruch, 1990). This is dependent of antigen (e.g. Parasites strongly invoke switching to IgE), route of immunisation (e.g. IgA induction to function at mucosa), the presence of regulatory cells, and cytokines produced by CD4 helper T cells, and genetic factors. Apart from difference in complement-fixing properties, IgG sub-class may differ in binding to Fc gamma receptors on immune cells such as macrophages, neutrophils and dendritic cells and thus have variable effects through antibody dependent cytotoxicity mechanism (Nimmerjahn et al., 2005; Nimmerjahn & Ravetch, 2005).

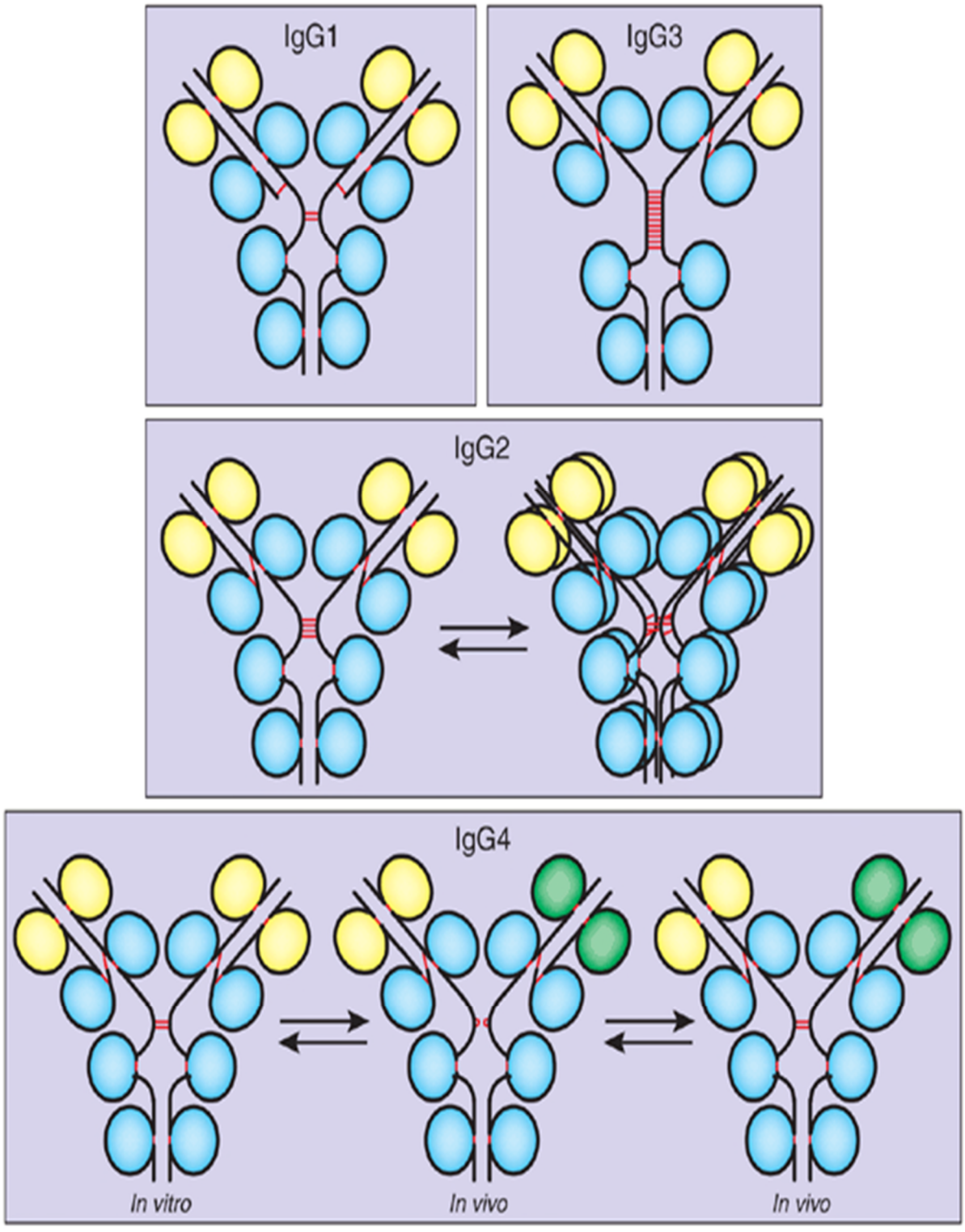


Figure 1-21 : Structure and properties of immunoglobulin G sub-classes (taken from (Salfeld, 2007))

1.3.6.3 *Affinity maturation*

Prolonged or repeated exposure to a specific antigen can lead to production of higher affinity antibodies through a process called affinity maturation. This is brought about by the introduction of somatic point mutations in V genes (Berek and Milstein, 1987) (Figure 1-22), and T cell help is essential for this process (Figure 1-23) (Rizzo et al., 1992). Affinity maturation takes place in germinal centres (Berek et al., 1991) through somatic hypermutation that is exclusive to B cell differentiation pathways (Baltimore, 1981). This leads to intra-clonal diversification and accumulation of memory B cells producing high affinity antibodies (Figure 1-22). The frequency of gene mutation is estimated to be one in 10^3 base pairs per cell per division, which is 100 fold higher than for other genes, hence it is called hypermutation. As a result, B cells of various clones are formed and the clones secreting higher affinity antibodies are selected, the rest are removed via apoptosis (Phan et al., 2006). This process is called clonal selection.

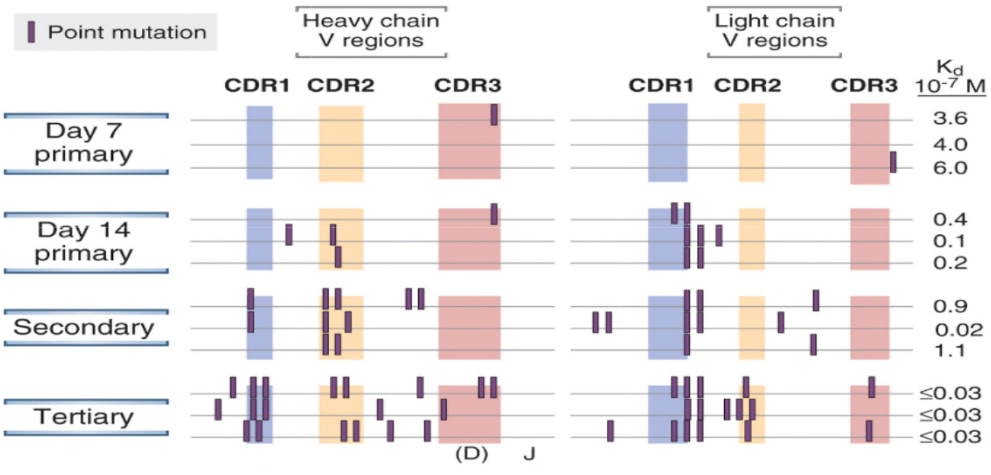


Figure 1-22 : Somatic mutations in Ig V genes and associated change in dissociation constant (K_D)

(Figure taken from chapter 11, Cellular and Molecular Immunology, Elsevier; ref Note here K_d is used to represent K_D)

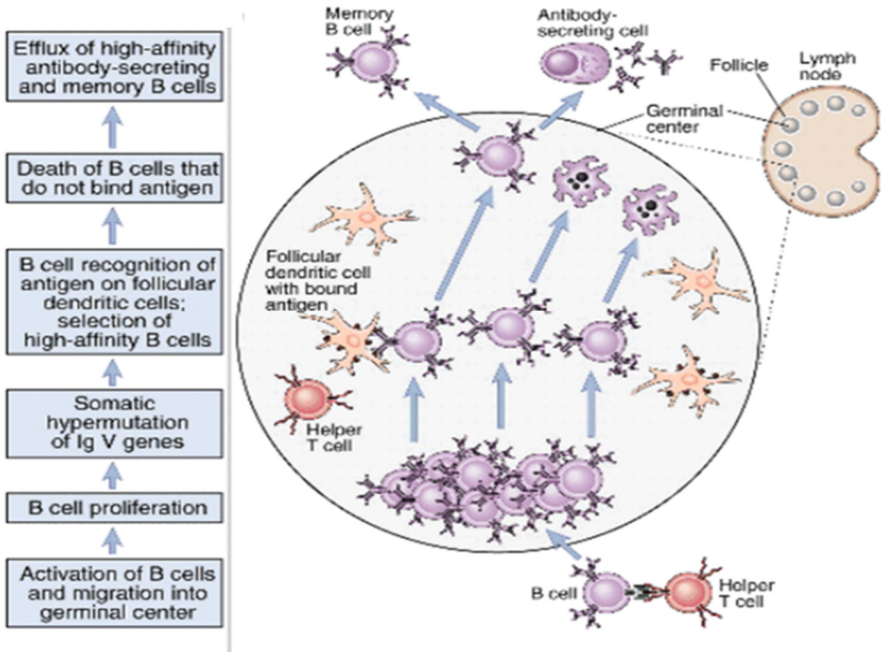


Figure 1-23: Somatic hypermutation and clonal selection

(Adapted from http://wenliang.myweb.uga.edu/mystudy/immunology/ScienceOfImmunology/index.html Elsevier Science)

The selection of high affinity antibody producing B cells could be due to number of mechanisms. The higher affinity immunoglobulin may act as cell surface receptor that binds to antigen and seeks T-cell help for survival, or the immunoglobulin is secreted as antibody which binds to antigen where the immune complexes (Song et al., 1998) are then displayed by follicular dendritic cells in the germinal centre, and binding to the B cell that is secreting higher affinity antibodies. The number of follicular dendritic cells is limited and high affinity interactions are preferred, with no interaction with B cell with lower affinity BCR. Apoptosis of B cells are ensured in absence of interactions with follicular dendritic cells. Follicular dendritic cells prevents B cell apoptosis and this action is maintained through Toll-like receptor (TLR)-4 activity (Garin et al., 2010). Thus antibodies produced by plasma cells can

give feedback to germinal centre B cell responses. Immunisation with pre-formed immune complex or administration of IgG shortly after antigen exposure results in augmented germinal centre formation and hypermutation compared to administration of antigen alone (Song et al., 1998). The T cell help may also drive clonal selection and this could be due to endocytic receptors other than BCR. The progeny of high affinity, isotype switched B cells differentiate into plasma cell and memory B cells.

The cycle of somatic hyper mutation could continue unchecked resulting in high affinity oligoclonal antibodies. But heterogeneities of affinity are observed and this could be due to B cell selection due to other favourable factors that enhances cognate interactions with helper T cells and not just affinity-driven selection (Eisen, 2014). Additionally there are regulation pathways that act as check points to arrest the process. A antibodies could form immune complexes in the circulation and limit antigen access and thus antigen uptake which can lead to inhibition of T:B cell interactions and ultimately lead to lymphocyte death by neglect (Zhang et al., 2013). Other possible mechanisms wherein immunoglobulin class switching may provide regulation is by reducing antibody potency once the response has advanced.

1.3.6.4 *Binding region for Fc-gamma receptors*

The Fc portion on the antibodies has binding sites for Fc-gamma receptors on immune cells (e.g. dendritic cells, NK cells and monocytes). The interactions between immune complex and Fc gamma receptors on dendritic cells allow the processing and presentation of antibody-antigen complexes leading to effective stimulation of helper T cells and effector cytotoxic T cells. Micro-array analysis from tissue of kidney biopsy with antibody mediated rejection, showed evidence of Natural Killer cell signalling mediated through FCyRIIIA (Hidalgo et al., 2010, JM et al., 2015). This may be another possible mechanism of antibody-mediated cell cytotoxicity that is independent of the complement system (see Figure 1-24).

There are different types of FcyR. The FcyRIIB receptor is inhibitory and has been shown to control plasma cell apoptosis (Xiang et al., 2007). This receptor is of lower affinity compared to other FcyR. Binding of IgG to FcyRII and cross talk with the BCR results in an increase threshold for B cell activation and reduction in antibody production (see Figure 1-23) In addition to this it has other inhibitory mechanism and thus results in regulation of humoral immune response (Smith and Clatworthy, 2010). Polymorphisms in FCyRIIB and underexpression increases risk of developing auto-immune disease such as systemic lupus erythromatosis (SLE) (Clatworthy et al., 2007). However, the polymorphism in FCyRIIB gene in a study of renal transplant recipients did not show association with higher pre-transplant PRA, acute rejection in first year and patient or graft survival at 10 years (Clatworthy et al., 2014).

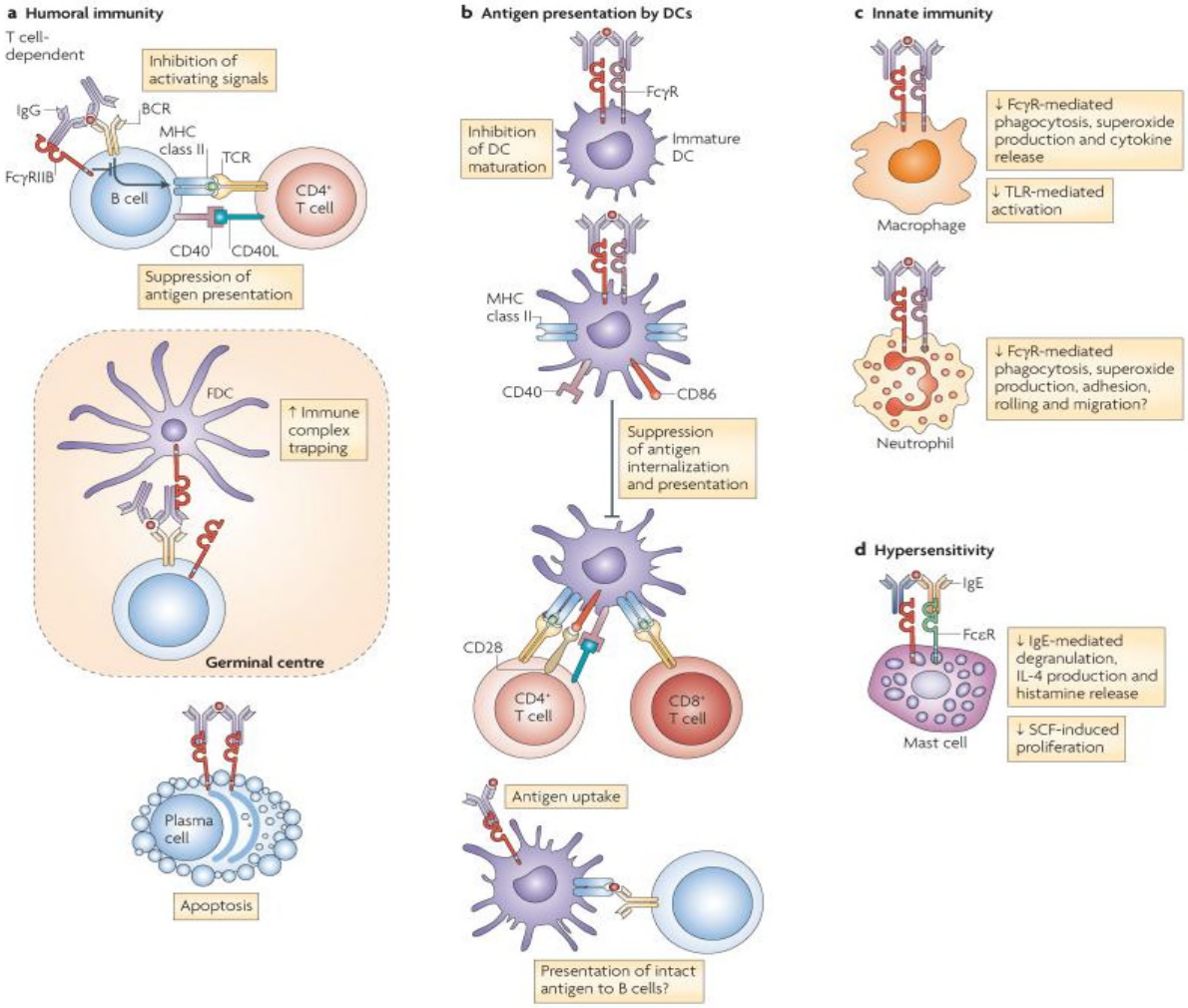


Figure 1-24: Function of inhibitory Fc-gamma receptor IIB (taken from (Smith and Clatworthy, 2010)

1.4 Antibody Mediated Rejection (AMR) of transplanted organ

Acute antibody-mediated rejection happens in about 5-7% of transplanted kidneys of standard immunological risk (Roberts et al., 2012, Colvin and Smith, 2005). In high immunological risks (Antibody incompatible transplantation) the prevalence is about 50% (Higgins et al., 2011b). Following antigen-antibody interaction, the Fc portion of the antibody activates effector mechanism such as Fc receptors and the complement system, and this ultimately results in a pathogenic response effecting graft function (Figure 1-25).

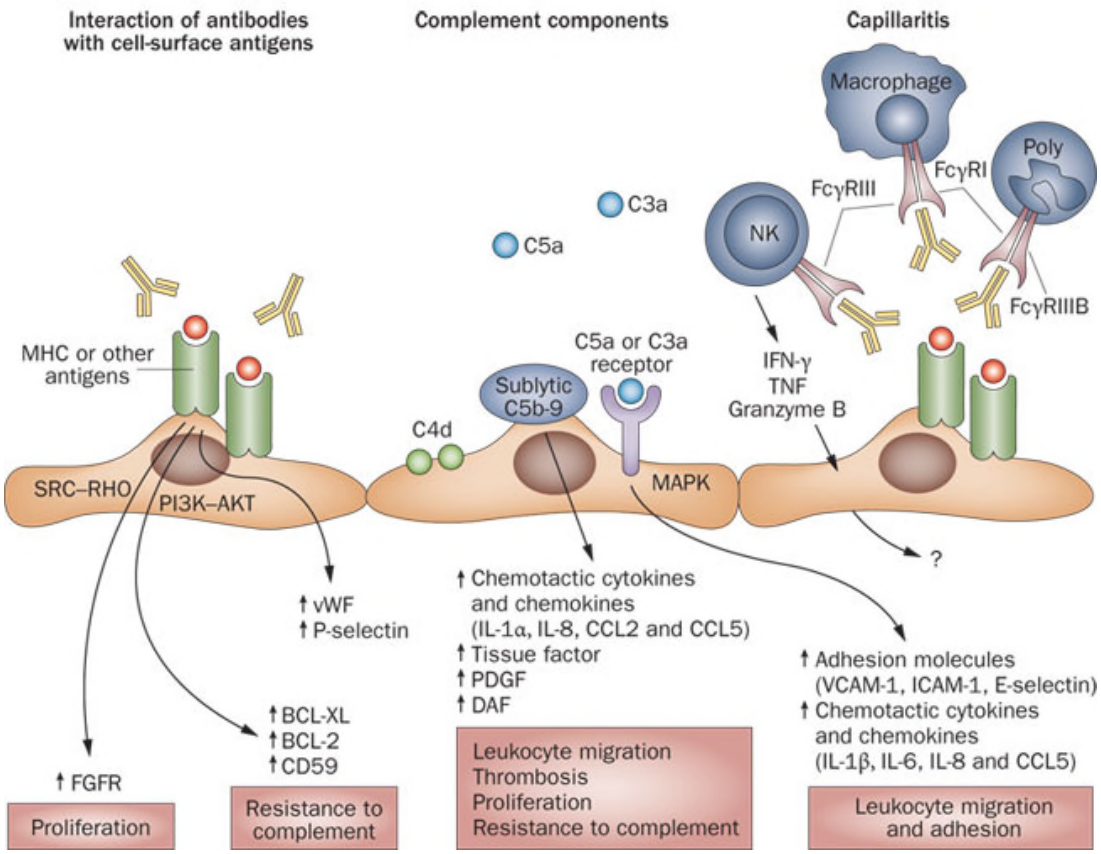


Figure 1-25 : Mechanisms of donor-specific antibody-mediated endothelial injury in renal allografts (Farkash and Colvin, 2012)

The histology of antibody-mediated damage is evolving and has been defined by a consensus of histopathologist across the world (leading to the Banff criteria) (Haas et al., 2014, Solez et al., 2007, Sis et al., 2010).The difficulties faced in making clear diagnoses include the inability to visualise immunoglobulin histologically and also the lack of specificity of complement binding and activation. Due to this there is a big gap between what we can see histologically and what might be happening immunologically.

1.4.1 Acute/active AMR; all three features must be present for diagnosis [clarify this is the current BANFF] see Figure 1-26

1. Histologic evidence of acute tissue injury, including one or more of the following:
    a. Microvascular inflammation (g>03 and/or ptc>0)
    b. Intimal or transmural arteritis (v>0)
    c. Acute thrombotic microangiopathy, in the absence of any other cause
    d. Acute tubular injury, in the absence of any other apparent cause
2. Evidence of current/recent antibody interaction with vascular endothelium, including at least one of the following:
    a. Linear C4d staining in peritubular capillaries (C4d2 or C4d3 by IF on frozen sections, or C4d>0 by IHC on paraffin sections)
    b. At least moderate microvascular inflammation ([g+ptc]>2)
    c. Increased expression of gene transcripts in the biopsy tissue indicative of endothelial injury, if thoroughly validated
3. Serologic evidence of donor-specific antibodies (DSAs) (HLA or other antigens)

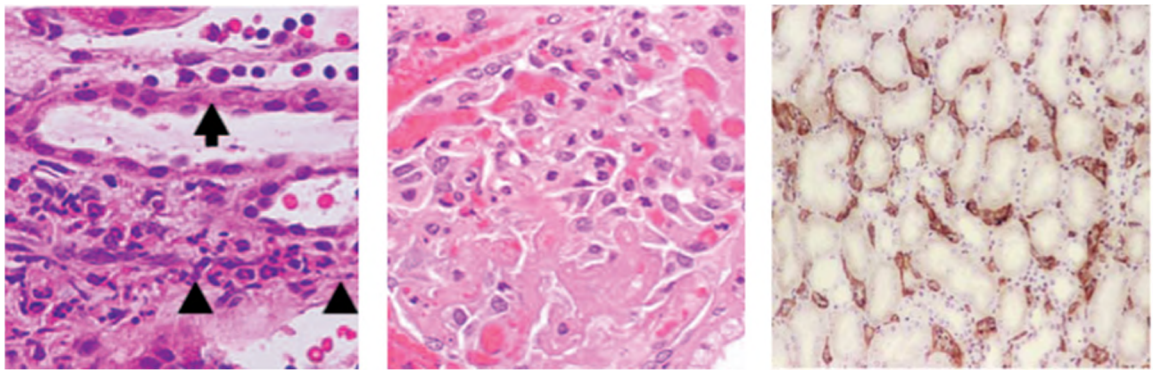


Figure 1-26: Histology of acute antibody mediated rejection.

Left – peri-tubular capillaritis (arrow showing mono-nuclear cell); middle – glomeruli capillary thrombosis; right – C4d staining positive**.**

1.4.2 Chronic, active AMR; all three features must be present for diagnosis

1. Morphologic evidence of chronic tissue injury, including one or more of the following:
    a. Transplant glomerulopathy (TG) (cg>0), if no evidence of chronic thrombotic microangiopathy
    b. Severe peritubular capillary basement membrane multilayering (requires EM)
    c. Arterial intimal fibrosis of new onset, excluding other causes
2. Evidence of current/recent antibody interaction with vascular endothelium, including at least one of the following:
    a. Linear C4d staining in peritubular capillaries (C4d2 or C4d3 by IF on frozen sections, or C4d>0 by IHC on paraffin sections)
    b. At least moderate microvascular inflammation ([g+ptc]>2)
    c. Increased expression of gene transcripts in the biopsy tissue indicative of endothelial injury, if thoroughly validated
3. Serologic evidence of DSAs (HLA or other antigens)

1.4.3 C4d staining without evidence of rejection; all three features must be present for diagnosis

1. Linear C4d staining in peritubular capillaries (C4d2 or C4d3 by IF on frozen sections, or C4d>0 by IHC on paraffin sections)

2. g=0, ptc=0, cg=0 (by light microscopy and by EM if available), v=0; no TMA, no peritubular capillary basement membrane multilayering, no acute tubular injury (in the absence of another apparent cause for this)

3. No acute cell-mediated rejection (Banff 97 type 1A or greater) or borderline changes

1.5 Varied pathogenic response to donor specific antibody

There has been intense debate with regards to pathogenicity of HLA-specific antibodies and the humoral immune theory in transplantation. We have observed varied graft responses in the presence of donor HLA-specific antibodies, in some instance causing acute rejection and in other instances existing with perfectly functioning grafts or resolution of rejection in the presence of high levels of antibodies (Higgins et al., 2007) (See Figure 1-27).

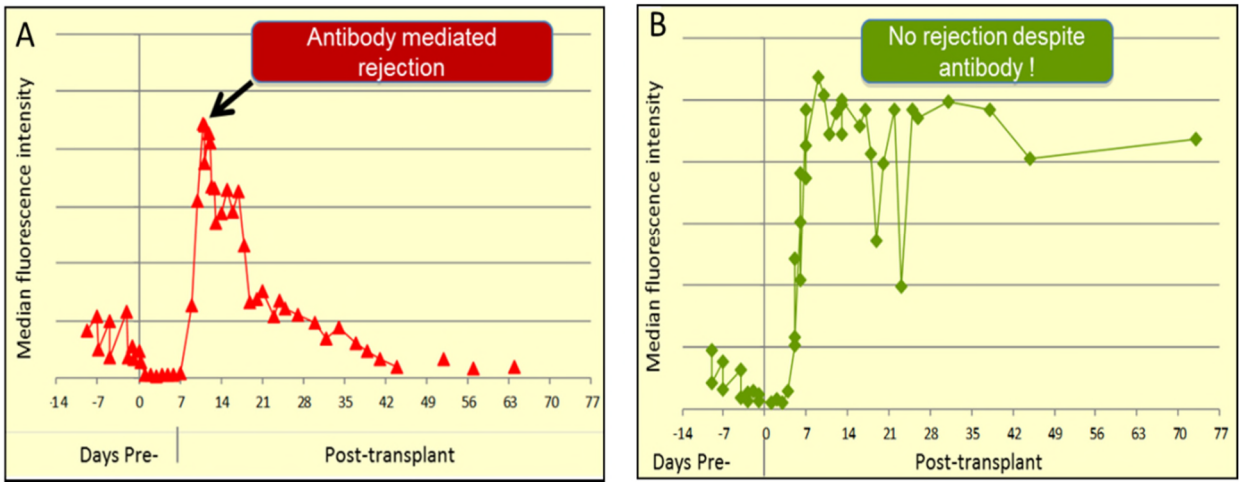


Figure 1-27 : Variable graft response to donor HLA-specific antibody (DSA)

A – Case who develops graft rejection accompanied with rise of DSA and B – Case whose graft is functioning perfectly normal despite a steep rise of DSA

Similar findings have been reported in prospective studies of *de novo* HLA-specific antibodies; the positive predictive value of rejection from *de novo* HLA-specific antibodies is poor (Cooper et al., 2011, Gill et al., 2010). However, these studies were only followed for short periods of time. Longer-term study demonstrated poorer outcomes of grafts that develop *de novo* DSA (Wiebe et al., 2012). They suggested a model for *de novo* HLA-specific antibodies (Figure 1-28). However there may be a complex overlap with the presence of antibodies and graft damage (Figure 1-29).

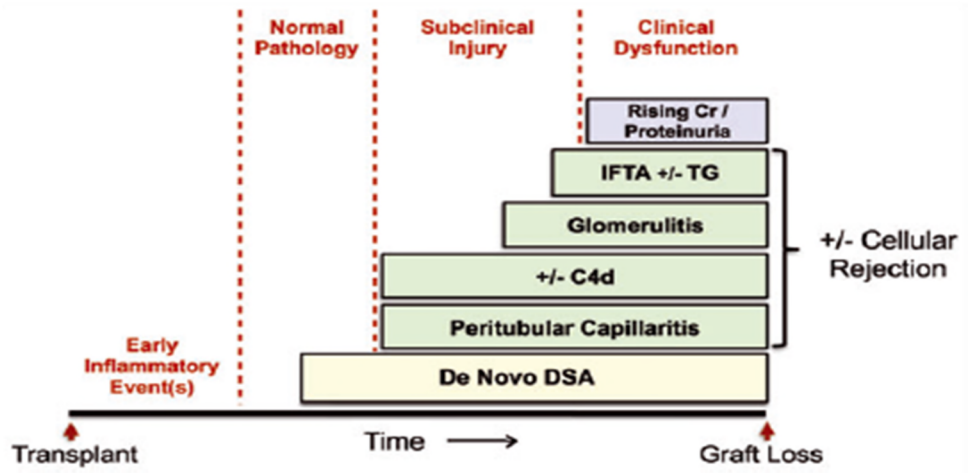


Figure 1-28 : Spectrum of Antibody mediated damage (Wiebe et al., 2012)

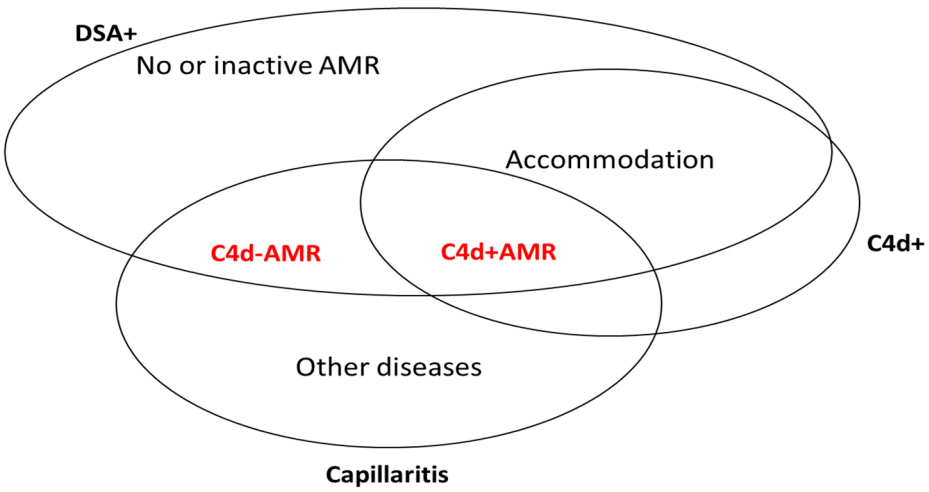


Figure 1-29 : Overlap of DSA and histology of antibody mediated rejection (AMR)

These variable responses could be due to antibody-specific factors (see Figure 1-33), and/or host responses. However, although the mechanisms that underlie these variable responses are complex, it is important to note that the mere presence or absence of antibodies does not determine adverse events; either there is change in the immunological milieu or graft environment, or there is change in the characteristics of these antibodies that determine immunological response and graft fate.

1.5.1 Mechanisms of variable responses of HLA-specific antibodies

1.5.1.1 *Class switching*

It is generally accepted that pre-formed IgM donor HLA-specific antibodies are not a contraindication for transplantation (Suzuki et al., 2009, Chapman et al., 1986, Taylor et al., 1989). The consequences of *de novo* HLA-specific IgM post-transplantation are variable and poorly studied. The development of donor-specific IgM in itself may not be damaging to allografts (Everly et al., 2014, Marcen et al., 1988) but subsequent class switching from IgM to IgG has been associated with poorer outcome (Lietz et al., 2005). Others have found that post-transplant donor-specific IgM responses are harmful (Stastny et al., 2009, Piatosa et al., 2011, Bentall et al., 2014). Variations in immunosuppressive drugs, and different methods for identifying the antibodies might in part explain these inconsistent observations. A larger study determining exact association is required.

IgG DSA may result in variable responses and this could be due to different IgG sub-class profiles. At our Centre in Coventry, we have shown significant heterogeneity of subclasses of IgG in cases with pre-formed HLA-specific antibodies (Lowe et al., 2013a) (See Figure 1-30). Studies on pre-formed HLA-specific antibodies have suggested that IgG (specifically IgG1 or IgG3 subclasses) are associated with rejection (Chapman et al., 1986, Griffiths et al., 2004, Lowe et al., 2011a). This may be due to different complement fixing abilities (Bindon et al., 1988). IgG4 DSA are noted to be higher in patients with long-term graft function (Gao et al., 2004). The effects of subclass variation pre-transplant is not significant in terms of outcome following kidney transplantation (Hoenger et al., 2011). Studies at our Centre showed high

total IgG4 DSA pre-transplantation is associated with higher rejection rates and the presence of IgG4 pre-transplant is associated with poor 10-year graft survival (unpublished, submitted). A study of *de novo* HLA-specific antibodies showed worse outcomes in cases that had exclusive IgG1 & G3 subclasses compared to mixed panels of subclasses (Arnold et al., 2013).

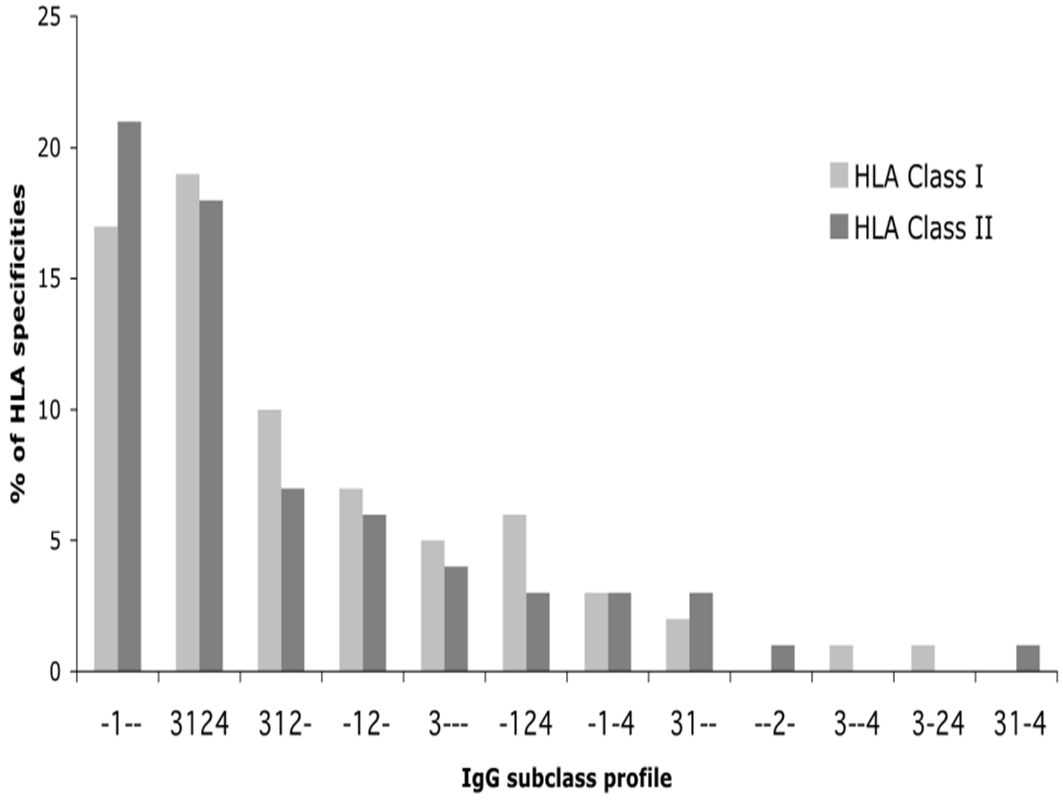


Figure 1-30: Heterogeneity in sub-class profile for 138 HLA-specific antibodies (presence is indicated by IgG subclass number (in order of class switch) and absence as ---)(Lowe et al., 2013a)

1.5.1.2 *Complement fixing ability*

Complement activation following the binding of antibodies to the HLA antigens on endothelial cells is considered to be one of the most potent mechanisms for antibody mediated rejection (see Figure 1-31). This can be studied further by C1q luminex binding (Chen et al., 2011), C4d or C3d flow cytometry or CDC assays. Complement fixing HLA-specific antibodies (C4d & IgG FC positive) have been associated with poorer graft outcome (Wahrmann et al., 2006, Yabu et al., 2011). However, complement-fixing HLA-specific antibodies (C4d & IgG FC) have also been observed in stable renal transplant recipients without mounting pathogenic responses (Bartel et al., 2008). We have shown worse graft survival in HLA-incompatible kidney transplantation recipients with a pre-desensitisation CDC positive status compared to Flow cross match positive or high MFI on single antigen beads alone (Higgins et al., 2011b, Higgins et al., 2014a). A study using a C1q binding assay protocol showed that the presence of C1q fixing antibodies are associated with worse graft function (Loupy et al., 2013). The C1q positivity, however, is related to higher IgG MFI levels (Schaub et al., 2014) and hence many don't feel it provides additional value over routine IgG MFI measurements. Recent study showed measurement of C3d was better at predicting worse graft outcome compared to C1q binding assays (Sicard et al., 2015). The complement fixing ability of an antibody is determined by its class, subclass and degree of affinity maturation. However, it is important to appreciate that antibodies can also mediate complement independent damage.

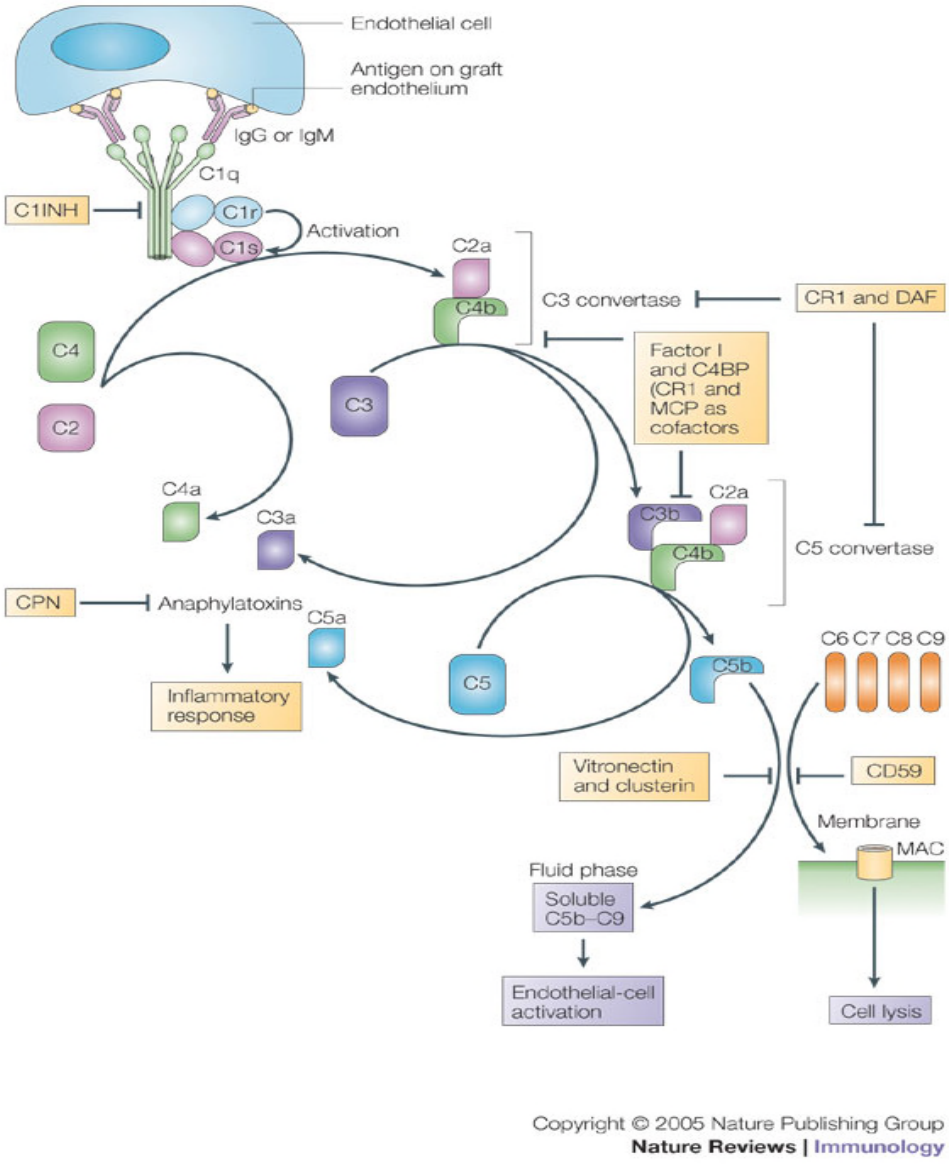


Figure 1-31: Classical pathway of complement activation by antigen–antibody complexes (Colvin and Smith, 2005)

1.5.1.3 *Affinity maturation*

Studies have shown that profiles of antibodies demonstrate heterogeneous affinity across the immune response, and that higher affinity antibodies cause increased pathogenic responses (Williams et al., 1999, Werblin et al., 1973). This has been studied for autoantibodies against double-stranded DNA, ANCA and GBM but not for HLA-specific antibodies (see section 1.6). There are no studies that have looked at this directly and a recent study speculated that higher binding strength of the interaction between the HLA epitope and HLA-specific antibody, results in higher binding energy release which brings conformational change to the immunoglobulin molecule; particularly to the complement fixing Fc portion. Thus it has been speculated that complement fixation could serve as a surrogate marker for higher affinity interactions (Duquesnoy et al., 2013). (See Table 1-4 where a monoclonal HLA-specific antibody is classed as higher affinity if it causes a reaction on all three assays (Luminex SAB, C1q and CDC assay)).

Reactivity	Binding energy	Interpretation
Negative in all 3 assays	0	No specific epitope recognition
Only Lum-Ig positive	+	Epitope is recognised but insufficient conformational change in antibody
Lum-Ig positive Lum-C1q positive CDC negative	++	Epitope is recognised and conformational change in antibody exposes C1q-binding site but no complement activation
Positive in all 3 assays	+++	Epitope is recognised and C1q-binds on antibody associated with conformational change in C1q that activates C1qrs complex and classical complement cascade

Table 1-4 : Mechanisms of reactivity of antigen-antibody interactions in various assay (Duquesnoy et al., 2013)

The authors have further postulated that the reactivity and strength depends on interaction of antibody paratope with eplets and other residues within 15 Angstrom of the eplet in the structural epitopes. The larger area of interactions will depend on the difference in presence of critical amino acid residues around the functional specific epitope, which gives further stability to the interaction. (Figure 1-32). The larger the area of interaction will give rise to stronger binding force / affinity. HLA epitopes are discussed in more detail in section 1.7.

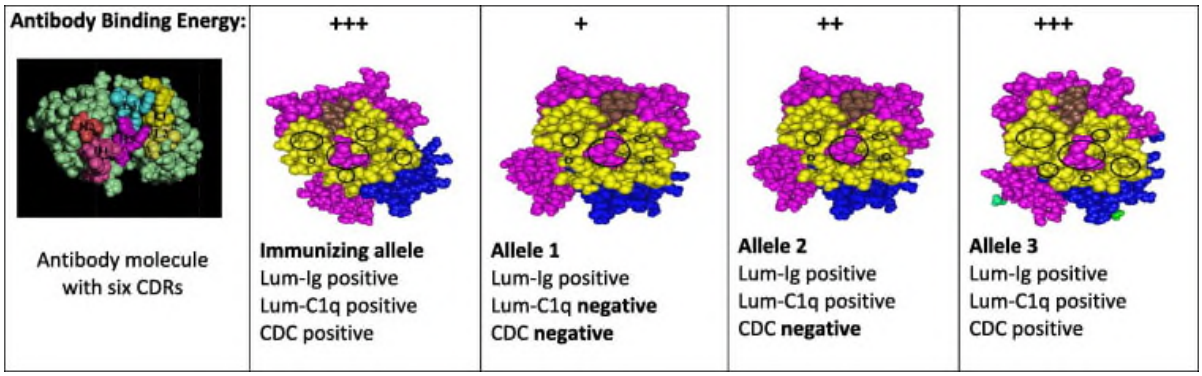


Figure 1-32: Mechanism of reactivity of antibody in various assays(Duquesnoy et al., 2013).

Note the reactivity is dependant of surface area of interactions. The central circle with pink residues is common to all the alleles but the area of interactions around the central area in the yellow region circled differs. Overall fits between the Allele 3 and the immunizing allele is same and hence highest reactivity compared to other alleles (Duquesnoy et al., 2013).

1.5.1.4 *Other factors*

Antibodies against HLA may have different effect depending on class of HLA; e.g. DSA antibodies to Class II HLA is considered as not higher risk if present pre-transplantation (Susal and Opelz, 2004). Levels of MFI values are associated with poor outcomes (Mizutani et al., 2007, Higgins et al., 2009b). However it is not clear if the association of high MFI levels is due to higher concentration or higher affinity of HLA-specific antibodies.

Glycosylation pattern of HLA-specific antibodies may play important role in pathogenesis. However, this has not been studied in a clinical setting. Similarly the effect of antibodies may be neutralised by the presence of soluble HLA secreted by donor kidneys or by the formation of anti-idiotypic antibodies (King et al., 1989, Suciufoca et al., 1991). Polymorphisms of the Fc gamma receptor on immune cells have been shown to affect certain immune responses. Fc gamma receptor III polymorphisms (158V/F) demonstrated varied effects on T cell depletion following administration of anti-thymocyte globulins (ATG) (Ternant et al., 2014). Regulation of immune response by IgG and activating Fc gamma receptor has not been studied in detail in the context of human transplantation.

Other than antibody characteristics, activity of cellular immune response primarily T cell cognate interactions with donor-specific B cells may drive chronic rejection. The cytokines (predominantly IFN-γ and IL2) released during cognate interaction can cause direct damage independent of antibodies (Shiu and Dorling, 2014). Figure 1-33 summarises antibody characteristics that has been investigated and in progress.

	Comment
HLA antibody	
Concentration	Not currently possible to measure antigen-specific concentration of antibody
Antibody binding to HLA in solid phase assay (readout a combination of avidity and concentration)	Measureable by microbead assays
Class	Mostly IgG, early reports of IgM mediated AMR and occurrence of IgA
Subclass	Early studies show heterogeneity in responses
Affinity	Measurement under investigation
Glycosylation	Measurement under investigation
Cellular binding	CDC and FC crossmatches Endothelial binding
Inhibitors	
Soluble HLA	Early reports of methods to quantify
HLA-E	Early reports of correlation with clinical outcomes
Idiotypic antibodies	Hard to measure with current tools but could be important
Immune complexes	
HLA-sHLA immune complexes	Not currently measureable

Figure 1-33 : Summary of antibody characteristics that may contribute to damage to transplant organ and current evidence (Higgins et al., 2014a)

1.6 Studies on antibodies in other renal disease

1.6.1 ANCA-associated Vasculitis (AAV)

Screening for this condition is performed via with immuno-fluorescence to show the presence of peri-nuclear or cytoplasmic ANCA. Following this, quantification of MPO or PR3 is done via quantitative ELISA using selective antiserum standards (Hagen et al., 1996). Higher or rising titres have been associated with relapse of ANCA-associated vasculitis. Similarly, IgG3 sub-class levels are particularly associated with active renal disease in ANCA-associated vasculitis (Jayne et al., 1991). A recent study has summarised all the studies that have looked at sub-classes of IgG – ANCA and clinical manifestation (Colman et al., 2007) Table 1-5.

Author, year	cANCA subclasses	pANCA subclasses	Subclass association with clinical effect	Subclass association with neutrophil function
Brouwer *et al*,[9] 1991	IgG1, IgG4*	IgG1, IgG4*	IgG3 associated with renal involvement	—
Esnault *et al*,[10] 1993	—	IgG1, IgG3, IgG4	IgG3 decreased during remission	IgG3 had highest relative functional affinity
Jayne *et al*,[11] 1991	IgG3*	—	IgG3 associated with acute phase of disease	—
Segelmark *et al*,[12] 1993	IgG1, IgG3, IgG4*	IgG1, IgG2, IgG4*	Higher IgG3 cANCA during acute disease	—
Mellbye *et al*,[13] 1994	IgG1, IgG4*	IgG1, IgG4 (no IgG3)	—	All cANCA sera caused deposition of C3c on neutrophils
Mulder *et al*,[14] 1995	IgG1, IgG3, IgG4 (IgG2 not done)	—		IgG3 ANCA were more potent at inducing a neutrophil respiratory burst
Locke *et al*,[15] 1999	—	IgG1, IgG4*	—	—
Harper *et al*,[16] 2001	IgG1, IgG4*	IgG1, IgG4*	—	No particular subdass linked to neutrophil activation
Nowack *et al*,[17] 2001	IgG1, IgG3, IgG4 (IgG2 not done)	IgG1, IgG3, IgG4 (IgG2 not done)	IgG3 cANCA associated with ANCA persistence, multiple organ involvement and grumbling disease activity	—
Holland *et al*,[18] 2004	IgG1, IgG3, IgG4 (IgG2 not done)	—	—	IgG4 cANCA activated a neutrophil respiratory burst

Table 1-5 : IgG-ANCA sub-classes and association with active disease (Colman et al., 2007)

The biophysical characteristics of anti-myeloperoxidase (MPO)-antibodies have been studied in cases with vasculitis (Kokolina et al., 1994). A study of 28 cases showed no association

between isotype, the level, or apparent affinity of anti-MPO antibodies and clinical symptoms and severity of vasculitis. However, higher affinity was observed prior to requirement of treatment and there was decreased apparent affinity following immunosuppressive therapy. During relapse, lower affinity serum IgG was observed. The affinity was studied using a competitive binding MPO-specific ELISA protocol. Higher affinity IgG molecules may not be detectable in serum as they may be adsorbed on to the tissue; causing active disease (Esnault et al., 1991). Using Inhibition binding on solid-phase radioimmunoassay (SPRIA) with 10/20mM DEA (Diethyl amine), the study observed affinity maturation of different sub-classes of IgG and postulated that higher affinity IgG3 antibodies may be more pathogenic than other sub-classes (Esnault et al., 1991). Surface plasmon resonance has been used to study binding interactions between cANCA-IgG from patient sera and purified PR3 antigen in the past to define epitope heterogeneity (Griffith et al., 2001, Colman et al., 2007, Short and Lockwood, 1997).

1.6.2 Anti-GBM disease:

Goodpasture's Disease or Anti-GBM (Glomerular Basement Membrane) disease is characterised by the presence of autoantibodies to the alpha-3 chain of class IV collagen. This is typically associated with alveolar lung haemorrhage and renal failure. The anti-GBM antibodies can be measured using quantitative ELISA. IgG1 class is predominantly associated with active clinical disease (Weber et al., 1988, Zhao et al., 2009). Unlike AAV this is usually a monophasic illness with rare cases of relapses (Levy et al., 1996a). A study using antigen inhibition ELISA defined avidity by EC_{50} (Effective Concentration required for 50% inhibition) and found that high avidity anti-GBM antibodies were associated with increased severity of

disease (Cui and Zhao, 2005). Surface plasmon resonance was used to study interaction of monoclonal antibodies and polyclonal sera from patients with GBM disease (Levy et al., 1996b).

1.6.3 Lupus Nephritis

Systemic Lupus Erythematous (SLE) is another auto-immune condition characterised by presence of anti-nuclear antibody (ANA), and antibodies to DNA (especially dsDNA) and smith (Sm) antigen. Studies have characterised pathogenic phenotypes of these antibodies and have noted similar conclusions as above. IgG1 anti-dsDNA antibodies have been associated with active lupus nephritis (Bijl et al., 2002). dsDNA-specific antibodies of higher relative avidity and cationic charge were associated with active nephritis (Suenaga and Abdou, 1993). Surface plasmon resonance has been used to detect and study the binding interactions of dsDNA-specific antibodies (Buhl et al., 2007). Antibodies against C1q have also been associated with active lupus (Wener et al., 1987) and surface plasmon resonance has facilitated the investigation of the kinetics and affinities anti-C1q antibodies binding to peptides on the C1q molecule (Vanhecke et al., 2012). A recent study in 99 patients showed higher risk of lupus nephritis if the avidity of dsDNA was higher (Andrejevic et al., 2013).

1.7 HLA antigens and epitopes

Human Leucocyte antigens (HLA) are proteins present on most of human cell surfaces and patterns of multiple HLA allele expression are unique to the individual person. These proteins help the Immune system differentiate self from non-self. The genes that encode the HLA molecule are located on chromosome 6. They are high polymorphic, with over one thousand variant alleles having been characterised (Marsh and HLA, 2011).

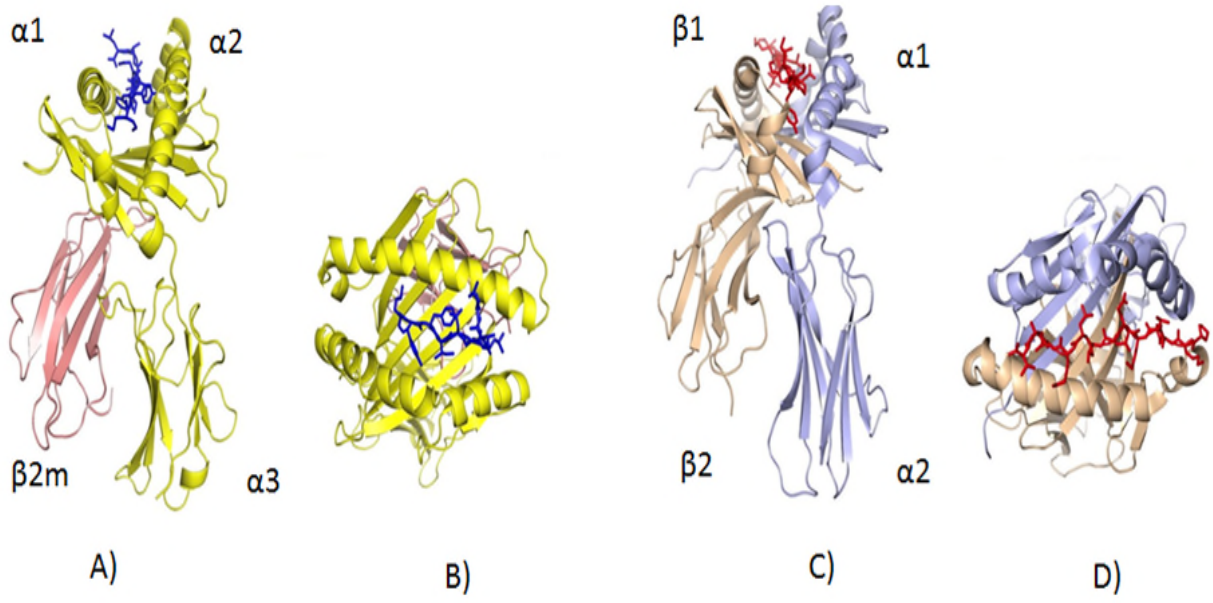


Figure 1-34: HLA molecule structure with peptide in the grove

(Class I on the left (with peptide (blue)) and Class II on right (with peptide (red)) (taken from Immunology lectures by E Gheradi, University of Pavia website). A & C – front view; B&D – top view

The HLA are divided into class I and class II molecules based on their structure and tissue distribution (Figure 1-34). The archetypal class I HLA molecule has one chain with three domains (α1-3) and forms a heterodimer with β2 microglobulin, whereas class II HLA has two homologous chains (α & β) with two domains each. Class I HLA is expressed on all nucleated

cells whereas Class II HLA is restricted to antigen presenting cells, some endothelial cells and the thymus. Each cell typically expresses six class I alleles (A, B and C) and six to eight class II alleles (DP, DQ & DR). These are further characterised based on haplotype. HLA proteins can also be found in soluble form (sHLA) circulating in blood, even in healthy individuals (Rebmann et al., 2009) and donor specific sHLA release is associated with rejection (Devito et al., 1993, Mathew et al., 1996).

Non-self HLA molecules on the donor kidney serve as trigger for intrinsic immune responses within the recipient, in addition to performing direct peptide presentation to T cells potentially causing activation of T and B effector cells. Peptide-dependent HLA-specific antibodies require peptide in the binding groove of the target HLA molecule in order to drive an immune response (Mulder et al., 2005). The ability to induce an antibody response is called HLA immunogenicity and ability to react with specific antibodies is called antigenicity (Duquesnoy and Marrari, 2009). In an antibody-mediated humoral response, the paratope on the variable regions of an antibody molecule binds to the antigenic determinant, called the epitope and subsequently, trigger a cascade of responses (Figure 1-25). More than one epitope can be present on a single HLA antigen (Figure 1-35).

1.7.1 HLA Epitopes

An epitope consists of specific amino acid residues on antigen molecule that interacts and binds to the CDR loops of variable region of antibody molecule. The structural epitope has 15-25 contact amino acid residues in an area of 700-900 mA. Within the structural epitope, certain amino acid residues (4-8) are responsible for specific binding to CDR loops of antibody, and these residues constitute the functional epitope. The accessible residues are called eplets and are responsible for HLA-specific antibody responses (antigenicity) and immunogenicity (Duquesnoy et al., 2012).

Epitope mapping improves our understanding of the protein structure-function relationship that is important for biological processes. As multiple epitopes can be present on one HLA-protein, and some are shared between HLA-proteins, epitope mapping allows one to understand whether the reactivity is due to shared epitopes or not. Additionally, epitope mapping may explain the development of *de novo* antibodies due to epitope spreading following kidney transplantation (Yang et al., 1999, Papassavas et al., 2000, Nainani et al., 2009). To date 110 epitopes to HLA class-I have been defined; 34 for HLA-A and 44 for HLA-B locus antigens (El-Awar et al., 2009). The epitopes can be defined using monoclonal antibodies or eluted alloantibodies using recombinant HLA class I cell lines (El-Awar et al., 2009) or by using computer algorithms (e.g. HLA-match maker) to predict epitopes from stereochemical modelling of epitope-paratope interfaces of antigen-antibody complexes (Duquesnoy and Marrari, 2009, Duquesnoy et al., 2012). Table 1-6 shows an example of an epitope against HLA-A locus antigens.

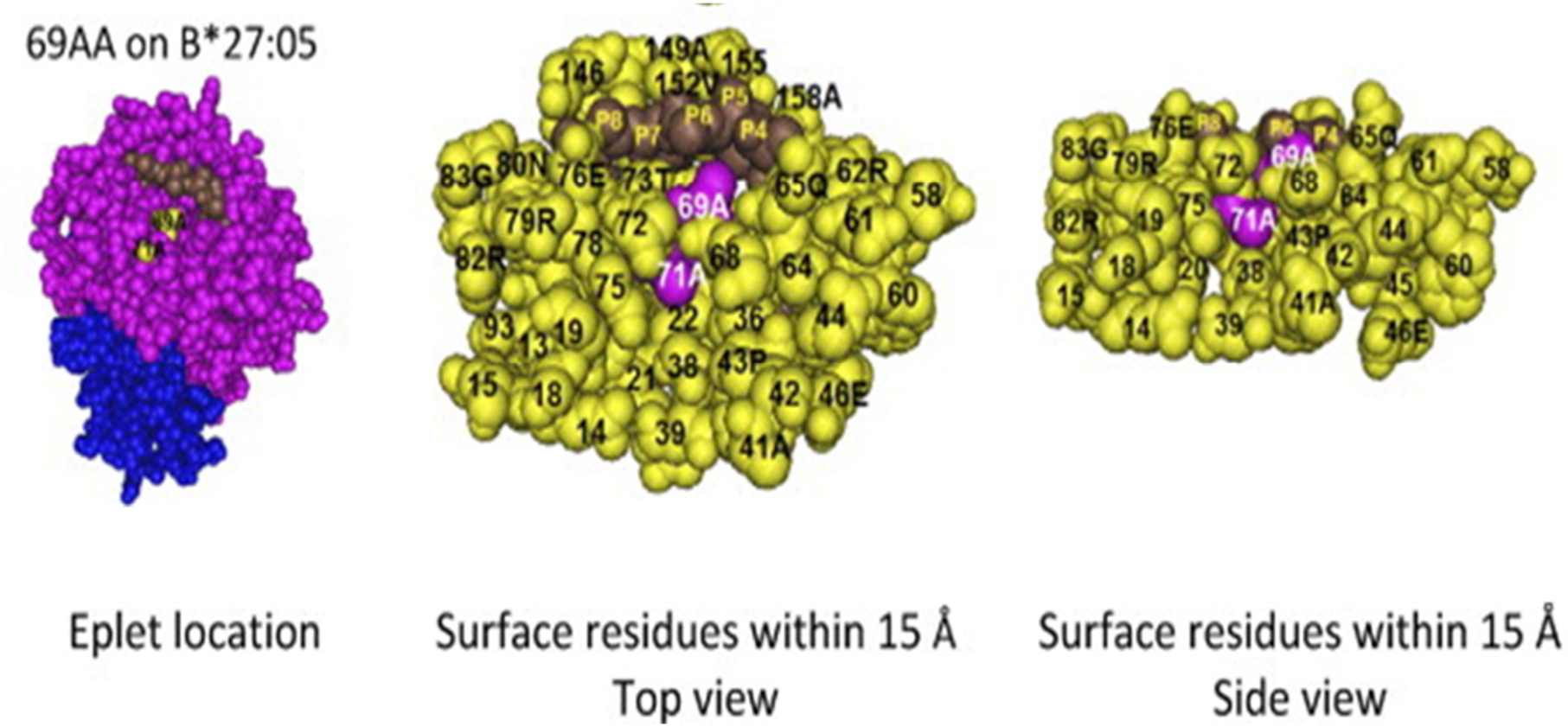


Figure 1-35: Molecular models of crystalline HLA structure (Duquesnoy et al., 2012)

Note: yellow area represents structural epitope on the antigen molecule (left) and pink areas (69A and 71A) within defines specificity (middle and right image - top and side view of surface residues within 15A radius.

Epitope Specificity	A1	A11	A2	A23	A24	A25	A26	A29	A3	A30	A31	A32	A33	A34	A36	A43	A66	A68	A69	A74	A80
44K+	■														■						
107W			■																■		
65G				■	■																
149T						■	■							■		■	■				
62L								■								■					
161D									■												
65Q																					■
163R	■	■				■	■									■	■				
144K	■	■	■		■				■						■			■	■		■
166D+	■			■	■																■
76A	■						■	■							■	■					■
90D	■	■				■	■							■	■	■	■				■
142T+			■															■	■		
127K			■	■	■													■	■		
80I				■	■	■						■									
82L+				■	■	■						■									
149T			■			■	■							■		■	■				
62E				■	■																■
56E+/62E+/163E+																					■
19K		■																			
56R										■	■										
167W		■	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	
109L												■								■	
56R+73T										■											
253Q			■			■	■	■			■	■	■	■		■	■	■	■	■	
62G+			■																		
127K+/151R+				■																	
156Q+					■																
109L+163T												■								■	
109L+131R												■								■	
158V															■						
144K+	■	■			■				■						■						■
163R+		■				■	■									■	■				
76V+144K		■	■						■									■	■		
125E+156W			■			■	■							■		■	■				
80I+				■	■							■									
138M+144Q				■		■	■	■		■	■	■	■	■		■	■			■	
62R+						■	■						■	■			■	■	■		
62R+163T													■	■				■	■		
90H+171H													■								
166E+																	■				

Table 1-6 : Class I HLA-A locus epitope specificities and the associated HLA antigen (adapted from Dave Lowe PhD thesis, University of Warwick)

1.8 Current assays to characterise HLA antigen and HLA-specific antibody interactions: advantages and limitations

There are a number of assays developed to measure and characterise HLA-specific antibodies in transplantation medicine (Figure 1-36). The assays can broadly be divided into two categories: 1) Cell based assay and 2) Solid phase assay.

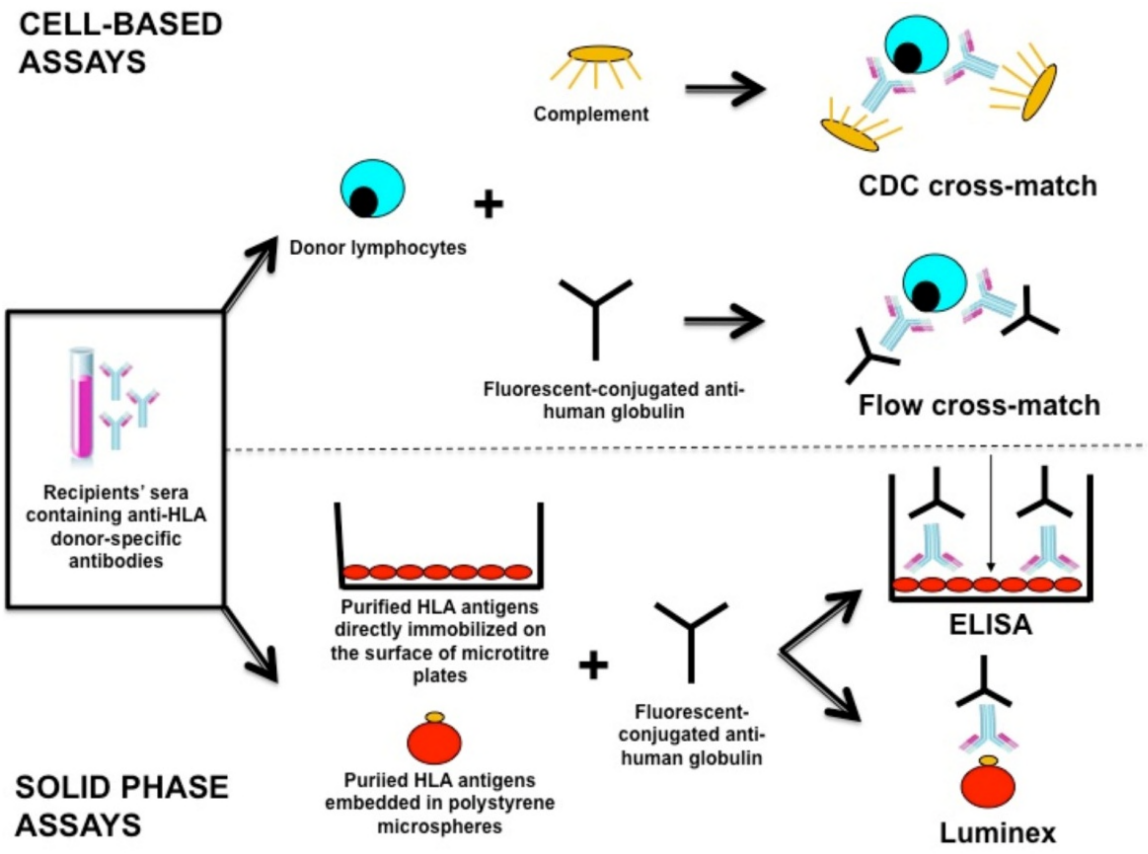


Figure 1-36 : Assays to measure and characterise HLA-specific antibodies

(Taken from Current Issues and Future Direction in Kidney Transplantation edited by Thomas Rath, Publisher: InTech February 13, 2013 DOI: 10.5772/45909)

1.8.1 Cell based assays

1.8.1.1 *CDC (Complement dependent cytotoxicity) assay*

First described in 1964 (Terasaki and McClelland, 1964, Terasaki and Rich, 1964), CDC has now become the international standard test prior to any organ transplantation (Patel and Terasaki, 1969). Donor lymphocytes are used to detect complement-fixing antibodies. Briefly, the donor lymphocytes are isolated from peripheral blood, spleen or lymph nodes. The donor lymphocytes are further separated in to T cell and B cell compartments and recipient's serum is mixed in the well followed by rabbit serum containing a source of active complement. If donor-specific antibodies are present, they binds to the donor cells and the terminal complement cascade is activated resulting in the lysis of lymphocytes.

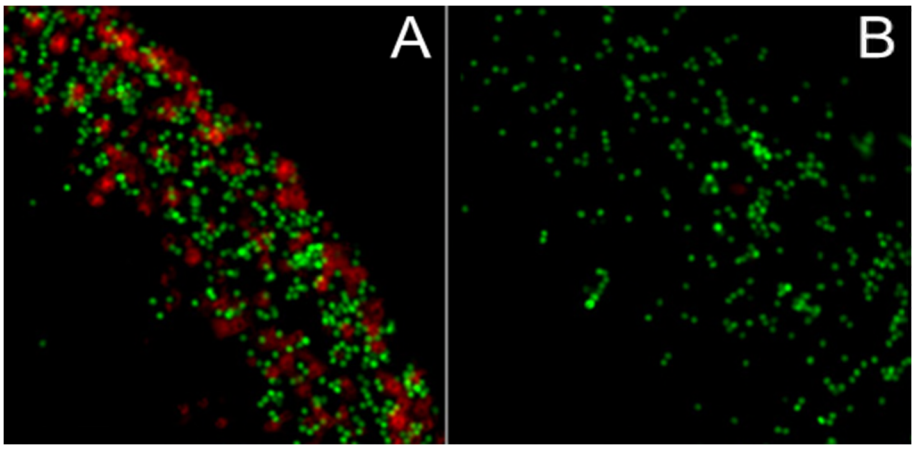


Figure 1-37 : Complement Dependent Cytotoxicity (CDC) assay

Note: A = cell lysis seen in red colour, positive results, B = absence of lysis (red colour) – negative result (Seen through microscope) (adapted from Dave Lowe PhD thesis, University of Warwick)

The results are expressed as percentage of cell lysis and the serum can be titrated down to obtain a titre at which there is no lysis of donor lymphocytes (Figure 1-37). The assay is not

specific for any particular anti-HLA antibody nor its class and is usually a summative result of all antibodies (HLA or non-HLA; Allo or Auto-antibodies; IgG or IgM) present in the serum of the recipient. However despite being relatively non-specific, a positive CDC result pre-transplant is associated with worse outcome (Higgins et al., 2011b) (see Figure 1-10). The CDC assay can be made more specific by treating sera with dithiothreitol (DTT) (removing autoantibodies of IgM class) and sometimes treating sera with autologous lymphocytes (to remove auto-antibodies). AHG (anti-human globulin) enhancement improves sensitivity of the assay by allowing detection of non-complement fixing antibodies.

1.8.1.2 *Flow cytometry*

This was first described in 1983 (Garovoy et al., 1983) and is more sensitive than CDC assay as it can detect both complement fixing and non-complement fixing donor specific antibodies. Additionally, it is able to detect the antibody class without any modification of test serum. Briefly, recipient's serum is mixed with donor lymphocytes. Fluorescence labelled anti-human IgG monoclonal antibody is added to detect the presence of donor-directed. The results are expressed as relative median frequency (relative to a negative control) and shifts to right on scatter plots suggest positive results (Figure 1-38).

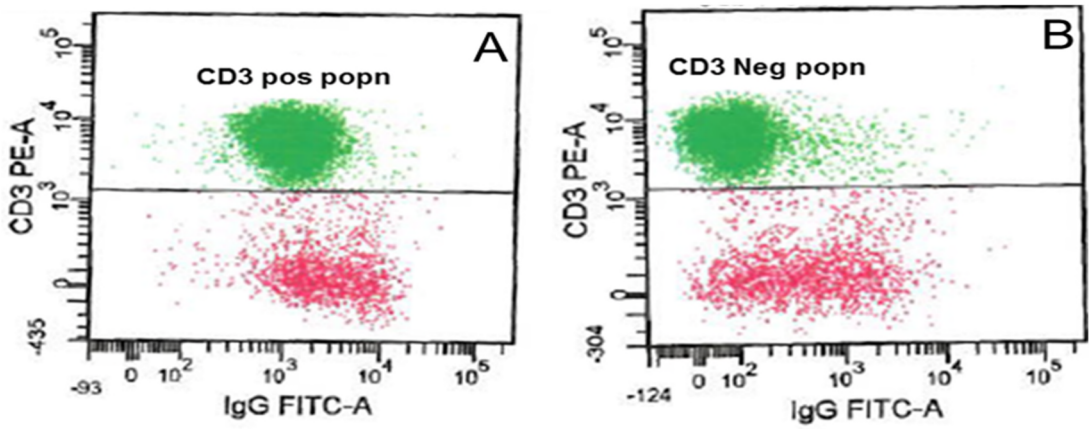


Figure 1-38 : Flow cytometry cross match

Note positive result suggested by rightward shift as shown in A. (adapted from Dave Lowe PhD thesis, University of Warwick)

1.8.2 Solid phase assays

With the advent of purified or recombinant- HLA molecules, solid phase assays using ELISA on microwell plates, and fluorescent beads (Flow and Luminex) were developed to detect HLA-specific antibodies. Currently, the Luminex platform based assays are used extensively in the UK.

1.8.2.1 *Screening and single antigen bead assay to determine HLA-specific antibodies:*

The commonest screen used is based on multiplex bead assay using the Luminex platform. Typically 100 different antigen coated beads can be used to identify HLA-specific antibodies. There are two types of beads – Mixed beads used to screen for HLA-specific antibodies and SAB (Single Antigen Beads) that characterise specificities against single antigens (Figure 1-39).

The Luminex assay is very sensitive and positive cut-off values vary between laboratories due to lack of clarity and consensus for clinically relevant thresholds. The cut-off could range from 500 to 3000 MFI and is based on experience and protocol of individual Centres. Even with standardisation of the protocol, the coefficient of variation is around 25% (Reed et al., 2013). MFI levels are affected by artefacts (Zachary et al., 2009) or presence of IgM HLA-specific antibodies (hence treatment with DTT (Kosmoliaptsis et al., 2010)) or presence of complement proteins (Weinstock and Schnaidt, 2013) (hence treatment with EDTA (Schnaidt et al., 2011)). Finally, care should be taken to interpret the results, as some would equate the MFI values to concentration of the HLA-specific antibodies.

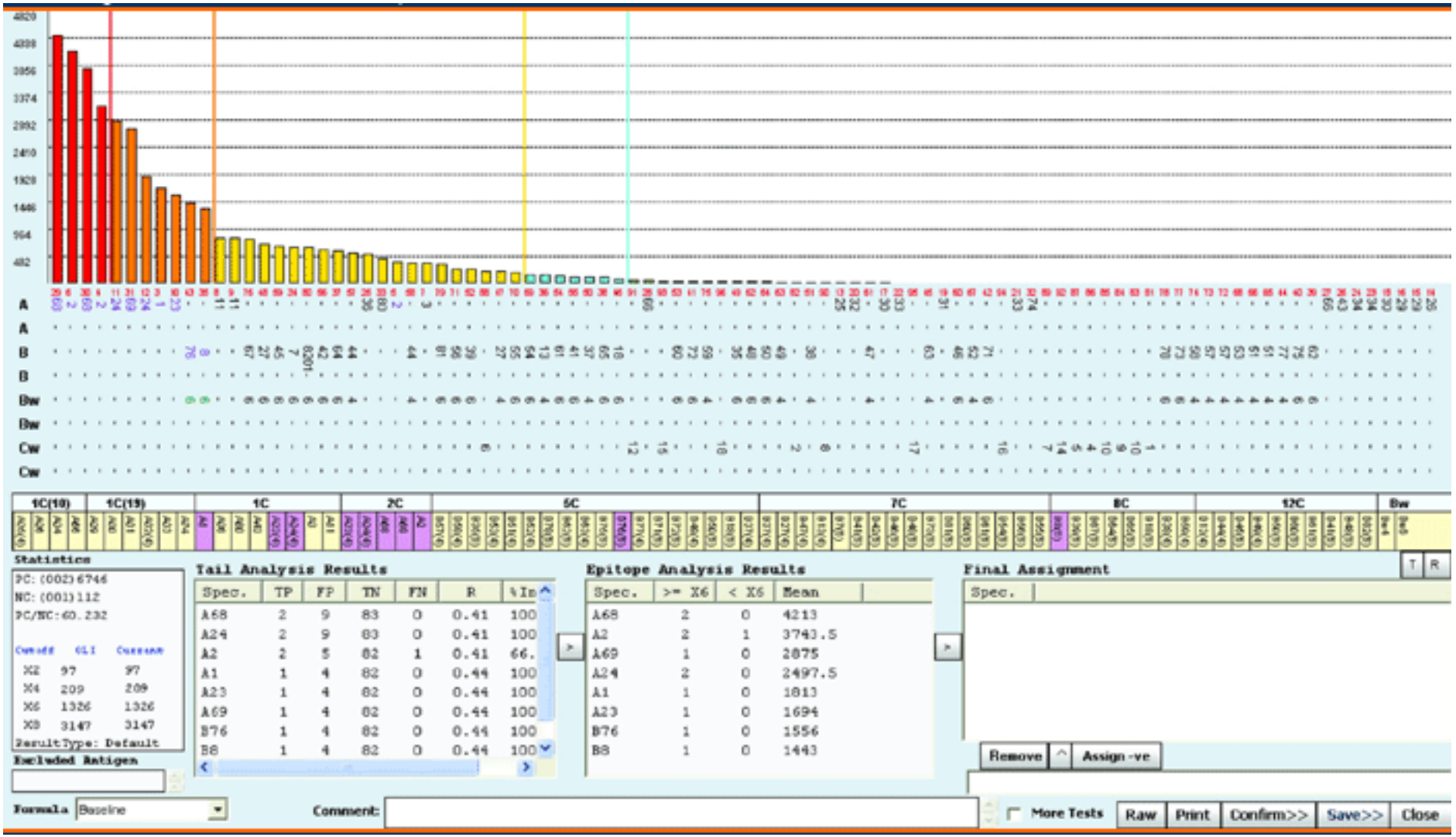


Figure 1-39 : Read-out from HLA fusion software on Single antigen bead Luminex assay (source – Medscape)

Work from our Centre has shown that there is a *'hook effect'* and the assay has a tight linearity range (Lowe et al., 2011b, Lowe et al., 2007) (See Figure 1-40). When the concentrations of antibodies are high, there is a competition for binding site resulting in false low levels, this phenomenon is called '*hook effect*'. Also as this is a protein-protein binding assay, the results are likely to be influenced by affinity in addition to the absolute concentration of the antibodies (Nimmo et al., 1984, Aldao and Vides, 1984).

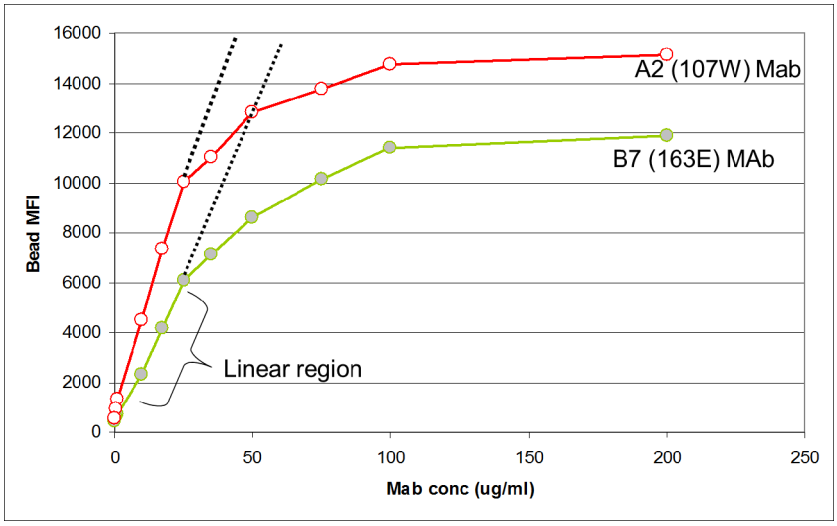


Figure 1-40: Linear range of Luminex assay (Note dose response curve obtained for two human monoclonal HLA-specific antibody (Lowe et al., 2011b).

1.8.2.2 *C1q assay to assess complement fixing properties of HLA-specific antibodies*

Single antigen bead (SAB) based assays described above were modified and an intermediate step is included in which C1q protein is added. C1q binds to the antigen-antibody complexes and is then detected by an anti-C1q PE conjugate in same way as with the normal SAB assay. Although specific for HLA, the results obtained could be due to other classes of HLA-specific antibodies (especially IgM) that are bound to the recombinant-HLA proteins. Additional characterising of HLA-specific antibodies detected by standard SAB Luminex assay has been shown to be associated with worse outcomes (Loupy et al., 2013, Yabu et al., 2011). However the additional value of this C1q assay is questioned as the positive result correlated with pan-IgG levels (Schaub et al., 2014).

1.9 Strength (affinity/avidity) of antigen-antibody interactions

The strength of binding between a single combining site of an antibody and an epitope of an antigen is called the affinity of the antibody. This has not been studied directly in clinical transplantation and is postulated that binding of antibodies to the epitopes can be inferred indirectly by its ability to activate complement cascade following binding to the HLA-antigen (Duquesnoy et al., 2013).

Binding interactions/ reactions can be described by set of equations and by curves (called kinetic curves). According to the model of "law of mass action"

$$[Ab].[Ag] \underset{K_d}{\overset{K_a}{\rightleftharpoons}} [Ab.Ag].$$

Where [Ab] and [Ag] are free antibody and antigen concentration and [Ab.Ag] is concentration of complex. K_d = Dissociation rate constant and K_a= Association rate constant.

Equation 1 $K_A = 1/K_D$

Rate of association = [Ab].[Ag]. K_a

Rate of dissociation = [Ab.Ag]. K_d

At equilibrium, the rate of association and rate of dissociation are same, so

Equation 2 [Ab].[Ag]. K_a = [Ab.Ag]. K_d

Equation 3: $K_D = K_d/K_a$ = [Ab][Ag] / [Ab.Ag]

The dissociation constant (K_D) is a measure of the stability of an interaction and the ease with which an antigen-antibody complex dissociates. A lower K_D value indicates higher affinity and vice versa. In general K_D values derived for antibody-antigen interactions range from between 10^{-7} M to 10^{-11} M. It is quoted that K_D values range between 10^{-7} M to 10^{-9} M in the primary response antibodies and between 10^{-9} M to 10^{-11} M in the secondary immune response. This upward trend in affinity is attributed to and associated with increases in somatic mutation events (Berek and Milstein, 1987) (see Figure 1-22).

It is important to distinguish avidity from affinity; especially when approaching the quantification of binding interactions; avidity is an overall binding strength at which an antibody binds to the antigen and usually is sum of binding of all the Fab arms to different epitopes on the antigen. Hence the avidity of IgM molecule which is pentameric and has ten identical antigen binding sites could achieve enhanced binding strength via this multivalency over a large surface area compared to other immunoglobulin molecules of lower order structure such as IgG. Affinity refers more specifically to the non-covalent interactions between defined, individual binding sites on two opposite binding partners and can be calculated with greater fidelity.

1.9.1 Binding forces involved in antigen-antibody interactions

Table 1-7 summarises the non-covalent forces involved in binding between epitope and paratope. The overall strength of binding depends on the fitness and surface area of the interfaces as defined by amino acid arrangements on the structural epitope. When studying the binding forces *in vitro*, temperature, pH and ionic strength may influence these non-covalent interactions. These binding forces can be manipulated and used for immuno-affinity purification of HLA-specific antibodies or antigens. Electrostatic potential over the HLA epitope can be measured (Kosmoliaptsis et al., 2011) and can be used to predict immunogenicity of epitopes (ability to mount alloimmune response) (Mallon et al., 2014).

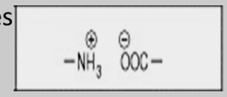
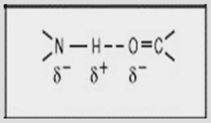
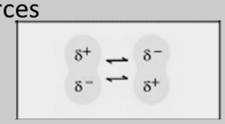
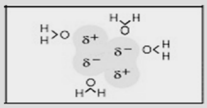
Non-covalent forces	Origin	Comments
Electrostatic forces 	Attraction between opposite charges	Long range forces Strengthens the over all interaction ? Determines antibody affinity Disrupts by extreme of pH and high salt concentrations
Hydrogen bonds 	Hydrogen shared between electronegative atoms (N,O)	
Van der Waals forces 	Fluctuations in electron clouds around molecules oppositely polarize neighbouring atoms	Close range forces. The strength is directly proportional to the surface area involved in binding
Hydrophobic forces 	Hydrophobic groups interact unfavourably with water and tend to pack together to exclude water molecules. The attraction also involves Van der Waals forces	

Table 1-7: Forces involved in Epitope-paratope bindings

(Modified from Janeway CA Jr et al. Garland Science; 2001)

The binding of antibody can also be influenced by spatial arrangement of different epitopes on a single HLA antigen (Table 1-8). At our Centre, using purified polyclonal HLA epitope-specific antibodies, we have shown the importance of the spatial arrangements of epitopes and their effects on CDC reactivity (Lowe et al., 2011b).

Spatial arrangement of different epitopes	Effect	Outcome on binding of second antibody molecule
Overlapping	Steric hindrance	Reduce binding
Non-overlapping	None	Additive
Allosteric	Conformational change in antigen structure	Positive or negative influence

Table 1-8 : Spatial arrangement of epitopes and effect on outcome of binding in a polyclonal antibody sample.

(Taken from book 'Cellular and molecular immunology by Abbas et al., Saunders publisher)

1.9.2 Methods to study affinity

There are various methods that can measure the dissociation constant (K_D) – the consensus quantitative expression of the affinity for a given protein-protein interaction. Broadly these can be divided in to two categories depending on the approach used (Neri et al., 1996):

1) **Equilibrium analysis**

   By measuring bound and free antibody (or antigen) at equilibrium,

   Equation 4 : K_D = [Ab][Ag] / [Ab.Ag]

   Where [Ab] and [Ag] are free antibody and antigen concentration and [Ab.Ag] is the concentration of the bound complex.

The time required to reach equilibrium is determined by the relative ratio of concentration of analyte and ligand. Generally 90% saturation is acceptable to attempt equilibrium analysis. It is suggested that concentration range of analyte should be between 10 x lower than the anticipated K_D to 100x higher.

Equilibrium analysis can be performed using various techniques (Table 1-9). In competition (ELISA) assay or indirect ELISA (Friguet et al., 1985), the antigen and antibody are incubated in solution to reach equilibrium. The antibody concentration is varied and antigen concentration is kept constant. Then ELISA is used to measure the amount of free antibody concentration. This ensures equilibrium is not severely affected by ELISA (Goldberg and Djavadiohaniance, 1993). Similar competition assay can be performed using surface plasmon resonance (SPR) assay (Heinrich et al., 2010).

Method	Measurement	Sample requirement	Sensitivity
Equilibrium dialysis	Equilibrium across a dialysis membrane, free and bound analyte measured	Small antigen that can cross the dialysis membrane	1 mM to 1 pM
Fluorescence quench	Fractional fluorescence quench upon binding	Pure material needed	1 uM to 1nM
Isothermal titration calorimetry	Heat produced on binding of increasing titration of antibody to concentrated antigen solution	Pure material needed in large quantities	1 mM to 100nM Higher affinity can be measured by increasing the temperature
Band-shift on Gel	Detection of free and bound antibody on gel	Radioactive or florescent labelling of either antibody or antigen	10 nM to 10pM
Physical separation	Antibody in the complex is separated by chromatography or magnetic beads and detected by fluorescence or electrochemiluminescence	Labelling of antibody is required	10 nM to 10 pM
Competition ELISA	After incubation of antibody with antigen, the unbound antibody is detected by ELISA	Purified antigen or antibody to immobilise on solid surface	50 nM to 1 nM
Surface plasmon resonance	Change in refractive index upon binding is measured	Purified antigen or antibody to immobilise on sensor chip	100mM to 1 nM (higher affinity can be measured by kinetic analysis)

Table 1-9 : Methods to determine dissociation constant using equilibrium saturation approach (Modified from ref- (Neri et al., 1996))

The K_D is determined at 50% fractional binding (F_R), when the concentration of [Ab] is equal to K_D.

Equation 5 : F_R = [Ab.Ag]/[Agtotal]= [Ab]/([Ab]+ K_D)

Linear or non-linear curve fitting computer programs are used to derive the relationship between analyte concentration and signal from antigen-antibody complexes (Glaser, 1993).

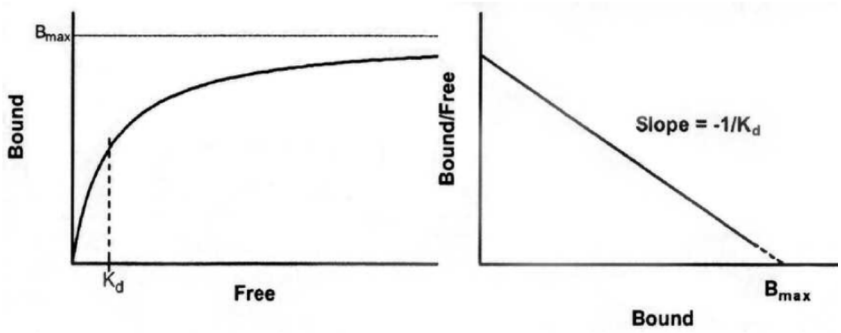


Figure 1-41 : Fitting linear regression model to data (Scatchard Plot)

Note– On left data obtained by plotting response (bound antibody) versus (free) antibody concentration and on right scatchard plot obtained after transforming data by linearising

Scatchard plots (Figure 1-41) can be used to determine K_D, by plotting the ratio of bound and free ligand against the bound complex. A linear regression fitting is applied to a obtain slope. (Slope = -1/ K_D). The assumption here is the involvement of univalent and homogenous interactions. With heterogeneous interactions, there is an increase in the degree of curvilinearity and in such cases the parameter is obtained by fitting to a hyperbolic curve. However linear regression assumptions are not met and the estimates are not accurate, so use non-linear regression analysis is required. The curve obtained requires mathematical

fitting to estimate accurate affinity readings (Delaage et al., 1992, Heinrich et al., 2010). Clinical samples contain antibodies that are polyclonal in nature and have a range of affinity values. Mathematical tools can be used to obtain two affinity values (Bobrovnik, 2003, Stevens and Bobrovnik, 2007).

There are some limitations to measuring dissociation constant via equilibrium analysis. Firstly, very low affinity antibodies will require prolonged periods to reach equilibrium and may result in denaturing the antigen depending on the experimental conditions. Secondly, larger samples (milligrams of protein) are required for competitive ELISA and SPR equilibrium assays that make comprehensive studies prohibitively expensive and labour-intensive.

2) **Kinetic analysis –**

This can be done in a single step, real time assay platform such as surface plasmon resonance or several separate assays where several incubations of antigen-antibody in liquid phase are interrupted after over a series of time points. (Table 1-10).

Method	Measurement	Sample requirement
ELISA	K_D and K_a is measured	Purified antigen or antibody to immobilise on solid surface
FP	Change in polarisation of antigen on antibody binding K_D and K_d is obtained	Antigen labelling with fluorescence
SPR	Change in refractive index on chip due to binding interactions K_a and K_d is obtained by fitting of curves	Purified antigen or antibody to immobilise on sensor chip

Table 1-10 : Methods to determine dissociation constant using kinetic analysis

Several separate assays done in parallel cover the range of time points until the reaction reaches its equilibrium as measured by ELISA. Alternatively, data can be obtained in a staggered fashion such as in fluorescence polarisation spectroscopy.

Fluorescence polarisation method:

This is performed entirely in liquid phase and is a highly sensitive method for studying molecular interactions in solution. The antigen labelled with fluorochrome is incubated with the antibody until equilibrium is reached. After this the baseline polarisation (P0) is measured. Following this unlabelled antigen is added to the solution and the change in polarisation (Pi) is measured every six seconds. Fluorescence polarisation can be measured by a plate reader

at 595 nm absorbance (depending on the fluorochrome used). The dissociation *rate* k_d is obtained using logarithm plot of (P0-Pi) against time. The dissociation constant K_D is measured using the conventional equilibrium method and the association rate K_a is derived using the values of K_D and K_d ($K_a = K_d / K_D$).

Competition/Inhibition ELISA:

This can be done in various ways. A mixture of antigen and antibody in stoichiometric proportion are incubated overnight. 1:100 dilution of the mixture is then added on to an ELISA plate coated with the antigen (fixed concentration) at different time points (0 to 90 minutes) and allowed to incubate for four minutes at room temperature and then the absorbance is read following later steps of the conventional ELISA protocol (Larvor et al., 1994). The K_d is calculated from the resulting curve derived from logarithmic plot of $((A_\infty)-(A_t))/ ((A_\infty)-(A_0))$ versus time. A_t = absorbance at given time, $A\infty$ = absorbance at 90 minute and A_0 = absorbance of the blank well. The slope of line is equal to $-K_d$.

In a another method, a mixture of equal volume of antigen and antibody is incubated at different time periods (such as 2, 4, 7, 10, 20 and 30 minutes)(Zhuang et al., 2001). ELISA with coated antigen is used to detect the free antibody concentration after incubation of one minute. The remaining steps are similar to conventional ELISA. A curve of binding fraction (ratio of bound antibody/total antibody) versus time of incubation allows determination of K_a using mathematical fitting. K_D is determined using competitive ELSIA described above and K_d is determined using values of K_D and K_a.

Surface Plasmon resonance (SPR):

The physical principle and methodology for this technique is described in detail in chapter 4. This is a bio-sensor platform that allows direct real time measurement of K_a & K_d of antibody-antigen interactions without any labelling or incubation steps. In the case of modern multi-channel SPR instruments, different concentrations of solution-phase analyte are flowed simultaneously over channels coated with immobilised ligand molecules injected to obtain multiple kinetic curves, and all the data sets are fitted simultaneously by global analysis software, typically built in to the manufacturer's operating software package. Global analysis gives good direct calculations of the kinetic parameters (K_a & K_d) unlike nonlinear fitting of individual curves. There are number of commercial software packages available to carry out curve fitting such as PRISM, MATLAB and the ProteOn XPR36 software, the latter being built in to the SPR operating package provided by Bio-Rad and containing a good range of interaction models from which to derive the best data fits. These use non-linear regression analyses, with aims of reducing the sum of squares of the vertical distances of the points from the curve.

1.10 Summary

The presence of donor-directed antibody alone or a single characteristic of the antibody does not determine humoral immune response. Various bio-physical properties of the antibody itself and interactions with components of immune system are responsible for a pathogenic immune response. Affinity/avidity of DSA has not been characterised before and correlation of the avidity or change in avidity with other characteristics of the antibodies and graft outcome will help delineate the characteristics of the humoral immune system.

1.11 Hypothesis and Objectives of the study

Equity of access to transplantation is a major challenge for highly sensitised patients. Based on current measurements in H & I laboratories, very high risk (CDC positive) and low risk (non-sensitised) are identified with good confidence and immunosuppressive therapy are tailored accordingly. Dilemma remains in a large number of patients on waiting list with low to moderate levels (defined by MFI values) and various approaches has been taken to achieve successful transplantation in these cases. With current strategies some cases may be denied transplantation or some cases may be put through higher risks inadvertently.

Studies have shown adverse outcomes in cases that had pre-formed donor specific antibodies and also cases that develops de novo donor specific antibodies. In the former, presence of DSA is not always associated with rejection and in the latter it may take few years before development of antibody mediated damage to the graft. Hence mere presence of antibodies is not useful in predicting adverse outcomes. There are two schools of thought, first supporting difference in biophysical characteristics of antibodies and second supporting change in activity of cellular component of immune system responsible for adverse outcome. In reality both these mechanisms will work in parallel to result immune damage. At our Centre, we have extensively studied different characteristics of antibodies such as specificities, dynamics of rise and fall, sub-classes of IgG and inhibitory factor such as soluble HLA in circulation. The current thesis looks at two biophysical properties of antibodies; class IgM and IgG of *de novo* HLA-specific antibodies and affinity of pre-formed IgG HLA-specific antibodies in detail.

1.11.1 Hypothesis:

The hypothesis to be examined herein is that HLA-specific antibodies within a highly sensitised patient's serum may possess varying affinities for relevant donor specific HLA molecules. Greater understanding of this phenomenon may help to explain discrepancies between HLA-specific antibody measurements made by current immunoassays such as Luminex and clinical outcomes and deepen antibody analysis in order to allow for improved patient prognosis, risk stratification and treatment.

1.11.2 Objective:

1.11.2.1 *Primary objective:*

To study the distribution of affinities of HLA-specific antibodies in highly sensitised patients and to group the HLA-specific antibodies in risk stratification groups based on strength of affinities.

1.11.2.2 *Secondary objectives:*

1. To study evolution of HLA-specific IgM and IgG antibodies in non-sensitised patients and study effects on graft outcome prospectively.

2. To develop and validate surface plasmon resonance as a technique for characterising HLA-specific antibody interactions with HLA proteins using available human monoclonal HLA-specific antibodies.

3. To characterise the binding kinetics of epitope – paratope interactions using same monoclonal HLA-specific antibodies against different HLA proteins with shared epitope.

4. To develop methods to quantify polyclonal HLA-specific antibodies

5. To develop protocols that allow for the study of polyclonal HLA-specific antibodies from human serum for affinity measurements in highly sensitised patients

2 Methods and materials

This project used a range of laboratory methods to identify and purify HLA-specific antibodies and carry out further analysis of their biophysical and chemical properties. The following section describes all the techniques and protocols used in detail. Commercially available kit-based assays were all performed as per the manufacturer's guidelines.

2.1 Materials and reagents

2.1.1 Soluble HLA proteins

Soluble HLA proteins were generously provided by Pure Protein LLC (Oklahoma City, USA) under the terms of an official collaborative agreement between University of Oklahoma, Pure Protein LLC, University of Warwick and University Hospital of Coventry and Warwickshire NHS Trust.

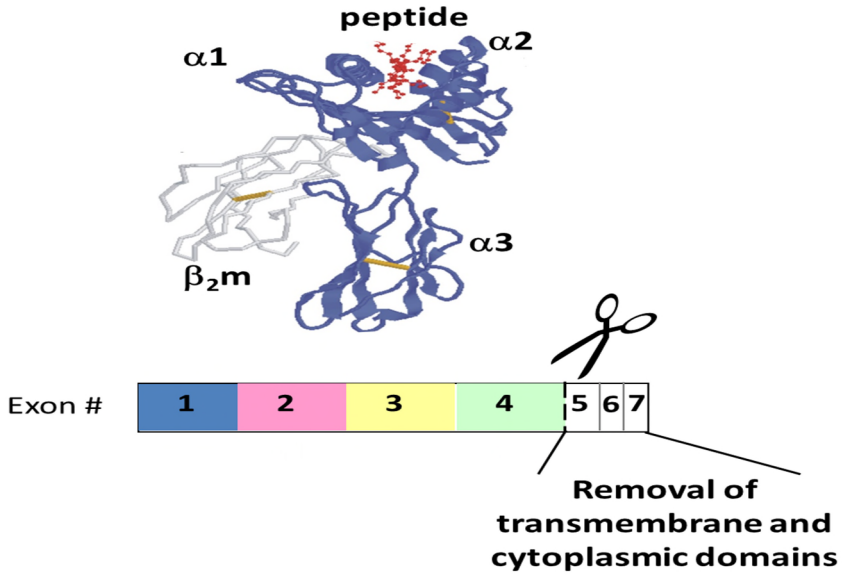


Figure 2-1 : Oklahoma protocol of sHLA production

In brief, soluble Class I HLA protein producing clones, produced by transfection of a construct that deleted the transmembrane and cytoplasmic domain of the protein (Figure 2.1) and replaced by VLDLr expression tag, were grown in serum free media. sHLA secreted by these clones were harvested and purified using W6/32 (antibody to pan HLA class I) affinity chromatography. The purity of the sHLA was assessed using mass spectrometry and quantified using W6/32 ELISA.

2.1.1.1 Stability of sHLA protein

Forced degradation studies were conducted to assess physical and chemical stability of sHLA proteins at Pure Protein LLC (Oklahoma, USA). sHLA-B7 was coated on beads and various conditions were tested on the Luminex platform. Monoclonal antibodies W6/32 and HC-10 were used to look for integrity and damage to HLA structure respectively.

2.1.1.1.1 Temperature stability

HLA-proteins were stable within the range of temperatures (4 to 45^{o} Celsius) up to one hour duration (see Figure 2-2).

2.1.1.1.2 pH range

HLA-proteins were stable between pH 5.5 and 10.5 for varied buffer composition. See Figure 2-3.

2.1.1.1.3 Buffer composition

Buffers with different ionic strengths and reducing agents were tested. As shown in Figure 2-4, buffer with reducing agent and SDS denatures HLA proteins.

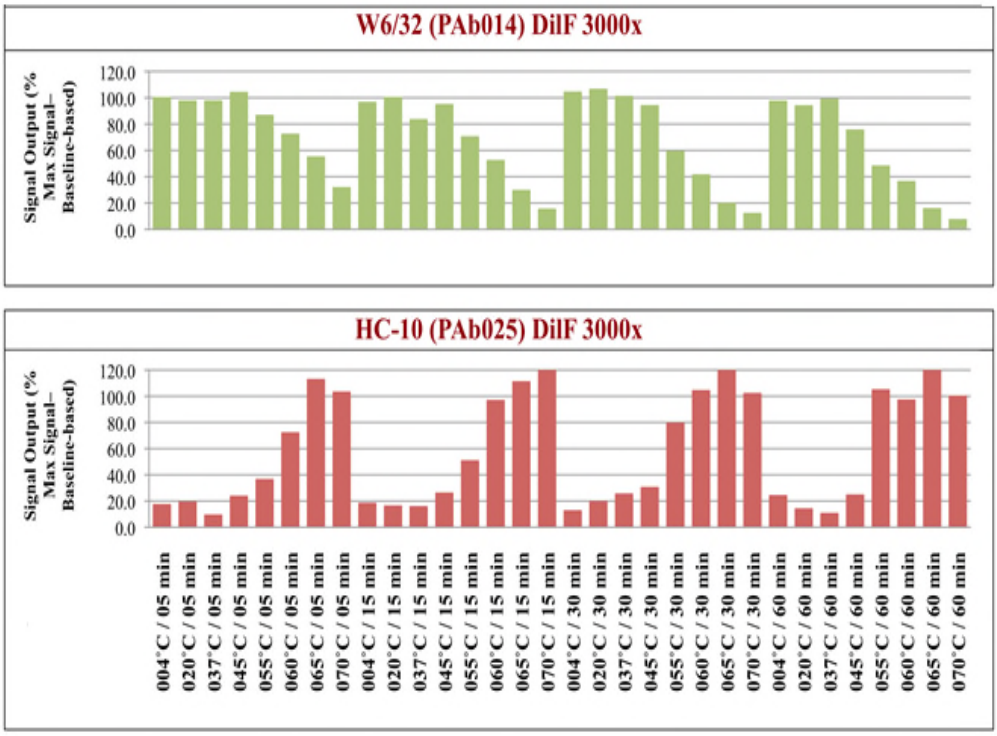


Figure 2-2 : Effect of temperature on HLA-protein; top panel shows reactivity towards W6/32 (pan-HLA class I monoclonal antibody) and bottom panel shows reactivity with HC-10 (binds to denature HLA-protein)

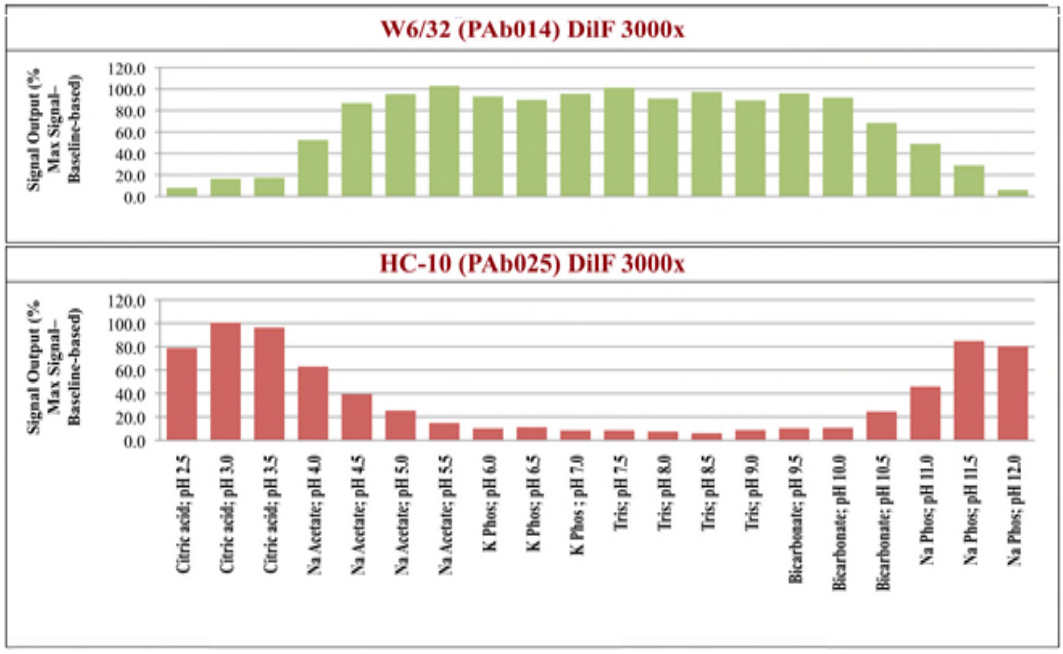


Figure 2-3 : Effect of pH on reactivity of HLA-protein Top panel showing reactivity of W6/32 at different pH values and bottom panel shows reactivity to HC-10 suggesting denaturation of HLA protein

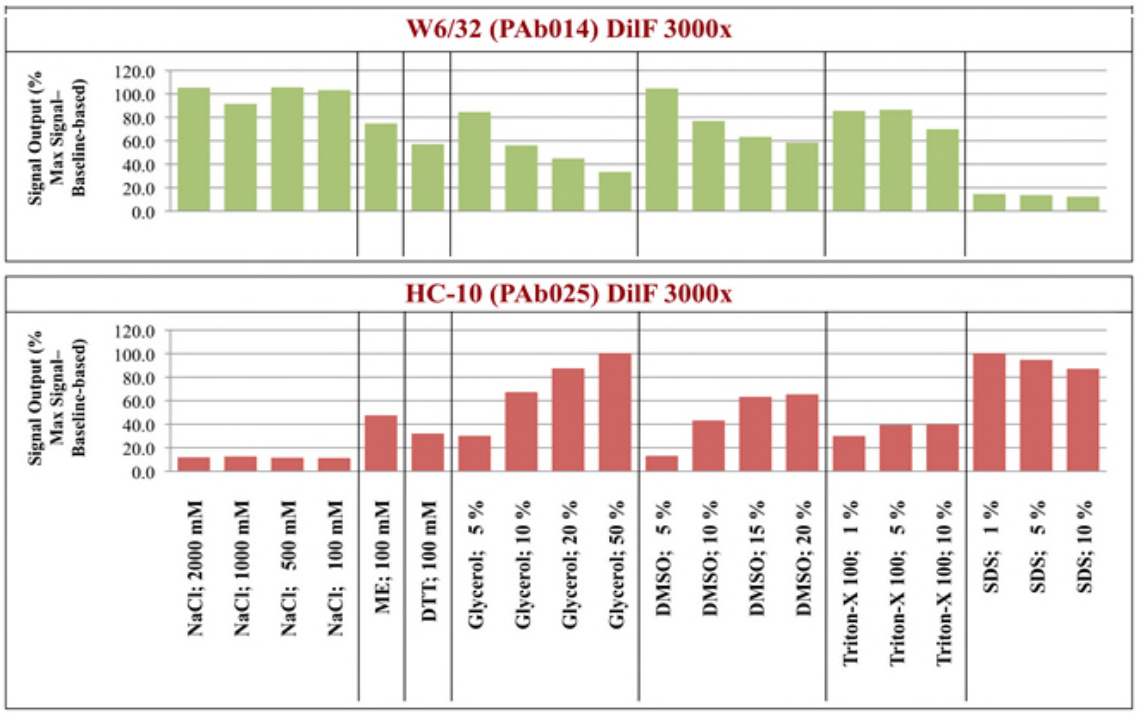


Figure 2-4 : Effect of buffer composition on reactivity of HLA-protein Top panel showing reactivity of W6/32 at different pH values and bottom panel shows reactivity to HC-10 suggesting denaturation of HLA protein

2.1.2 **Biotinylated HLA proteins**

HLA proteins were tagged to biotin so that they can be used to immobilise on assay platform to study interactions with HLA-specific antibodies. Biotin forms a strong irreversible binding to neutravidin on typical Surface Plasmon Resonance (SPR) sensor chips. The biotin conjugation reagent NHS-PEG12-Biotin (Thermo Fisher Scientific; 21312, Rockford, IL) was used, NHS esters readily react with free primary amines on HLA proteins to form stable covalent linkages. A 54 Angstrom PEG spacer was attached to the sHLA protein to allow for maximum protein bioavailability when captured on neutravidin/streptavidin surfaces.

The biotin conjugation of sHLA was performed at Pure Protein laboratories at Oklahoma by chemical biotinylation. Briefly, a small-scale protocol was used starting with a 1 mg sHLA probe in PBS. The reaction was initiated by adding sodium bicarbonate buffer, pH 9.0 (final 0.1 M) and NHS-PEG12-Biotin at a final concentration of 393 µg/ml. The final mixture was incubation at room temperature for 2 hours at a total volume of 1 mL and a final sHLA concentration of 1 mg/ml. To complete the procedure, the reaction was terminated by adding Tris-HCl, pH 8.5 at a final concentration of 50 mM. An empirically determined molar ratio of 20:1 (Biotin-NHS : sHLA protein) was applied to all Class I molecules to assure that on average, not more than one biotin became attached to the sHLA protein to prevent disturbing the binding capability of the targeting antigen by excess biotinylation. The extent of biotin incorporation was determined using a HABA assay. To remove excess biotin, sHLA molecules were buffer-exchanged with PBS at pH 7.2 and concentrated using 10-kDa cut-off Macrosep centrifugal concentrators (Pall Filtron, Northborough, MA). The final product was filter-sterilized and stored at 4 °C until further use. The concentration of the purified biotinylated molecules was determined using the Micro BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL) using BGG as a standard.

2.1.3 **Human monoclonal HLA-specific antibodies**

These monoclonal antibodies were kindly provided by the A Mulder/ F Claas group at University of Leiden. Briefly, the human monoclonal HLA-specific antibodies were secreted by human hybridoma produced by EBV transformation of B-lymphocytes from HLA antibody seropositive multi-parous women and sub-cloning of antibody producing EBV lines. Only human monoclonal antibodies of the IgG class were used.

Clone (ID)	Immunising Ag	HLA-specificity defined by CDC	Epitope	Isotype
SN203G6 (IHB-Hu-033)	A2/B57	A2/B17 (B57,58)	62G	IgG1, L
SN607D8 (IHB-Hu-081)	A2/B57	A2/A28 (A68,69)	142T --H	IgG1, K
WK1D12 (IHB-Hu-059)	B27	B27,7,60	163EW+self73TE	IgG1, K
VTM9A10 (IHB-Hu-093)	B7	B7, 27	69A,71A	IgG1, K
VTM1F11 (IHB-Hu-045)	B7	B27,7,60	163E	IgG1, K
WAR5D5 (IHB-Hu-074)	B27	B7,27,42,55	62RN-QIY and 62R—QIC	IgG1, K
OUW4F11 (IHB-Hu-041)		Bw6	80I	IgG1, L

Table 2-1 : Characteristics of Human monoclonal HLA-specific antibodies (provided by A Mulder)

These monoclonal antibodies (MAb) were provided as culture supernatants in RPMI / IMDM with 10% FCS, 50 uM 2-ME; 0.02% sodium azide and stored at 4^{o}C. The MAb were isolated and purified from the supernatants by techniques described latter (section 2.2).

2.1.4 Mouse anti-human HLA-class I specific monoclonal antibody

W6/32 monoclonal antibodies were generously provided by Pure Protein PLC (PAb028), Oklahoma, USA.

2.1.5 Human serum / plasma samples

Samples containing HLA-specific antibodies were obtained from subjects with Chronic Kidney Disease (CKD) and kidney transplant recipients from two transplant centres. Ethical approval has been obtained for the use of samples towards this project (Table 2-4). Both the cohort were well defined with complete sets of clinical and outcome data. Samples collected include serum, plasma and plasma effluent (following plasmapharesis therapy). For validation of assays, control serum and plasma samples from NHSBT, Birmingham were used. Details of the samples and cases used can be found in the relevant sections.

Cohort	Ethics reference	Stored at
UHCW, Coventry, UK	CREC-055/01/03 and 13/WM/0090	CSRL, University of Warwick, UK
UHB, Birmingham, UK	CREC-08/H1204/103	NHSBT Birmingham UK

Table 2-2 : Clinical cohort used for the project

2.1.6 **Reagents / buffers**

Chemical reagents and buffer used in this project are detailed in the relevant section. Ultra-pure water (ddH2O) was obtained from Super-Q ™ water system (Millipore). Buffers were filtered through 0.45 micron size filter and de-gassed prior to chromatography or surface plasmon resonance assays.

2.2 **Purification of HLA-specific IgG antibodies**

Serum or plasma is a complex material that contains many components that may interfere with analysis of specific component. This effect is often called as "matrix effect" which can inhibit specific signal and cause non-specific signal (noise). Similarly, culture supernatant can contain impurities too. Culture supernatant containing human monoclonal HLA-specific antibodies has albumin and/or transferrin as contaminants (added as supplements). Sometimes, foetal calf serum is used that has other contaminants such as alpha-2 macroglobulin, serum proteins etc.

Various strategies has been utilised to overcome this interference such as treatment with Dithiothreitol (DTT) to obliterate IgM interference, EDTA to obliterate interference by C1q and complement. Similarly albumin and lipid molecules can stick to the matrices of affinity columns and SPR chips, causing interference with assays.

2.2.1 Preparation of sample prior to purification

Samples were centrifuged at 10,000 to 15,000 rpm for 30 minutes and pellet containing debris, particulate matter and lipids was separated. If samples were still cloudy, filtration using 0.45 um pore size filter was used.

Buffer exchange, phenol red removal and desalting was done prior to liquid chromatography. This was done by dialysis into lower salt buffers or via spin concentrators.

Dialysis:

Sample was decanted into dialysis tubing with 8,000 MWCO (Spectra/Por ® Dialysis membrane) and incubated with stirring in a cylinder of the desired buffer (e.g. PBS) overnight at 4^{o}C.

Viva Spin concentrator:

The sample were filled in 3000/ 10,000 MWCO cut off container (Vivaspin 2/20, Sartorius Stedim, UK) and centrifuged for 15 minutes, further three cycles were done by adding PBS and centrifugation. This is added advantage of concentrating and buffer exchange at the same time.

2.2.2 **Sequential fractional precipitation**

This method is especially effective for IgG enrichment from serum and works on the principle of differential protein solubility, whereby increasing salt concentration enhances hydrophobic interactions between proteins and causes precipitation. Differential precipitation of proteins can be achieved by adding varying concentrations of octanoic acid and ammonium sulphate in defined stages. Octanoic (Caprylic) acid selectively precipitates bulk proteins and some α2 macroglobulins and immunoglobulins are left in the solution. This step is followed by addition of ammonium sulphate (45 % saturation) which precipitates immunoglobulin selectively. The concentration of immunoglobulin should be at least 1 mg/ml for this protocol to work. Protocol described by an earlier study (Perosa et al., 1990) was utilised (Protocol 2.1).

1. Centrifuge the sample at 10 000 x g for 30 minutes at 22 degree.
2. Mix X ml fluid with 2 -4 times X ml acetate buffer 60 mM, pH 4.0.
3. Adjust to pH 4.5 with 1M TRIS-HCl/Base
4. Slowly add **Caprylic acid 25 ul / ml** drop by drop, stirring constantly. Continue stirring for 30 minutes room temp
5. Centrifuge at 10 000 x g for 30 minutes at room temp
6. Remove supernatant and filter through 0.8 um filter
7. Adjust pH 7.4 with 1 M Tris-Base
8. Cool the sample to 4 degree (10 minutes)
9. Add **ammonium sulphate 0.277 g/ ml** and stir for 30 min at 4 degree
10. Centrifuge at 10 000 x g for 15 minutes at 4 degree
11. Discard the supernatant – re-suspend in PBS pH 7.4 (equal volume as starting serum vol)

Protocol 2-1: Sequential precipitation using Octanoic acid and Ammonium sulphate

2.2.3 **Affinity chromatography**

Affinity purification using specific ligand coupled to Sepharose matrix allows for selective and high capacity purification of desired proteins. Protein G and HLA protein linked to Sepharose matrices were used to affinity purify total IgG and HLA-specific antibodies respectively.

Cibacron Blue and alginate depletion chromatography were tested but not used subsequently. Cibacron blue adsorption resulted in removal of albumin but also 30 to 50% reduction in MFI levels of HLA-specific antibodies. Alginate affinity chromatography did not result in reduction of non-specific binding to sensor chips used for SPR assay.

2.2.3.1 Protein-G affinity chromatography

Protein G is a bacterial protein obtained from Group G streptococcus and has high affinity for the Fc region of IgG-type antibodies. Protein G has a greater affinity for IgG than Protein A and minimally binds to albumin. The binding capacity of commercial Protein G Sepharose resin is about 17 to 20 mg/ml. Protein G Sepharose ™ 4 Fast flow (Sigma) was packed in 2-4 ml columns and used to purify IgG (protocol 2.2). Protein G chromatography was adapted to automation using the Bio-Rad Duo Flow Fast Protein Liquid Chromatography (FPLC) rig.

1. Equilibrate the column with 20 ml (5 column volume) of 20 mM sodium phosphate pH 7.0 (running buffer).
2. Flow through: Pass 250 ml of dilute plasma effluent / culture supernatant (1:1) in 20 mM sodium phosphate pH 7.0 and allow the flow under the gravity slowly. Collect the flow through and store at 4°C.
3. Wash through: Wash the column with 80 ml (20 column volume) of 20 mM sodium phosphate pH 7.0 until the OD on Nanodrop is less than 0.02.
4. Elution in acidic condition with 20 ml of Glycine (100mM pH 2.5) and collect the elution fractions of 500 ul and analyse on Nanodrop. 1M Tris pH 9 was added to the elution fraction to neutralise the antibody fractions. The fractions corresponding to the peak were pooled together and concentrated
5. Regenerate the column by washing with 20 column volumes (80 ml) of glycine 100mM pH 2.8 followed by 0.5 M NaCl 10 column volume (40 ml). Store the column with 20 % Ethanol at 4°C.

Protocol 2-2 : Protein-G Affinity Chromatography

2.2.3.2 HLA affinity chromatography

HLA protein coupled to Sepharose was obtained from Pure Protein PLC. Oklahoma USA. Columns of 4 ml and 60 ml size were prepared and the binding capacity of swollen matrix determined as 150-200ug of HLA-specific antibodies per ml of HLA-protein resin. These columns have been tested for specificity and efficiency for enrichment of HLA-specific antibodies (McMurtrey et al., 2014). Column efficiency following repeated runs of antibody elution was tested by depletion of a standard concentration of W6/32 monoclonal antibody. For the 60 ml column, a peristaltic pump (Watson Marlow, Portsmouth, UK) was used to drive sample at a flow rate of 50 ml/min. Protocol 2.3 was used to purify/ enrich for HLA-specific antibodies.

1. The column was first equilibrated with running buffer (PBS pH 7.4) – 10 column volume (40 ml)

2. Flow through: 20 ml of precipitated protein dissolved in PBS (pH 7.4) obtained from sequential precipitation step (2.3.4.5) or 250 ml of dilute plasma effluent (1:1) was loaded into the column slowly. The fluid was let to drip through by gravity by realising the flow tap at the bottom. Flow through was collected in bottle and stored (as there may over-flow of interested and other HLA-specific antibodies)

3. Wash through: The column was then washed with PBS until the OD at 280λ (Nanodrop) less than 0.02. Depending on the preparation up to 20 Column volume of wash buffer was required.

4. Elution: HLA-specific antibodies bound to the immobilised HLA protein in the column were eluted by elution buffer (Glycine 100mM pH 11.0). Four column volumes (24 ml) were passed through and the 250 ul elution fractions were collected and analysed on Nanodrop. 1M Tris pH7 was added to the elution fraction to neutralise the antibody fractions. The fractions corresponding to the peak were pooled together and concentrated. Protease inhibitor tablets (Sigma) and 0.01 % NaN3 was added in the eluted antibody solution and stored for further characterisation studies.

5. Recycle the column by washing with 20 column volume (80 ml) glycine 100mM pH 11.0 followed by 0.5 M NaCl 10 column volume (40 ml) and then equilibrate with 20 column volume (80 ml)of PBS(pH 7.4). Store the column with PBS and 0.01 % NaN3 at 4^{o}C.

Protocol 2-3 : HLA-protein Affinity Chromatography

2.2.4 Ion exchange chromatography

Ion exchange chromatography separates proteins based on their net charge as they bind to oppositely charged resin (Figure 2.5). It exploits the principle that the net surface charge of proteins change according to the surrounding ionic strength and pH, so the bound proteins are eluted differentially by increasing salt concentration or changing pH in a controlled gradient (Figure 2.5). This allows for high capacity and high resolution purification steps. Another advantage is that the target proteins are very often concentrated in the elution fractions. Ideally, the pH of the loading buffer is selected as 0.5 to 1 pH unit away from the isoelectric point (pI) of the target protein. At a pH above its isoelectric point, a protein typically binds to a positively charged medium (Anion exchange, e.g. quaternary amine, or Q-Sepharose) and, at a pH below its pI, a protein will bind to a negatively charged medium (Cation exchange, e.g. sulfonic acid, or S-Sepharose). The pH of the running buffer is determined by the isoelectric point (pI) of the desired protein, and generally the pI for IgG pools is between 4 and 10 and it varies depending of the subclass (Prin et al., 1995).

The ion-exchange chromatography is best suited for separation of monoclonal HLA-specific antibodies (most of them are IgG1 subclass) as they will be expected to elute as single peak. This was used as final step in purification following HLA-protein affinity chromatography.

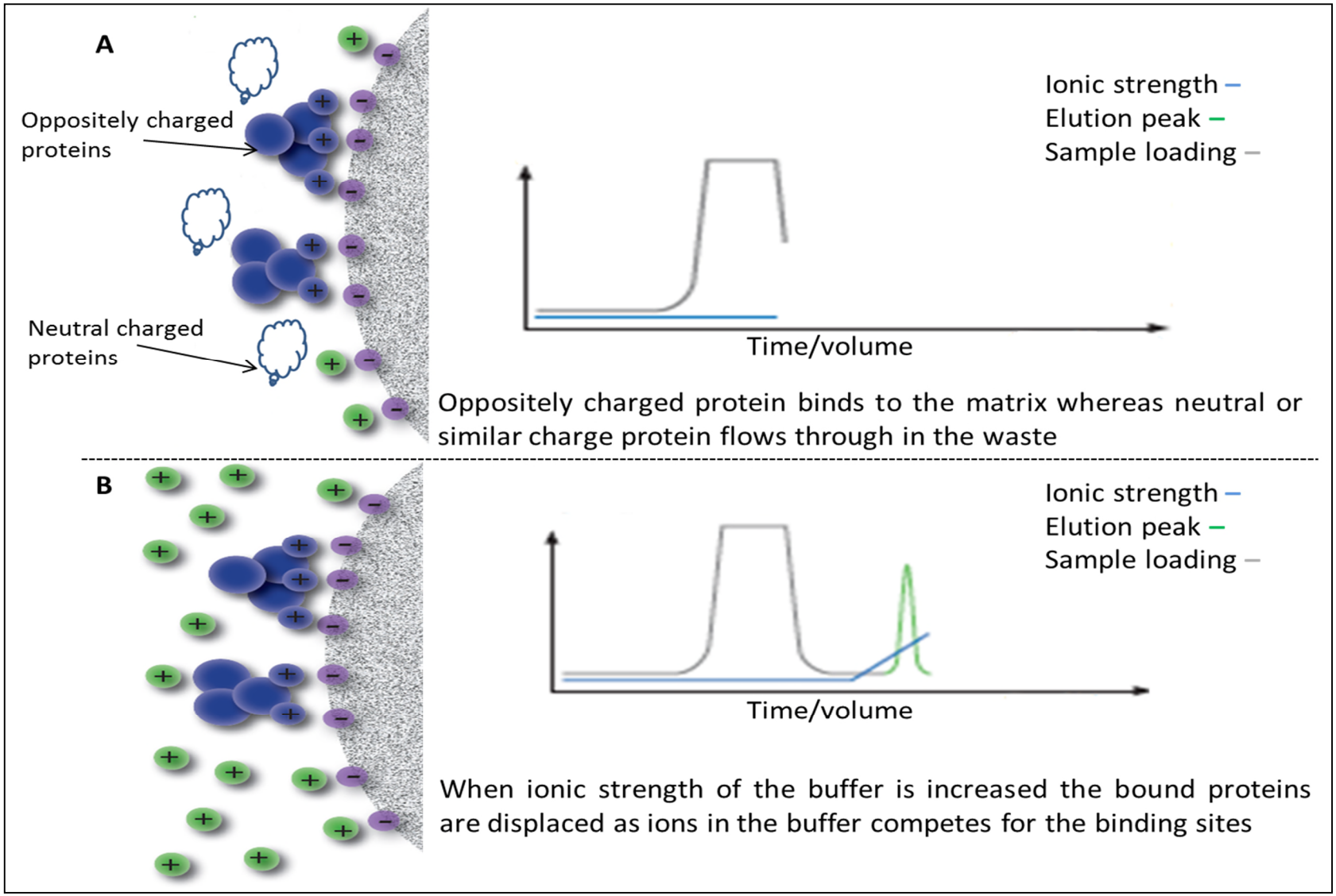


Figure 2-5 : Principle of Ion-exchange chromatography

Step 1 – Buffer exchange using dialysis of samples overnight against HEPES 10mM pH 8.0 for Anion exchange and Acetate 10mM pH 5.2 for Cation exchange chroma tography (section 2.2.1)

Step 2 – Equilibrate the column with 20 ml of buffer A (HEPES or Acetate) until the baseline pH and conductivity is stable. (Flow rate – 3 ml/min) This will wash away the storage buffer (usually Ethanol),

Step 3 – Flush 2 ml of Buffer A in the loading loop and then load the sample using a peristaltic pump on the column (watch for any leaks). Following this connect the column to the FPLC rig (GE AKTA or BioRad DuoFlow).

Step 4 – Elute the bound protein by increasing the ionic strength using Buffer B (B uffer A + 1 M NaCl) in a linear gradient fashion. The pH is maintained constant and is same as the Buffer A. Peaks of proteins separated differentially based on the charge. The flow rate and pressure is watched (limit – Resource Q pressure ≯ 0.6 mPa, Q-Sepharose not much issue.) The eluted proteins in buffer are collected in fraction collector (Frac- 900). On-line pH, conductivity, gradient and UV are obtained on the software (UNICORN or BioRad) manager.

Step 5 – Wash the column with 100% Buffer B to elute any remaining ionically- bound material. Store the column in 20 % Ethanol.

Protocol 2-4 : Ion Exchange Chromatography (Anion and Cation Exchange)

2.2.5 Size exclusion chromatography or Gel filtration

Size exclusion chromatography (SEC) separates the protein on basis of differences in the molecular size, specifically the Stokes radius of a specific protein. It is an effective final polishing step in the purification protocols when the sample volume is low or if sample is polyclonal in nature. Samples are eluted isocratically, without salt or pH gradients. Low sample volume requirement is a major limitation and hence prior steps of concentrating are required if samples have not gone through sequential precipitation protocols.

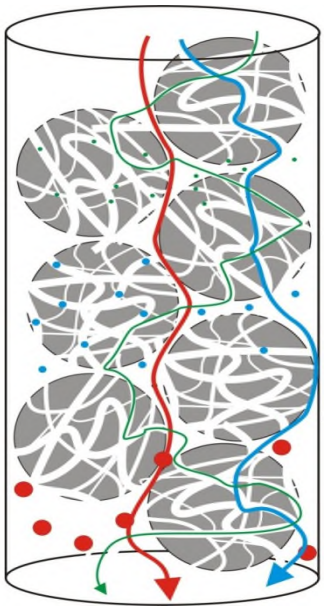


Figure 2-6 : Size exclusion chromatography – large size molecule runs through space between the media by a shorter path compared to smaller size that travels through longer path via pores / channels.

(Adapted from chapter 2, book Protein Engineering - Technology and Application, edited by Dr Ogawa; Published InTech 2013)

A Superose 12 column was used for the purpose of preparing improved IgG samples. The AKTA purifier (GE Healthcare) or BioRad Duo Flow fast protein liquid chromatography workstations were used to support the column and perform automated liquid chromatography and accurate fraction collection.

Step 1: Concentrate the material or if precipitate following sequential precipitation technique dilute in 500 microlitre of PBS (pH 7.4). Centrifuge 13.5/15 K rpm prior to loading to the loop on the rig.

Step 2: Superose 12 column is equilibrated with PBS (pH 7.4) and then sample is injected automatically by the FPLC rig.

Step 3: PBS (pH 7.4) is run through isocratic flow 0.5 ml/min through the column. The samples fractionated as per size are collected by fraction collector.

Step 4: 40 ml of PBS (pH 7.4) is run through the column and finally is stored in 20% Ethanol.

Protocol 2-5 : Size exclusion chromatography

2.2.6 **Multi-step purification protocols**

To study the biophysical and chemical properties of IgG, it has to be substantially purified from complex mixtures such as tissue culture medium, serum or plasma effluent. There is no one-step purification process that is satisfactory; hence a combination of protocols within a strategy of combined is required and adapted according to complexity and other properties of the starting mixture. Table 2-3 summarises the choice depending on the characteristics of IgG molecule and Table 2-4 lists the chromatography columns used.

IgG property	Technique
Charge and isoelectric pH (pI)	Ion exchange chromatography
Size	Size exclusion chromatography
Hydrophobicity	Ammonium sulphate precipitation
Ligand specificity	Affinity chromatography

Table 2-3 : Properties of IgG molecule and technique that can be used to purify/ separate accordingly

(Adapted from Antibody purification Handbook (18-1037-46, Amersham Biosciences)

Column	FPLC principle	Compositions	Binding capacity	Max back pressure
Q Sepharose	Anion Exchange	Q – quaternary ammonia group linked to sepharose		
Resource Q (Amersham Bioscience)	Anion Exchange	Q – quaternary ammonia group linked to polystyrene/divinylbenzen	1 ml – 45 mg BSA 6 ml – 270 mg BSA	1 ml – 220 psi 6 ml – 87 psi (0.6 MPa)
SP Sepharose	Cation exchange	Sulfopropyl (SP) linked to sepharose		
Resource S	Cation exchange	Methyl sulfonate linked to polystyrene/divinylbenzen	1 ml – 80 mg lysozyme 6 ml –480 mg lysozyme	1 ml – 220 psi 6 ml – 87 psi (0.6 MPa)
Superose 12 (10/300 GL)	Size exclusion chromatography	Cross linker agarose 9-13um	NA	435 psi (3 MPa)

Table 2-4 : Various columns used in Ion exchange and size exclusion chromatography

2.3 Quality check

SDS PAGE gel electrophoresis was done after each step of purification described in section 2.2 to look for specificity or any impurities following a particular step. At the end of purification protocol Luminex Single antigen bead assay was used to describe specificities of HLA-specific antibodies. During the early stage of protocol development, mass spectrometer was used to describe the proteins separated.

2.3.1 SDS PAGE Gel electrophoresis

Gel electrophoresis was used to assess the complexity of samples and efficacy of purification techniques used for this project. SDS-PAGE (sodium dodecyl sulphate – polyacrylamide gel) 4 – 12% was used to separate proteins according to their size. A protein ladder (2-color SDS marker, RunBlue^{TM}) was used. Gel was performed in native and reducing (DTT) conditions (Protocol 2-6). Silver staining protocol was used as per manufactures instructions, if the bands were not visible or faint following SDS PAGE Gel electrophoresis and staining with coomassie blue stain (protocol 2-7).

Acetone precipitation was used on samples with very low concentration of proteins. 1 ml of sample was mixed with 9 ml of cold (4^{o}C) acetone and left in the freezer for one hour. Following this the sample was centrifuged at 4.5k rpm for 30 minutes and the supernatant was discarded. The test tube with pellet was left on heat block (60^{o}C) for 20 minutes prior to suspending in 20 ul of LDS-DTT buffer for reducing condition gel electrophoresis.

Step 1: Sample preparation

Stock buffer (LDS-DTT)	Reducing	Non-reducing
NuPage LDS sample buffer (4X) (Invitrogen CA, USA)	5 microliter	5 microliter
0.25 M Dithiothreitol (DTT) (Sigma)	2 microliter	None
Sample	13 microliter	13 microliter

Step 2: Samples and controls were vortex mixed and then placed over heat block (70°C) for 10-15 minutes. Following this sample was cooled and vertexed. Control Human serum Albumin (Sigma A8763), Human IgM (Sigma I8260) and Pierce ® Human IgG (Thermo Scientific) were used to correlate the bands from the test samples.

Step 3: SDS Precast gel (4-12 % 12 well, 10x10cm RunBlue™) was removed from the packaging and preservative was thoroughly rinsed with ddH2O. The gel was then placed in the electrophoresis chamber and locked in to the place. The gel chamber was filled with 600 ml of MES (2-(N-morpholino) ethanesulfonic acid) buffer (30 ml of 20X MES SDS (Invitrogen CA, USA) mixed with 570 ml of ddH2O).

Step 4: 20 ul of sample were then loaded into the wells of the gel. 5 ul of Protein size ladder (2-color SDS marker, RunBlue™) was added in the first lane.

Step 5: Electrophoresis was run at 170 voltages or 121 current for 35 minutes. After this cassette was taken out and carefully broken by the knife to get the gel out. The gel was immersed in coomassie blue stain (Instant BlueTM) solution on a plate shaker until protein bands were clearly visible.

Protocol 2-6 : SDS PAGE gel electrophoresis

Step 1 - De-stain: If stained with coomassie stain, de-stain it with destaining solution (100 ml of 40% Ethanol, 10% acetic acid) for 1 hour in rocker/shaker (or microwave on high power for 40 seconds to 1 minute and incubate for 10 minute). Decant the solution and wash the gel with 100ml of 30% Ethanol for 10 minutes (shaker). Decant the ethanol

Step 2 - Sensitiser: Add 100 ml of sensitizing solution (30 ml Ethanol+ 10 ml Sensitiser and dH2O up to 100 ml). Incubate for 10 minutes. Decant the solution and wash the gel with 100ml of 30% Ethanol for 10 minutes (shaker). Decant the ethanol followed by wash the gel with 100ml of dH2O for 10 minutes (shaker). Decant the dH2O

Step 3 - Stainer: Add 100 ml of staining solution (1 ml stainer in 100 ml dH2O) for 15 minutes. Decant the solution and wash the gel with 100ml of dH2O for 30-60 seconds (shaker). Decant the dH2O

Step 4 - Developer: Add 100 ml of developing solution (10 ml developer, 1 drop of developer enhancer to 100 ml of dH2O) for 4-8 minutes until bands start to appear and the desired band intensity is reached

Step 5 - Stopper: Add 10 ml of stopper directly in above. Gently agitate the gel for 10 minutes. Colour changes from pink to colourless (indicating that the development has stopped). Decant the solution and wash the gel with 100ml of dH2O for 10 minutes (shaker). Decant and immerse in dH2O to keep it wet

Protocol 2-7 : Silver staining of SDS PAGE gel

2.3.2 **Luminex micro beads assay**

Microbead coated with target HLA proteins are used in the Luminex platform. It requires very small amount of sample usually 5-20 microliters and is high throughput. Simultaneously over 90 HLA-proteins and HLA-specific antibody interactions occur within each microwells containing the micro beads. This is a very sensitive assay and can detect antibodies at very low concentrations. Hence it is used extensively in both clinical and research laboratories.

Screening assays were used to detect the presence of HLA-specific antibodies in a given sample. This was used as first stage in characterisation samples followed by single antigen beads to characterise the specificity of HLA-specific antibodies. Single antigen bead Luminex assay was also used to confirm specificity profiles of purified material following protocols described in section 2.2.

Samples were analysed as per protocol 2-8. MFI readouts of Luminex runs were considered positive if the MFI values were over 500 for the One-Lambda kit. Whilst Life code SAB assay, the positive assignment is based on company's recommendation of two of more positive adjusted ratio relative to control beads. The assay readout was used in qualitative and not in quantitative terms.

Step 1: The samples were centrifuged at 13,000 rpm for ten minutes prior to the test. The reagents and beads were sonicated for 5 minutes, centrifuges for 10 seconds and vortex mixed for 30 seconds prior to use. The 96 well Luminex plate (Millipore UK) was washed with 200 ul of wash buffer by vacuum suction method. Finally, 20ul of buffer was pipetted in to each well.

Step 2: Add beads (For screening of the samples, 2.5 microliter of Labscreen beads (One Lambda Inc, CA, USA) were used and for defining specificities 2.5 microliter of Single Antigen Beads (One Lambda Inc, CA, USA) or 20 microliter of Gen-Probe single antigen beads (Gen-Probe, CA, USA) were used.

Step 3: Add sample – 10 ul (if using one-lambda beads) or 5 ul (if using Gen-Probe beads) of sample was added to the beads and incubated for 30 minutes at room temperature (22 degree Celsius). Positive and negative controls were used with each experiment.

Step 4: Add fluorescence conjugated secondary antibodies – Wells were washed three to four times with wash buffer and then 50 microliter of 1:100 dilution phycoerythrin (PE) conjugated goat anti-human IgG (supplied with One Lambda kit) or 25 µl of 1:10 phycoerythrin (PE) conjugated goat anti-human IgG (supplied with Gen-Probe kit) was added and incubated for 30 minutes in dark at room temperature (22 degree Celsius). For IgM analysis, phycoerythrin (PE) conjugated goat anti-human IgM was used in similar dilutions.

Step 5: Following above, the wells were washed four times using vacuum filtration and then 90 microliter of wash buffer was added to each well. No wash steps were performed if using Gen-Probe kit.

Step 6: Analysis were carried out using the Luminex® Xmap 100/200 platform Fluoroanalyser (Luminex Corp, USA). HLA Fusion software (One Lambda Inc, USA) was used to obtain raw median fluorescence intensity of different alleles. Life-match Quicktype analysis software version 2.2 was used for Gen-probe kit.

Protocol 2-8 : Luminex bead assay to detect and measure HLA-specific antibodies

2.3.3 **Mass spectrometry**

This was carried out at Waters/Warwick Centre for BioMedical Mass Spectrometry and Proteomics by Susan Slade. The Coomassie stained gel pieces were processed and digested with trypsin using the manufacturer's recommended protocol on the MassPrep robotic protein handling system. The extracted peptides from the sample were analysed by means of nanoLC-ESI-MS/MS using the NanoAcquity/Synapt HDMS instrumentation (Waters) using a 30 minute LC gradient. The data were used to interrogate the UniProt human reference proteome database (http://www.uniprot.org/) using ProteinLynx Global Server v 2.5.1.

The mass spectrometry was performed to assess the efficacy of purification protocols and identify any contaminant proteins to guide further steps of purification. Hence it was only used as a qualitative tool in the earlier stages in order to establish protocols.

2.4 Quantification of antibody

Quantification of antibody content is required during purification/ enrichment protocols and studying binding kinetics on biosensor platform. The methods to detect can be divided in to two categories – 1) Methods that estimates protein concentration and 2) Methods that estimates HLA-specific IgG concentration.

2.4.1 Methods to measure protein concentration –

Depending on the purification technique, this could estimate either IgG or HLA-specific IgG content specifically however as described in section there are impurities following any methods of purification and the aim of these methods is to enrich as pure as possible. Methods that measure concentration either does this by detection of particular amino acids by absorbance or by reaction of dyes or copper with amino acids and peptide bonds. Various methods were used during this project and the choice was influenced by sample volume and lower limit of detection of the methods.

2.4.1.1 Nanodrop® 1000 Spectrophotometer

The NanoDrop® 1000 (ND-1000) spectrophotometer (Thermo Scientific, Loughborough UK) can measure concentration of protein at range of 100ug/ml to 100mg/ml of BSA. This is based on principle that protein containing tryptophan (Trp), Tyrosine (Tyr) or cys-cys disulphide bonds will absorb strongly at 280nm. The absorbance analysis is performed and concentration is derived by the software. This is effective for purified

preparations only. The advantage of Nanodrop is that it requires very small volume of sample and is quick.

To assess protein concentration, select 280 nm absorbance on the ND-1000 3.7.1 software and initialise the Nanodrop with 2.5 ul ddH2O. A blank measurement using 2.5 ul of the sample buffer alone is performed prior to testing the samples. The blank measurement is subtracted from the measured absorbance of each sample to give concentration of sample protein.

2.4.1.2 Jenway® 6505 Spectrophotometer

This equipment is more sensitive than the Nanodrop, however requires larger volume for analysis. 1 ml of neat sample (if low concentration) or 10 ul of sample in to 1 ml of 50mM NaOH (if high concentration) is placed in a quartz cuvette and absorbance measured at 280 nm. Similar to the Nanodrop Spectrophotometer, a blank reading with dilution buffer is done to subtract background absorbance from the test samples.

2.4.1.3 Calorimetric plate assay

These assays are based on biuret reaction (reduction of Cu2+ to Cu+ by protein in an alkaline medium). Cu+ reacts with reagent (folin in Lowry and bicinchonic acid (BCA) in BCA assay) resulting in change in colour which is proportional to the concentration or protein in solution. This is measured at 595 nm absorbance.

Micro-plate assay	Linear range	Sample requirement
Lowry's	0.2 to 1.5 mg/ml	5 ul (in duplicate)
BCA	0.02 to 2 mg/ml	25 ul (in duplicate)

Table 2-5 : Colorimetric micro-plate protein quantification assays

A standard curve is obtained using protein standard – Bovine serum Albumin (BSA) and fitting is applied on the data points to obtained relationship between X and Y axes. If the data are not linear then non-linear regression analysis (such as four-point parametric or polynomial regression models) are applied to determine the concentration of protein in the sample.

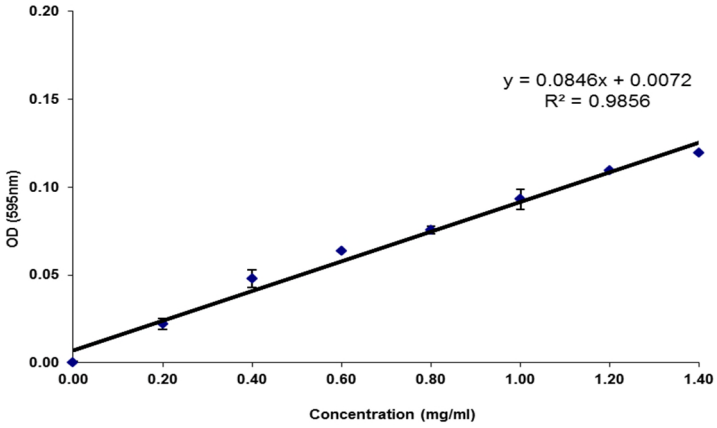


Figure 2-7 : Dose response curve / standard curve for Lowry's assay showing linear fit and equation used to derive concentration of unknown samples

Reagents used in Lowry's assay were obtained from BioRad (DC protein assay Reagent A, S & B) and for BCA assay are obtained from Pierce Thermo scientific (Reagent A & B). Lowry's assay was used for samples with higher concentration as it consumes lesser amount of volume of sample (protocol 2-9); whereas BCA for more dilute samples sample (protocol 2-

10). If the concentration is lower than the lower limit of linear range of BCA assay, then they were re-tested following concentrating using viva spin concentrators.

Step 1: Vortex the protein standard and test samples. Transfer in to the plate (Microtest ™ 96 Falcon, Becton Dickinson) in duplicates. Samples are diluted if too concentrated and placed in duplicates

Protein standard / Ladder titration for Lowry's assay -

Conc of standard	**1.4 mg/ml**	**1.2 mg/ml**	**1 mg/ml**	**0.8 mg/ml**	**0.6 mg/ml**	**0.4 mg/ml**	**0.2 mg/ml**	**0 mg/ml**
BSA (ul) from stock	28	24	20	16	12	8	4	0
PBS pH 7.4 (ul)	172	176	180	184	188	192	196	200

Step 2: Place 5 ul of protein standard in the column in duplicate and samples in row in duplicate

Step 3: Prepare a mixture of 1 ml of reagent A and 20 ul of reagent S in a reagent reservoir. Add 25 ul of mixture in to each well with the standard and the samples.

Step 4: Following above step add 200 ul of Reagent B in to each well with the standard and the sample

Step 5: Place the plate on the shaker (Labsystems Automix) for 10-20 seconds and then incubate at room temperature for 10-15 minutes.

Step 6: Read the plate in (Therma Electron Corporation, MULTISKAN ASCENT) at absorbance of 595 nM absorbance using Ascent software version 2.6.

Step 7: The Optical density (OD) read out is transferred on to the Microsoft excel to work the relationship equation for the standard curve and to derive the concentration of test samples by using linear fitting.

Protocol 2-9 : Micro plate Lowry's assay

Step 1: Vortex the protein standard and test samples. Transfer in to the plate (Microtest ™ 96 Falcon, Becton Dickinson) in duplicates. Samples are diluted if too concentrated and placed in duplicates

Protein standard / Ladder titration for BCA assay -

Conc of standard	2 mg/ml	1.5 mg/ml	1 mg/ml	0.75 mg/ml	0.5 mg/ml	0.25 mg/ml	0.125 mg/ml	0.025 mg/ml	0 mg/ml
BSA (ul) from stock	40	30	20	15	10	5	2.5	2	0
PBS pH 7.4 (ul)	160	170	180	185	190	195	197.5	800	200

Step 2: Place 25 ul of protein standard in the column in duplicate and samples in row in duplicate.

Step 3: Take 5 ml of BCA reagent A and 100 ul of reagent B (50:1) and mix in the large well → add 200 ul of mixture in to each well with the standard and the sample

Step 4: Place the plate on the shaker (Labsystems Automix) for 10-20 seconds and then incubate at room temperature for 10-15 minutes.

Step 5: Read the plate in (Therma Electron Corporation, MULTISKAN ASCENT) at absorbance of 595 nM absorbance using Ascent software version 2.6.

Step 6: The Optical density (OD) read out is transferred on to the Microsoft excel to work the relationship equation for the standard curve and to derive the concentration of test samples by using linear fitting.

Protocol 2-10 : Micro plate BCA assay

2.4.2 HLA-specific IgG quantification

HLA-specific IgG quantification can be achieved using monoclonal HLA-specific antibodies as standard on ELISA or Luminex based assay. However titration Luminex assay is very costly, so we adapted ELISA assay developed by Rico Buchli at Pure Protein LLC, Oklahoma USA.

2.4.2.1 ELISA

Solid phase sandwich ELISA, whereby W6/32 monoclonal antibody was coated and then specific soluble HLA protein was incubated in the microwells. Human monoclonal HLA-A2 and HLA-B7 specific antibodies with known concentration were used as standards to derive concentrations of unknown samples containing HLA-A2 or B7 specific antibodies.

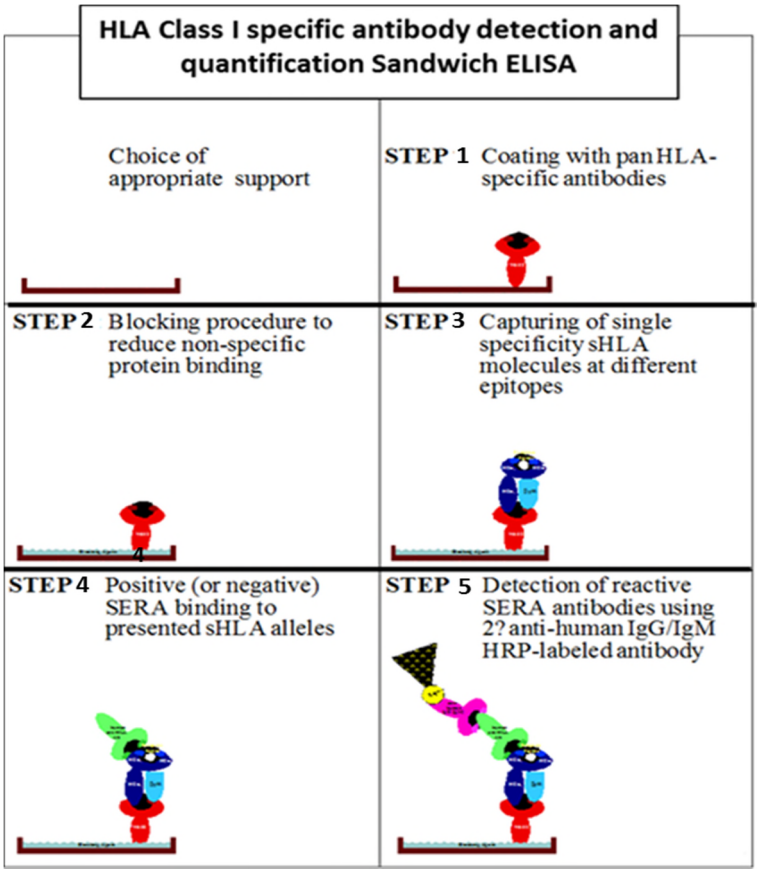


Figure 2-8 : Sandwich ELISA for quantification of HLA-specific antibodies

Step 1 – Coating plate:

Mix W6/32 capture antibody in Phosphate buffer to a final 30ug/ml (changes= 4.59 ul in 5 ml) working solution and vortex well. Add 50 ul coating antibody per well. Put an adhesive film cover on the plate and incubate overnight at 4 C

Step 2- Blocking plate:

Wash plate with PBS-T (on plate washer) and grip plate firmly, invert and slap excess liquid. Add 200ul 3% BSA per well and incubate at RT for 2 hr.

You could do the step 1 for 2 hours at RT followed by step 2 – leave it at 4 ^{o}C overnight incubation.

Step 3- Adding sHLA protein

Wash plate with PBS-T three times and grip plate firmly, invert and slap excess liquid. Add 50 ul of 250ng/ml of sHLA (HLAA*2:01 or HLAB*7:01) and incubate at room temperature for 1 hour.

Step 4 -Adding sample and standards

Human Monoclonal HLA-specific antibodies (SN607D8 for sHLA-A2 and WK1D12 for sHLA-B7) were used as standard. Make mAb standard, stock conc of 1000ng/ml – make dilution (480ng/ml, 240, 120, 80, 40, 20, 10 and 0 ng/ml). Set up ELISA template and make dilutions for samples in 3% BSA (Neat, 1:10, 1:100 & 1:1000). Wash the ELISA plate with PBS-T (on plate washer) and get rid of the excess water (slap). Finally add 100 ul standards and samples to their corresponding wells based in your template and incubate at RT for 1 hr

Step 5 – Add detection antibody (Anti-human IgG HRP)

Make a dilution of 1:30000 of anti-human IgG HRP antibody in 3% BSA (10 ml needed per plate). Wash the ELSIA plate with PBS-T & slap the excess fluid. Add 100ul per well of dilution of detection antibody and incubate 1 hour at RT (away from light)

Step 6 – Adding substrate & developing

Add 2 tablet of OPD (o-Phenylenediamine dihydrochloride) in 10 ml Phosphate-citrate buffer and vortex. Add 4 ul of H2O2 (hydrogen peroxide) right before use and vortex. Wash the ELSIA plate with PBS-T (on plate washer) & slap the excess fluid. Add 100ul per well of substrate and incubate 1-10 min at RT (away from light). Terminate the reaction by adding 100 ul of 3N H2SO4 per well.

Step 7 – Read plate on plate reader (Therma Electron Corporation, MULTISKAN ASCENT) at absorbance of 490nm using Ascent software version 2.6. A standard curve is made and curve fitting is done by the software SoftMax Pro 5.0.1 and concentration of HLA-specific antibodies in the serum is derived.

Protocol 2-11 : Sandwich ELISA for quantification of HLA-specific antibodies

2.4.3 **Protein concentrators**

The HLA-specific antibodies in the samples following purification protocols are usually very dilute and difficult to quantify accurately using protein assays described above. Hence methods to concentrate the protein were utilised in this project using 3000 and 10000 molecular cut-off (MWCO) 2 or 20 ml centrifugal spin concentrators (Vivaspin 2/20, Sartorius Stedim, UK). Another advantage of these concentrators is that they allow for buffer exchange at the same time as protein concentration.

The concentrators are first washed by centrifugation in 2 ml of PBS pH 7.4 (4500 rpm for 15 minutes.) The solution is collected in the filtrate vessel and discarded. The sample is added and then centrifuged again at 4500 rpm for 30 minutes. Proteins larger than MWCO are retained in the upper vessel and 250 ul of PBS pH 7.4 was topped and carefully removed using a fine tipped pipette. For larger sample volumes, 20 ml concentrators were used.

2.5 Statistics and data analysis

Statistical analysis and data presentation were done using Microsoft excel (Windows®), SPSS IBM® and Graphpad ® online quick calculator. Mathematical modelling for kinetics data was done using inbuilt XPR ProteOn software (BioRad®). Non-linear curve fitting on ELISA experiments were done using SoftMax Pro software by collaborators at Oklahoma, USA. The statistical functions used are mentioned in individual chapters.

3 IgM and IgG *de novo* HLA specific antibodies

3.1 Introduction:

The detection of HLA-specific antibodies is used as a risk stratification tool to guide the suitability of transplantation and the diagnosis of rejection. Post-transplantation, *de novo* HLA specific antibodies (Tenhoor et al., 1993) can be of prognostic significance. The most important general characteristic of these antibodies is donor specificity. Other characteristics include quantity, antibody class and subclass, HLA isotype (HLA-A, B, C, DR, etc.) specificity, and Fc mediated function such as complement activation. These have variously been shown to be associated with transplant outcomes (Griffiths et al., 2004, Kushihata et al., 2004, Arnold et al., 2013, Lowe et al., 2013a, Hoenger et al., 2011).

Pre-transplant donor HLA-specific IgG carries a high risk of hyper-acute rejection when determining a positive complement-dependent cytotoxicity crossmatch and medium term graft failure for lower levels. Post-transplant appearance of these antibodies is associated with rejection (Higgins et al., 2009a, Mohamed et al., 2011, Reinsmoen et al., 2008, Wiebe et al., 2012). It is generally accepted that preformed IgM donor HLA-specific antibodies are not a contraindication for transplantation (Chapman et al., 1986, Taylor et al., 1989, Tenhoor et al., 1993) and hence removing IgM by DTT treatment is recommended during sample preparation in order to reduce false positive results. The consequence of HLA-specific IgM developing post-transplantation may be more complicated. The development of donor-specific IgM in itself may not be damaging to allografts (Everly et al., 2014, Marcen et al., 1988) but subsequent class switching from IgM to IgG has been associated with poorer outcome (Lietz et al., 2005). Others have found that post-transplant donor-specific IgM

responses are harmful (Stastny et al., 2009, Piatosa et al., 2011, Bentall et al., 2014). Variations and changes to immunosuppression, and different methods for identifying the antibodies might in part explain these inconsistent observations.

The potential significance of donor-specific IgM is two-fold. Firstly, this immunoglobulin when complexed with antigen is highly effective at activating the classical complement pathway. Secondly, immunoglobulin heavy chain switching is a T-cell driven process, so a change from IgM to IgG implicates specific T cell activity. Conversely, persistence of IgM without IgG implies a certain lack of specific T cell activity. These phenomena will be influenced by the immunosuppression used in organ transplantation. Sequential testing for HLA-specific IgG and IgM may therefore provide a useful biomarker of *in vivo* T cell activation (Lietz et al., 2005).

The purpose of this study was to describe the evolution of immune response early following kidney transplantation in a prospectively collected cohort receiving triple therapy immunosuppression (Tacrolimus, Mycophenolate and Prednisolone) and relate this to adverse outcome during initial years post-transplantation.

3.2 Materials and Methods:

3.2.1 Clinical samples

A group of 85 transplant recipients with no pre-transplant donor specific HLA antibodies (as measured by CDC, Flow cross match and Luminex screen) followed prospectively at Queen Elizabeth University Hospital, Birmingham UK were included in this study. These patients were consented and blood samples and clinical data were collected by Dr Borrows's team. I designed the laboratory work and analysis of the samples and collected follow-up clinical and biochemical data. Ethical approval was obtained (CREC-08/H1204/103) for this study. Five cases were excluded, one case withdrew consent and four had low level pre-transplant IgG donor specific HLA antibodies on subsequent analysis.

All cases received standard triple immunosuppressant therapy consisting of tacrolimus, mycophenolate and prednisolone. Basiliximab was used as induction agent. For ABOi, antibody removal with Glycosorb immunoadsorption column (Glycorex, Sweden) was carried out pre-transplantation with the aim of lowering the haemagglutination titre below 1:8 prior to transplantation. Additionally, Rituximab was given at day -20 and day 0 in cases with ABOi.

3.2.2 Diagnosis and treatment of an rejection episode

Transplant recipients were biopsied to determine cause and the rejection was diagnosed as per Banff criteria (Sis et al., 2010). Rejection episodes were treated with a course of methylprednisolone, and for steroid resistant cases antithymocyte globulin (ATG) was given. Additionally, cases with AMR had plasma exchange and IVIg treatment.

3.2.3 Identifying and defining HLA-specific antibodies

For serology, blood samples were taken weekly for the first month, then fortnightly for four months, monthly until seven months, six weekly until twelve months and thereafter three monthly. For non-sensitised cases, latest samples were screened using mixed screen bead Luminex assays (One lambda) and if positive, the earlier samples were screened until they became negative. Samples were also screened for day 0, 7, 30 to observe for early responses. All the screen positive samples and a preceding negative sample were tested for HLA specificity using single antigen beads (Life code). For sensitised cases, the pre-transplant and latest samples were tested for HLA specificity using single antigen beads and if the profiles differed the samples were tested backwards in order to find the specific time point for theof appearance of de novo HLA-specific antibodies.

Luminex bead assays described in Cchapter 2 were used. Briefly, for screening 2.5 µl of lab screen beads were incubated with 10 µl of serum at room temperature for 30 minutes. Wells were then washed four times and incubated for a further 30 minutes with 1:100 phycoerythrin (PE) conjugated goat anti-human IgG or IgM. Samples were then washed a

further four times and analysed using the Luminex® Fluoroanalyser. HLA Fusion software was used to obtain raw median fluorescence intensity values of different alleles. Positive cut-off was MFI of 500 for IgG (laboratory cut-off) and& 1000 for IgM HLA-specific antibodies. Increased stringency for the IgM assay wasere introduced, as at the time of testing to ensure that specificities were called only if significant increases in MFI were observed at the potential risk of missing genuine but very weak reactions. Thus any effects of IgM anti-HLA responses are therefore likely to be underestimated in this analysis.

HLA class I & II specific antibodies were analysed on Luminex Xmap 200 platform using LIFECODE single antigen bead assays manufactured by Gen-Probe/Immucor/ QUEST. Briefly, 20 µl single antigen microbeads were incubated with 5 µl patient serum at room temperature for 30 minutes. Wells were then washed three times and incubated for a further 30 minutes with 25 µl of 1:10 phycoerythrin (PE) - conjugated goat anti-human IgG or IgM. Following this 75 ul of wash buffer was added and then the sample was analysed on Luminex® analyser. Life-match software was used to obtain the results. The positive assignment is based on company's recommendation of two of more positive adjusted ratio relative to control beads.

3.2.4 Statistics:

The data were analysed using Microsoft excel and Ggraph pad Prism software. Fisher exact test and un-paired t-test were used to compare outcomes between groups using SPSS IBM® software. Groups were compared using one- way Aanova for parametric data, Kruskal-Wallis

test for the non-parametric data and Pearson chi-square test for the categorical data using SPSS IBM® software. Survival curves were generated using SPSS IBM® software.

3.3 Results:

The demographic characteristics of the recipients are summarised in Table 3-1. Baseline characteristics of cases between the groups were not significant except PRA levels (≤ 5%) where the Pearson chi-square test was significant df = 3, P = 0.002). Thus PRA >5% associated with development of *de novo* HLA-specific responses. None of the cases that received rituximab (for ABO-incompatible kidney transplantation, N = 18) developed a *de novo* IgM response (P = 0.018, 0/18 versus 15/47**).** There was no difference in *de novo* IgG response according to prior Rituximab therapy (P = 0.37, 3/18 versus 18/62).

3.3.1 Prevalence and timing of de novo antibody responses

In eighty patients, NDSA were found to be more prevalent than DSA for both IgM (P = 0.0046) and IgG (P = 0.0061) classes. NDSA tended to be seen earlier than DSA for both antibody classes. IgM antibodies appeared earlier than IgG, but not statistical significance was seen (data given in Table 3-2). IgG DSA appeared after a median of 362 days compared to IgM DSA with median period day of 140 days. During the median follow-up of two years, the prevalence of IgG de novo DSA was 8.8%. There was an apparent larger breadth (number of antibody specificities) of responses for IgM compared to IgG (median no 4 (1-26) versus 2 (1-6). However these differences did not reach statistical significance.

	All	IgM^-/IgG^-	IgM+IgG-	IgM-/IgG+	IgM+/IgG+
No	80	50	9	15	6
Age (mean)	45	47.5	35.67	44.2	43.5
Female (no (%))	32 (40)	21 (42)	2 (22)	7 (47)	2 (33)
Race (Caucasian no (%))	58 (73)	40 (80)	7 (78)	8 (53)	3 (50)
PRA (≤ 5%) (no (%))*	72 (90)	48 (96)	9 (100)	12 (80)	3 (50)
HLA mm (mean)	3	3	3	2.8	2.3
DR mm ≥ 1 (no (%))	49 (61)	32 (64)	6 (67)	9 (60)	2 (33)
DR mm >2 (no (%))	14 (18)	7 (14)	3 (33)	3 (20)	1 (17)
First Transplant (no (%))	72 (90)	45 (90)	9 (100)	13 (87)	5 (83)
Pre-emptive (no (%))	21 (26)	15 (30)	1 (11)	3 (20)	2 (33)
ABOi (no (%))	18 (23)	15 (30)	0	3 (20)	0
Deceased donor (no (%))	39 (49)	21 (42)	5 (56)	8 (53)	5 (83)
SGF/DGF (no (%))	19 (24)	12 (24)	1 (11)	4 (27)	2 (33)

Table 3-1 : Baseline characteristics of transplant recipients with different immunoglobulin isotype profile

(PRA – Panel reactive antibody; mm – mismatch; SGF/DGF – Slow/delayed graft function)

	a. Prevalence (N (%))			b. Time of appearance (days post transplantation)	
	IgM	**IgG**		**IgM**	**IgG**
DSA	**3 (3.8%)**	**7 (8.8%)**		**140 (73-433)**	**362 (225-542)**
NDSA	**15 (18.7%)**	**21 (26.3%)**		**54 (19-137)**	**141 (36-485)**

Table 3-2 : *De novo* HLA specific antibodies in 80 renal transplant patients.

a. For both IgM and IgG there are more cases of NDSA than DSA (p=0.0046 and p=0.0061, respectively). b. In general DSAs appear later than NDSAs (median (IQR)) (but statistically not significant - Mann-Whitney U test p =0.654; and 0.219 respectively) and IgM appears earlier than IgG (but not statistically significant p= 0.105 Mann-Whitney U test)

The majority of first time IgM responses were seen within six months post-transplantation (12/15 (80%) versus 10/21 (48%) for IgG responses). The overall difference in the first appearance of IgG and IgM was likely to be due to more *de novo* IgG cases after one year although the difference was not significant (Figure 3-1a). There were more cases with Class I-specific compared with Class II –specific antibodies for IgG (P = 0.016, Figure 3-1b). For IgM, the appearance of antibodies against HLA Class I was similar to that for IgG but there were no detectable *de novo* Class II-specific IgM (P < 0.001, Figure 3-1c).

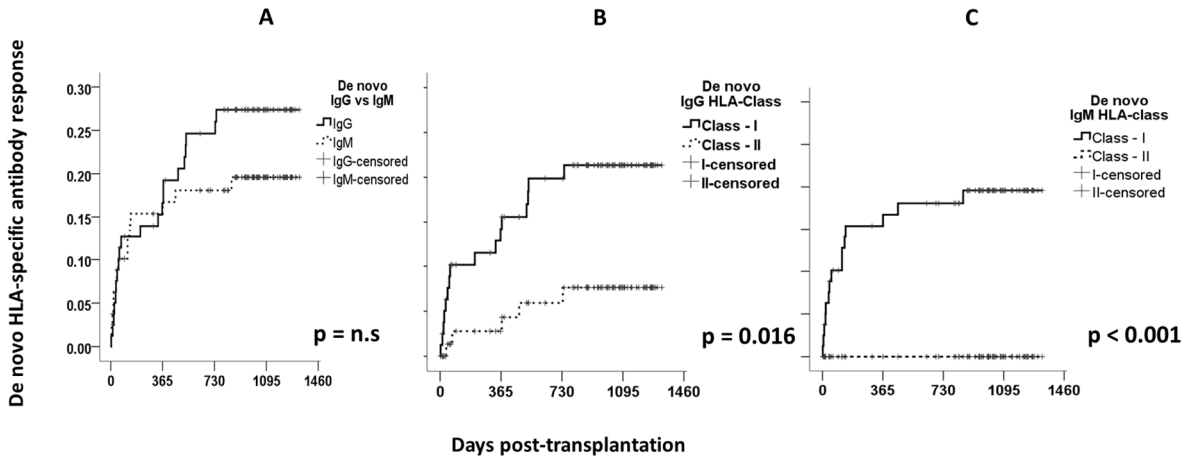


Figure 3-1: Kaplan-Meier analysis comparing the first appearance of HLA specific IgG and IgM

A – De novo IgG versus IgM appearance; B – HLA class I versus class II appearance for IgG and C – HLA class I versus class II appearance for IgM

3.3.2 *De novo* antibody responses class switch

In the six cases with dual IgM and IgG responses, both IgG and IgM against same HLA-specificities were demonstrated in two cases. In the first case there was evidence of class switching from IgM to IgG against HLA-Cw-7 as described in Figure 3-2a. Whilst, in the second case IgG HLA-A2 DSA preceded IgM (Figure 3-2b); this was a case of non-compliance leading to graft loss with AMR.

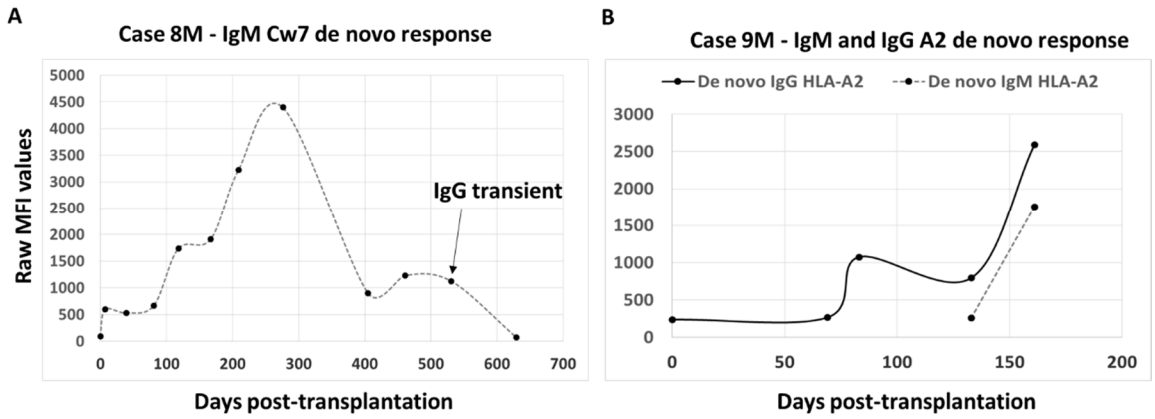


Figure 3-2 : Trend of MFI values of specificities that had class switch from IgM to IgG (A) and case with DSA for same allele (B) panel A

3.3.3 Outcomes

3.3.3.1 *Rejection –*

The post-transplant events associated with each antibody group are shown in Table 3-3. Rejection episodes were seen in 24 (30%) transplant recipients. Of these 5 (6.25%) were antibody mediated rejection (AMR) and 19 (24%) were acute T cell mediated rejection (ACR). There were no episodes of AMR in the IgM+/IgG- group. There were two episodes of AMR in IgM-/IgG- group but both of these had ABOi kidney transplantation. Although there is greater IgG positivity (DSA and NDSA) amongst the rejection group of patients, this did not reach statistical significance. Indeed the majority of IgG-positive cases (13/21, 62%) did not experience rejection.

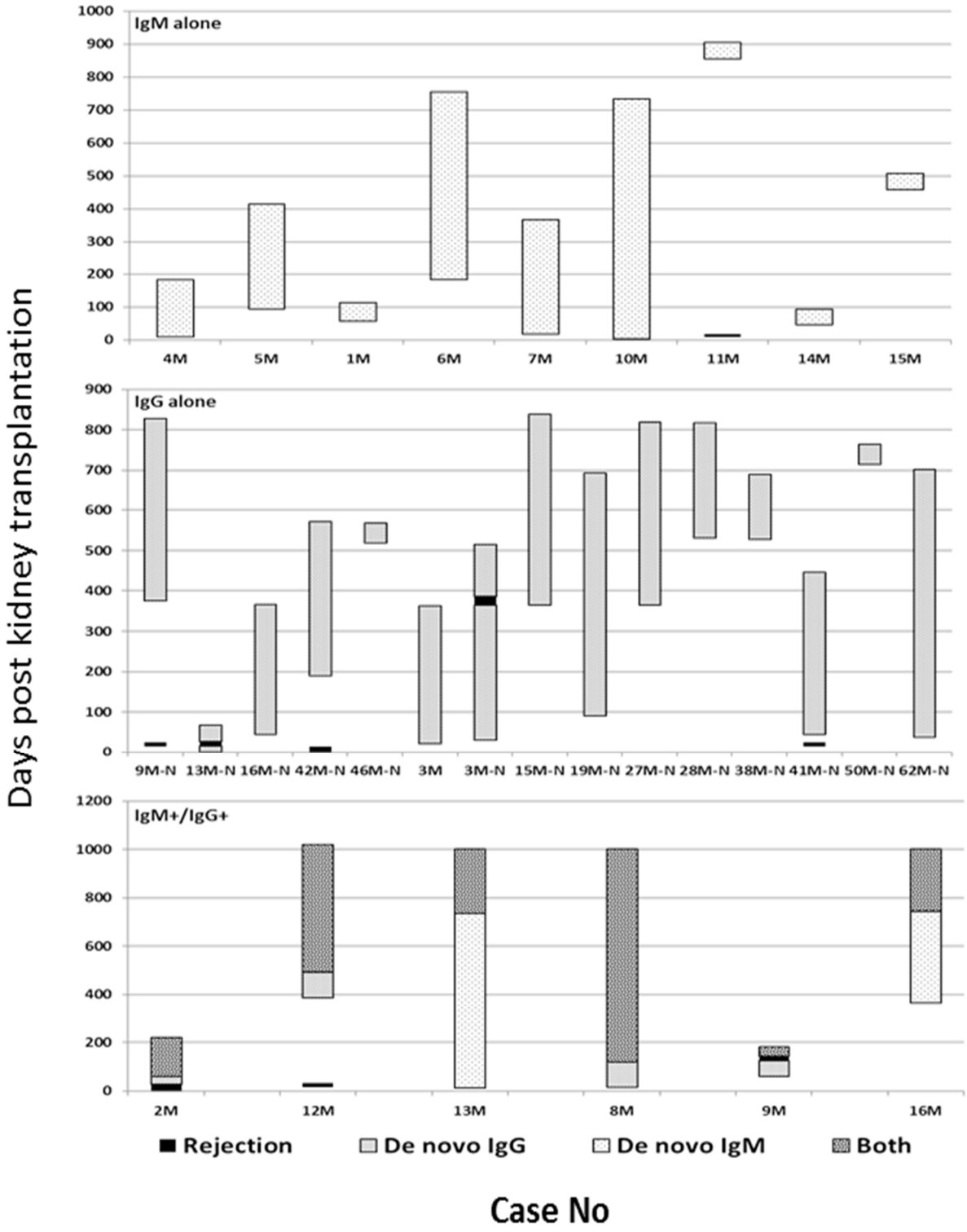


Figure 3-3: Relationship of rejection episode and HLA-specific antibodies evolution along time post Kidney transplantation

Figure 3-3 shows the temporal relationship between rejection episodes and *de novo* HLA-specific antibody appearances. *De novo* HLA-specific IgG preceded an episode of biopsy proven antibody mediated damage in just three cases (2 AMR and 1 transplant glomerulopathy (TG)). Only one of these had a detectable DSA response (case 9M; Figure 3-2B). In 6 cases with an episode of rejection, there was a de novo antibody response detected after the episode with a median of 263 days (IQR = 69 to 359 days). In the remaining 15 cases with rejection there were no HLA-specific antibody responses during median follow-up of 671 days (IQR = 211 to 757 days).

3.3.3.2 ***Graft dysfunction –***

Graft function measured by eGFR (MDRD) following transplantation was compared between group IgM-/IgG-, IgM+/IgG- , IgM-/IgG+ and IgM+/IgG+ groups (Figure 3-4). The trend analysed by Anova was not significant between the groups. Further analysis comparing the groups revealed that, eGFR was significantly lower at month-3 (P = 0.039) and month-36 (P = 0.037) in the IgM+/IgG+ group compared to the IgM-/IgG- group (Games-Howell test). Early graft function tended to be superior in the IgM+/IgG- group but did not reach statistical significance. Within the IgM-/IgG+ group , year three eGFR was significantly lower in those with a history of rejection compared to those with no rejection episodes (n = 5 versus 10; P = 0.03, Kruskal-Wallis test).

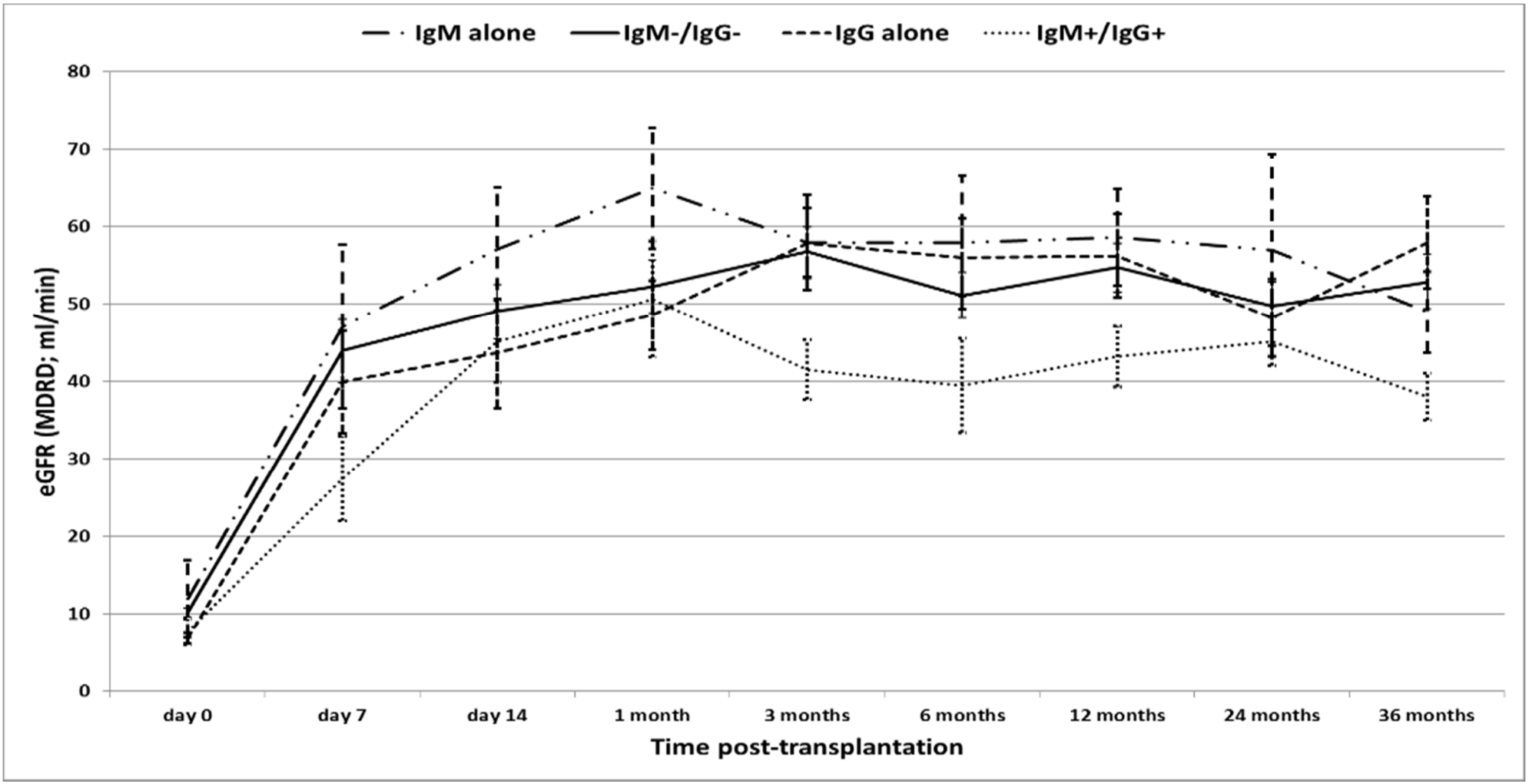


Figure 3-4 : eGFR (MDRD) following kidney transplantation in groups with *de novo* IgM+ (N=9) / IgG+ (N=15)/ IgM+/IgG+ (N=6) versus IgM^-/IgG^- (N=50). No significant difference in mean eGFR (ANOVA test). However post-doc analysis showed significant difference between IgM+/IgG+ and IgM^-/IgG^- at month-3 and –36 (Games-Howell test).

3.3.3.3 *Other outcomes –*

All other outcomes such as new onset proteinuria, death, and graft failure did not show any significant association with any of the antibody groups. (Table 3-3)

Groups	All	IgM-/IgG-	IgM alone	IgG alone	IgM+/IgG+
N	80	50	9	15	6
None events N (%)	49 (61.25)	30 (60)	10 (77)	10 (67)	2 (33)
Graft Failure N (%)	6 (7.5)	4 (8)	0 (0)	1 (7)	1 (17)
Death N (%)	4 (5)	2 (4)	0 (0)	1 (7)	1 (17)
Rejection N (%)	24 (30)	15 (30)	1 (11)	5 (33)	3 (50)
ACR N (%)	19 (24)	13 (26)	1 (11)	3 (20)	2 (33)
AMR +/- ACR N (%)	5 (6.25)	2 (4)	0 (0)	2 (13)	1 (17)
Proteinuria N (%)*	13 (16.25)	10 (20)	1 (11)	1 (7)	1 (17)

Table 3-3 : Outcomes in relation to *de novo* development of HLA Ig class-specific antibodies showed no significant difference between the groups **

(*Proteinuria – defined as Albumin Creatinine Ratio over 30mg/mmol persistent on two occasions, and after 1 month of transplantation, ** Fisher exact 2-tail test).

3.4 Discussions:

This prospective study looked at evolution of HLA-specific antibody (IgM & IgG) responses following kidney transplantation in a cohort receiving standard triple immunosuppressant regimen (tacrolimus, mycophenolate and prednisolone). The sampling was more rigorous compared to other studies. The median time between the last negative sample and the first positive was 21 (2-240) days for *de novo* IgM and 140 (3 to 497) days for *de novo* IgG responses. The patients in this study were receiving a homogenous maintenance therapy, so that the prevalence of HLA-specific antibodies relates to commonly used triple immunosuppressive therapy (Tacrolimus, Mycophenolate Mofetil and Prednisolone).

Previous studies on IgM HLA response are limited, smaller in size, indicate conflicting results and have used non-specific CDC or Flow cytometry assays (that use donor cells or panel of cells to assess the response). (Worthington et al., 1998, Suzuki et al., 2009, Tardif and McCalmon, 1995, Piatosa et al., 2011, Chapman et al., 1986, Taylor et al., 1989, Lietz et al., 2005). Studies using microbeads coated with purified specific HLA proteins have focused on pre-transplant sera from kidney transplant recipients. Studies by Khan et al, used the FlowPRA (One Lambda) assay and analysed sera of failed transplant cases and revealed the presence of DSA-IgM HLA class I-specific antibodies (Khan et al., 2003). Similarly, a study looking at pre-transplantation sera from 34 kidney transplant recipients using Luminex assay (One Lambda beads) noted that IgM+/IgG- DSAs were detected in higher proportion of cases with rejection compared to cases without rejection ($P < 0.02$) (Stastny et al., 2009). They observed similar findings in cardiac transplant recipients. Contrary to above, studies in 49 lung transplant

recipients showed that the presence of both pre and post transplantation IgM HLA-specific antibodies detected by screening beads was not associated with poor outcome (Paantjens et al., 2011). Recently studies (Everly et al., 2014) using Luminex single antigen bead assay and suggested that IgM DSA are significant as they were associated with higher grade of rejection but overall graft survival was not affected. However, in this study the majority (60%) of cases were receiving cyclosporine-based immunosuppressive treatment and proportions on mycophenolate are not mentioned.

In the current study, we have shown that in a prospective cohort of transplant recipients on standard triple immunosuppressive therapy, there were greater non-donor specific *de novo* responses for both IgM and IgG classes. IgM *de novo* responses appeared to emerge earlier than IgG but the difference in the median time was not statistically significant. IgG DSA responses were noted mainly one year post-transplantation (5/7 cases) except in a case with non-compliance at day 34 (class I) and in another case at day 90 (class II). All three cases with IgM DSA, the *de novo* DSA response was within the first six months following kidney transplantation. Responses to HLA class are interesting as no *de novo* class II responses were observed for IgM class.

Another interesting finding was the lack of *de novo* IgM anti-HLA responses in cases with ABOi kidney transplantation (p=0.018, Fisher's exact test). The rate of *de novo* IgG anti-HLA response in ABOi was no different from standard kidney transplantation (P = 0.37, Fisher's exact test). It is possible that Rituximab therapy was responsible for the reduced rate of IgM

production, as this was the only systematic difference in therapy between the groups, apart from ABO specific immunoadsorption therapy pre-transplant. Similar to the novel studies described here, other studies have shown no effect on IgG response with prior Rituximab therapy (Lobashevsky et al., 2013, Ashimine et al., 2014). Some of these *de novo* IgG responses could be re-call responses from a previous sensitisation. So although they were not present pre-transplantation, they may have been there historically due to prior sensitisation events. The HLA types of donors were not routinely tested, so it is difficult to assess the re-call responses. A non-donor specific re-call response could be due to generalised immune up-regulation and may drive non-specific inflammation in allograft causing adverse outcomes. Endogenous molecules released during tissue injury such as damage associated molecular patterns (DAMPs) can activate cellular receptors and lead downstream inflammation (Rosin and Okusa, 2011). TLRs mediate some of these effects (Krueger et al., 2009). Hence NDSA may still serve as biomarkers for poor transplant outcome.

Similar to the previous study (Everly et al., 2014), this study also observed that IgM alone is not associated with adverse outcome. Cases with dual positive (IgM+/IgG+) were small in number but had significant poor graft function at month 3 and month 36 compared to group with no antibody responses (IgM-/IgG-). Cases with *de novo* IgM alone (IgM+/IgG-) with no observed class switch tend to have better graft function (eGFR; MDRD). This suggests a better prognostic group. This finding is similar to a previous study on cardiac transplant recipients (Lietz et al., 2005). In 267 primary heart transplant recipients with a mean follow-up of 3.1 years, 20 % produced IgM antibodies. The study observed the persistence of IgM (particularly

anti-HLA class II) and the lack of IgG isotype switching, as measured by CDC, were associated with protection against acute rejection.

IgG HLA-specific responses studied here confirm findings of previous prospective studies. Particularly, that the de novo IgG response was not predictive of episodes of rejection and that in most cases with rejection, the antibodies appeared following the episode (Gill et al., 2010). Similarly, the present study noted that the group with rejection and IgG *de novo* responses had worse 3 year eGFR (MDRD) compared to group with IgG *de novo* response without any episodes of rejections (Cooper et al., 2011). Another similarity was the higher incidence of AMR in the *de novo* IgG group (Heilman et al., 2014) but numbers were too small. In contrast to the other prospective study, the *de novo* DSA response was earlier (Wiebe et al., 2012). In keeping with observations by Wiebe et al, a patient developed *de novo* DSA responses due to poor compliance resulting in AMR and graft failure.

A limitation of this study is the absence of protocol biopsy samples to detect sub-clinical rejection. This was considered in the initial protocol, but was dropped because of the high rate of failure to consent. The second limitation is the shorter clinical follow-up period (three years) and it may be in the long-term that the *de novo* HLA-specific antibody response does have significant effects similar to those observed by Wiebe *et al*. The third limitation is that the antibodies were defined by Luminex assay alone post transplantation and some of the positive results may be false positive (Gombos et al., 2013). However, the positive threshold

for IgM was raised to 1000 MFI on screening to increase the confidence of genuine anti-HLA reactivity.

3.5 Conclusion:

This three year prospective study of 80 cases following kidney transplantation showed that early *de novo* responses for both IgM and IgG *de novo* HLA-specific antibody production did not increase adverse events in the short term in cases receiving standard triple immunosuppressant therapy, except in a group of cases with dual positive responses. Rituximab therapy abolished *de novo* IgM responses but had no effect on *de novo* IgG responses. Prospective screening for IgM or IgG HLA-specific antibodies does not predict rejection or adverse events in early years post-transplantation in cases on the standard triple immunosuppressant regimen.

3.6 Acknowledgement

Staff at H&I laboratory, NHSBT and Queen Elizabeth University Hospital, Birmingham UK.

4 Biosensor assay to study interactions of HLA-specific antibodies and HLA-proteins

Biosensor assay based on surface plasmon resonance has advantage of studying the binding of antibody to the antigens in label free and real time. Additional biophysical characteristics such as dissociation constants (K_D) and kinetic rates (on rate K_a and off rates K_d) can be quantified. These additional characteristics have not been studied in transplantation medicine.

4.1 Surface plasmon resonance assay

This is an optical biosensor technique based on surface plasmon resonance principle.

4.1.1 Principle

Electrons within metallic surfaces such as a thin gold film/layer can be excited upon shining light over them. This excitation creates plasmons that travel along the metallic layer (Figure 4-1). Also, at same time an electromagnetic wave field is created in the fluid above the metallic film called Evanescent Wave (EW) field. The EW decays exponentially from the surface, resulting in energy being transferred from the plasmon creating a resonance. Hence it is called surface plasmon resonance. These resonance occurs on the interface between the gold surface and the liquid medium in the instrument flow cell, hence it is very sensitive to changes in surface proximal to the gold following adsorption of any molecules. When the refractive index changes, the energy and momentum of the plasmon also changes, resulting in the shift of the sensorgram dip/ curve. The reflected light can be plotted on a reflective curve (Figure 4-2).

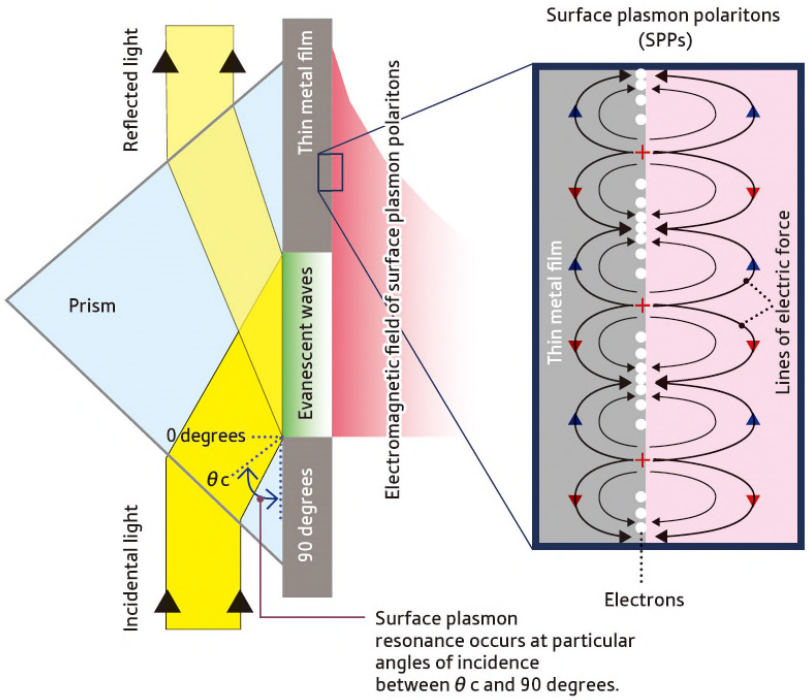


Figure 4-1 : Principle of surface plasmon resonance (taken form http://phys.org)

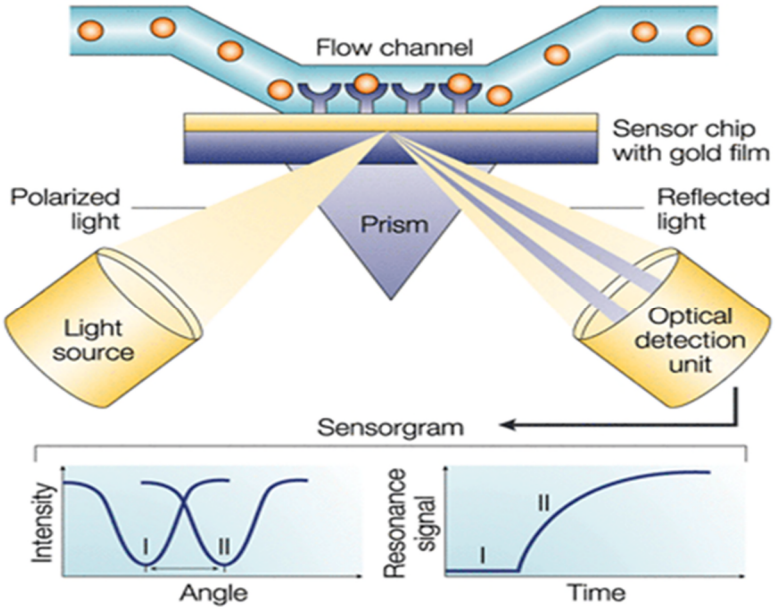


Figure 4-2: Sensorgram obtained during change in mass over the surface layer. This is monitored real time as a plot of resonance signal versus time

(Adapted from Cooper et al, Nature Reviews Drug Discovery 1, 515-528 (July 2002).

The BioRad Proteon XPR36 platform was used for this project. It allowed simultaneous study of 36 spot interactions. Hence a range of concentrations of analyte (HLA-specific antibody) could be studied against different immobilised ligands (HLA protein) or different ligand densities simultaneously. These high throughput features meant that different combinations of interactions could be studied together using the same solution-phase sample (Figure 4-3).

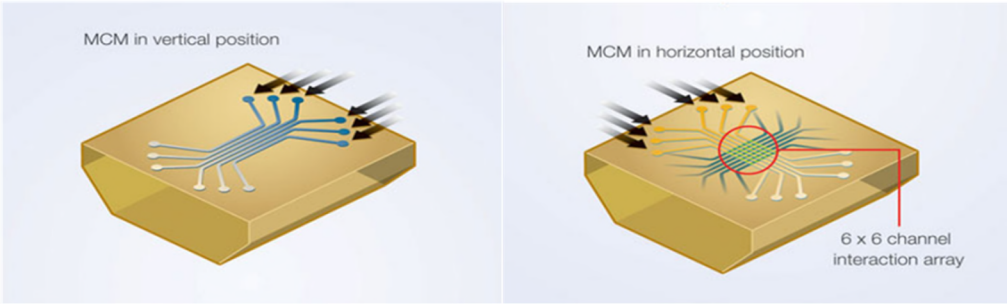


Figure 4-3 : Multiplex array set-up on XPR proteon platform

4.1.2 Mass transport and diffusion limitation

The analyte is transported to the chip surface initially by convection and then diffusion. The diffusion rate depends on the diffusion constant of the molecules. The larger molecule usually takes few seconds to diffuse through the unstirred layer. Hence the concentration will be lower than the sample; this is called mass transport limitation. (Figure 4-4).

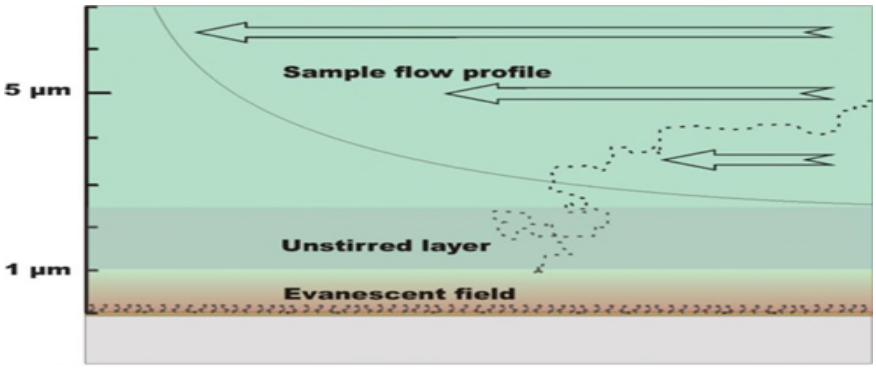


Figure 4-4: Convection and diffusion of analyte through the fluidic system (Adapted from the handbook of surface Plasmon resonance, page 179, chapter 6)

Ways to overcome mass transport limitation –

a) Flow rate – Increasing flow rate will increase the convection

b) Lowering ligand density and increasing analyte concentration

This will allow constant supply of analyte as the binding sites on ligands are limited and thus there will no depletion of antibody in the layer. Another advantage of lower ligand density is that it facilitates the resolution of interactions in affinity-dependent terms rather than avidity-dependant.

c) Compact matrix layer on the sensor chip

Matrix layers on the chip can vary in depth. The 2-D matrix provides a compact layer (10nm) and allow only one mono-layer of immobilised ligand. The analyte in the diffusion layer rarely gets depleted. This is beneficial for kinetic analysis as mass transport limitation and re-binding of analyte in the dissociation phase is reduced (Figure 4-5).

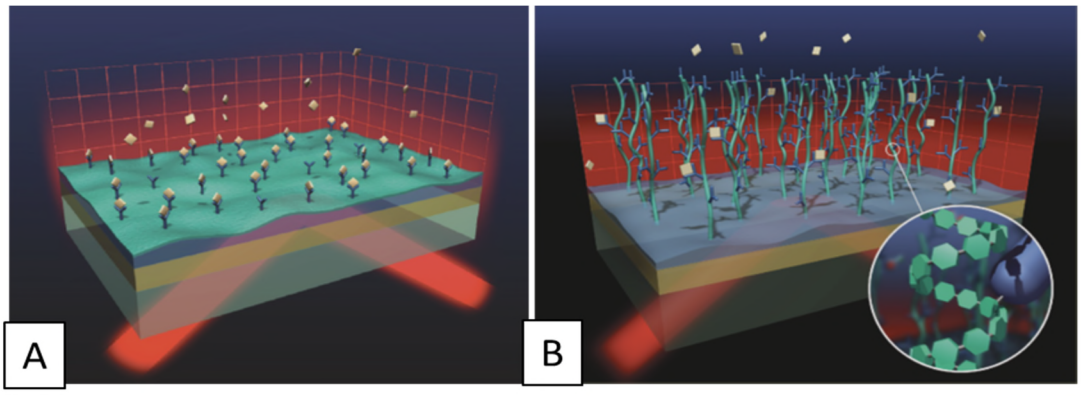


Figure 4-5 : matrix layer on the sensor chip (A – 2D matrix and B – 3D matric)

(Adapted from the handbook of surface Plasmon resonance, chapter 6)

The NLC chip (NeutrAvidin) has a compact layer of modified alginate polymer and utilises neutravidin to capture biotinylated proteins directly (biotinylated HLA proteins). See Figure 4-6.

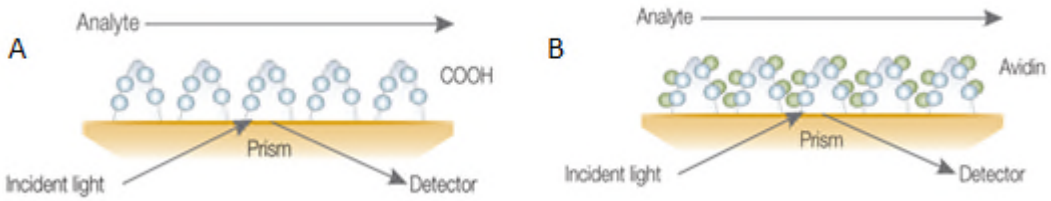


Figure 4-6 : Sensor chip for XPR platform (A – GLC chip with carboxyl group and B – NLC chip with avidin)

4.2 Optimisation of experimental conditions

In first instance to develop the assay, we studied optimal conditions and protocols using human monoclonal HLA-specific antibodies which were generously provided by University of Leiden (A Mulder and F Claas). This chapter describes the purification and accurate quantification of human monoclonal HLA-specific antibodies, use of equilibrium and kinetic analysis and ELISA to quantify dissociation constants, advantage and limitations of current approaches to quantify binding parameters. Although only two HLA-A2 specific monoclonal antibodies were later used, this chapter also describes purification of other human monoclonal HLA-specific antibodies used for experiments in Chapter 5. The insights obtained from work shown in this chapter were latter used to translate the assay for testing of clinical samples in chapter 6. Optimal conditions for monoclonal HLA-specific antibodies were latter used to study and distinguish reactivity for same epitope shared between different HLA-proteins and competitive binding in chapter 4.

4.3 Sample preparation

4.3.1 Purification of human monoclonal HLA-specific antibodies

Supernatants from hybridoma cultures containing secreted human monoclonal HLA-specific antibodies underwent series of upstream purification steps as described in the Methodology (Chapter 2.2). High quality protein purification was required due to strong evidence of impurities in the raw supernatants and even IgG-enriched fractions that resulted in non-specific binding to the highly sensitive sensor chip matrix that is inherent to the SPR experimental scheme (Figure 4-7).

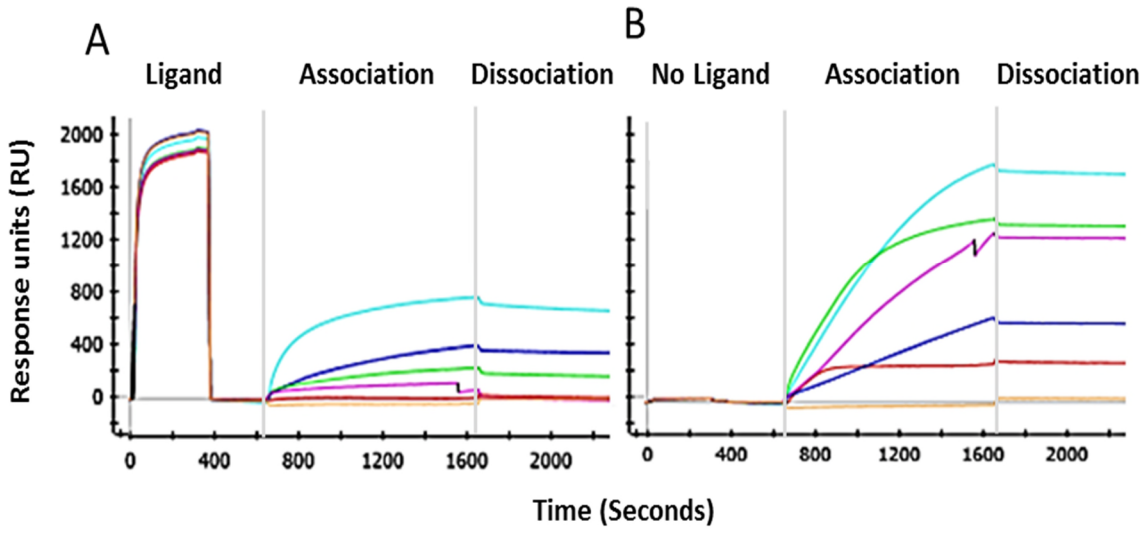


Figure 4-7 : Non-specific binding from un-purified monoclonal HLA-A2 specific antibody (IHB-HU-081 (SN607D8)) to empty reference channel as shown in B.

Whilst it was noted that despite some non-specific binding, a distinguishable signal could be obtained for monoclonal antibodies without rigorous multi-dimensional purification, the accurate quantification of monoclonal antibodies by BCA or Lowry protein assay was

hampered due to the presence of bystander protein impurities. This was evident even after subjecting raw HLA-specific antibody preparations to HLA-protein and Protein G-Sepharose affinity chromatography. Hence two different protocols were used to purify monoclonal HLA-specific antibodies (Figure 4-8).

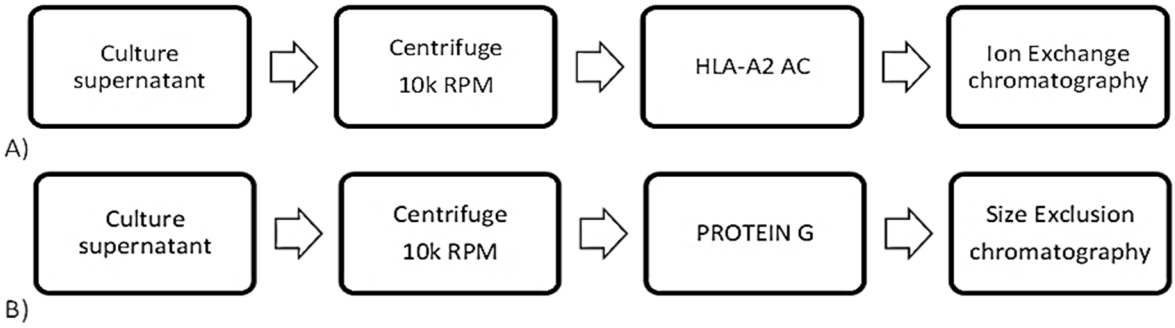


Figure 4-8 : Protocols for purification of human monoclonal HLA specific antibodies

Human monoclonal HLA-specific antibodies were recovered from hybridoma culture supernatant kindly supplied by Arend Mulder and colleagues at the University of Leiden. All the human monoclonal antibodies in the culture supernatants underwent dialysis against PBS overnight prior to centrifugation and affinity chromatography. HLA-protein and Protein G affinity chromatography were used as intermediate enrichment steps prior to either ion-exchange or size exclusion chromatography. The choice of combination of purification steps was determined by the biophysical and chemical characteristics of antibodies, in addition to potential for maximum yield of highly purified protein.

4.3.1.1 *Purification of Monoclonal Antibody SN607D8 (IHB-Hu-081)*

Culture supernatant (100 ml) containing SN607D8 (IHB-Hu-081) was dialysed overnight against PBS (pH 7.4) to achieve buffer exchange and remove dyes and culture medium solutes and then centrifuged at 10,000 rpm for 10 minutes to remove particulates. The material was subsequently diluted in an equal volume of PBS (pH 7.4). The total volume was then run over a HLA-A2-Sepharose column (50 ml settled volume) and semi-automated affinity chromatography performed with a peristaltic pump at a flow rate of 50 ml/min. A 500 ml volume of PBS was used to wash the affinity column prior to eluting with one litre (20 column volumes) of 100 mM Glycine, pH 11.0. The elution material was immediately neutralised with 125 ml of 1M TRIS (pH 7.0) and tested with Luminex single antigen beads giving MFI levels of over 20,000 for HLA-A2, -A68 and –A69 beads and over 1000 but less than 2000 MFI for HLA-B57 & -B58 beads.

The eluted and pH-neutralised material was dialysed against 10 mM HEPES pH 8.0 overnight prior to Anion exchange chromatography. Figure 4-10 shows separation of various protein species (peaks of absorbance at 280 nm) based on anion exchange chromatography runs using a 20 ml Q-Sepharose column with a 0 – 500 mM sodium chloride gradient. The top half shows multiple peaks demonstrating presence of multiple impurities in the raw culture supernatant containing monoclonal HLA-specific antibody, to be expected due to the use of foetal bovine serum in hybridoma culturing. Whereas the bottom part shows material undergone purification protocol-A (summarised in Figure 4-10). Gel electrophoresis (Figure 4-9) and Luminex by Single Antigen Beads (SAB) were performed to confirm the purity of the material and reactivity to specific HLA alleles respectively. Luminex testing gave MFI values

>20,000 for HLA-A2, -A68 and –A69 beads. The samples corresponding to the IgG elution peak was quantified using Lowry's assay and the concentration was 1 mg/ml.

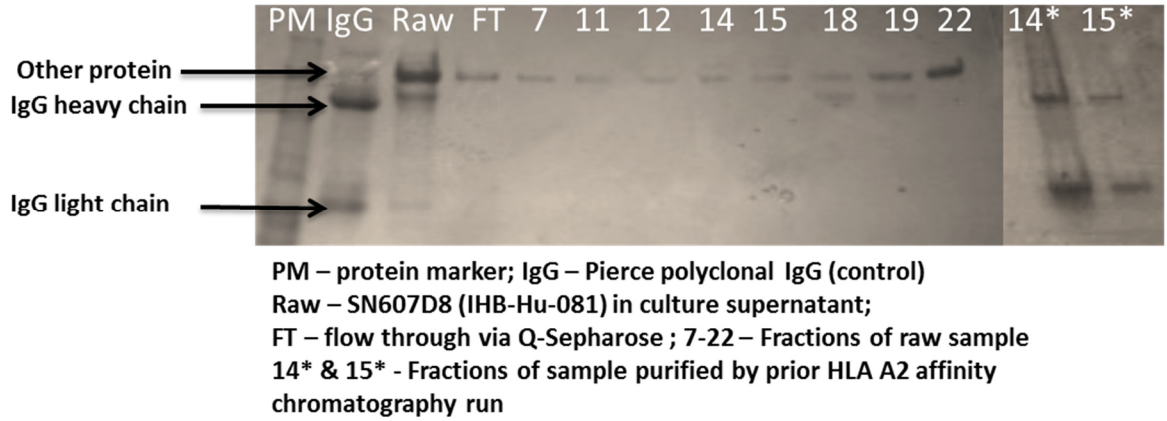


Figure 4-9: SDS PAGE gel electrophoresis done in reducing condition (DTT) shows bands of proteins in different fractions during anion exchange chromatography of raw and affinity purified SN607D8 (IHB-Hu-081).

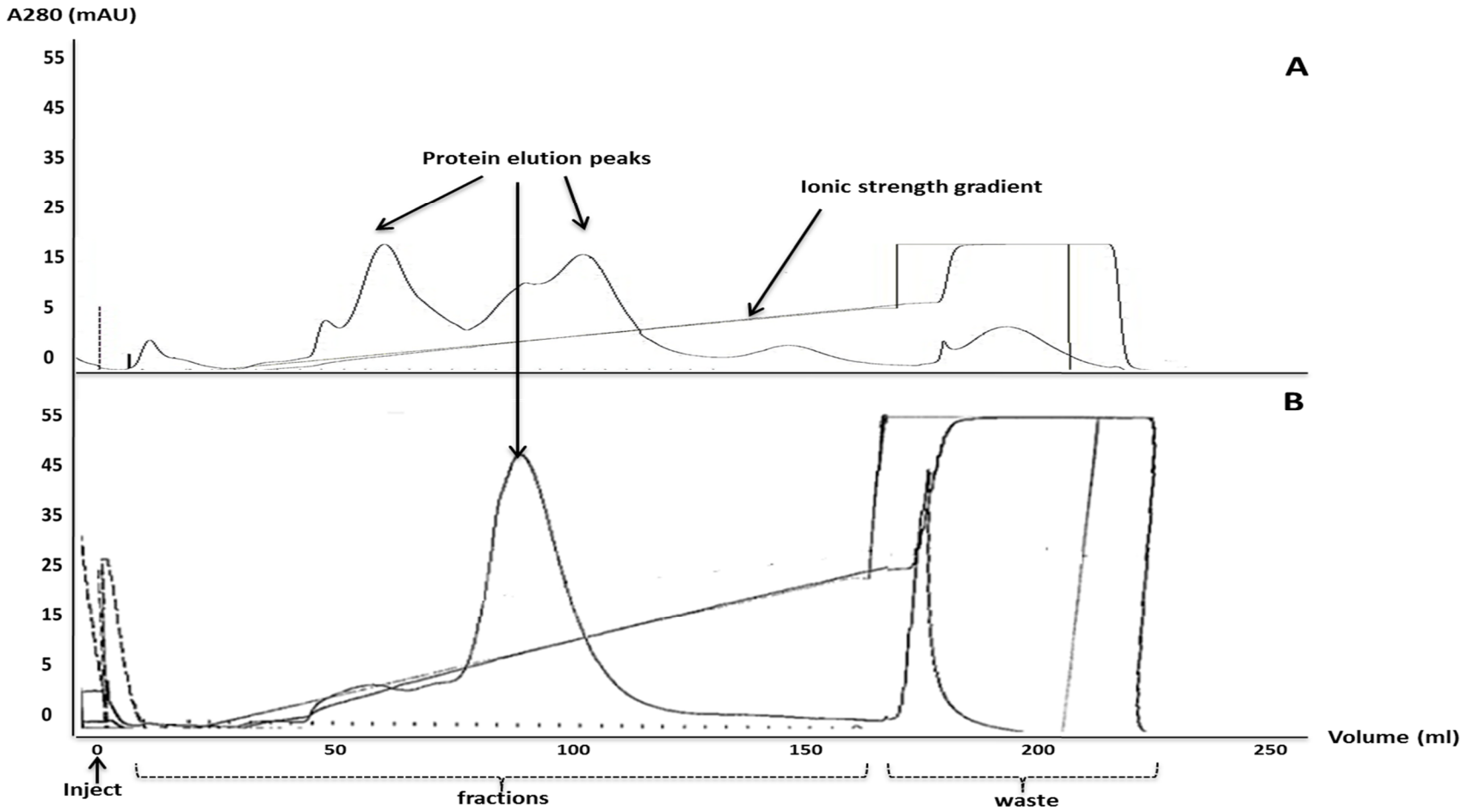


Figure 4-10 : Ion-exchange chromatography (Q-Sepharose column) for purification of SN607D8 (IHB-Hu-081) (A – raw supernatant containing antibody and B – supernatant through HLA-A2 affinity chromatography)

3.1.1.1 *Purification of Monoclonal antibody SN230G6 (IHB-Hu-033)*

Similar to the procedures above, the IHB-HU-033 (SN230G6) was purified via multi-dimensional liquid chromatography (protocol A, Figure 4-8). Luminex single antigen beads show MFI values over 10,000 for HLA-A2, -B57 & -B58 beads and negative for all other beads following the selective HLA-A2 affinity chromatography step. Anion exchange chromatography failed to purify SN230G6 (IHB-Hu-033) as there was no binding either to neither Q-Sepharose nor Resource-Q (Figure 4-12 (A)). The flow through was then buffer exchanged with 10 mM sodium acetate pH 5.5 overnight and *cation* exchange using a negatively-charged SP-Sepharose column was performed. Cation exchange chromatography was able to purify SN230G6 (IHB-Hu-033) with a distinct peak as seen in Figure 4-12 (B). Gel electrophoresis of fractions pre- and post-cation exchange chromatography is shown in Figure 4-11.

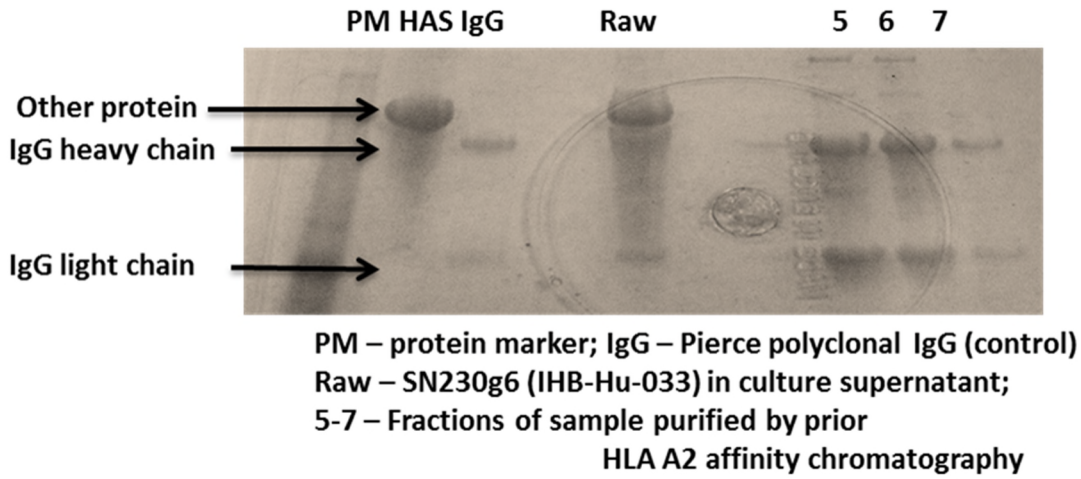


Figure 4-11 : SDS PAGE Gel electrophoresis showing protein bands pre and post Cation exchange chromatography of SN230G6 (IHB-Hu-033).

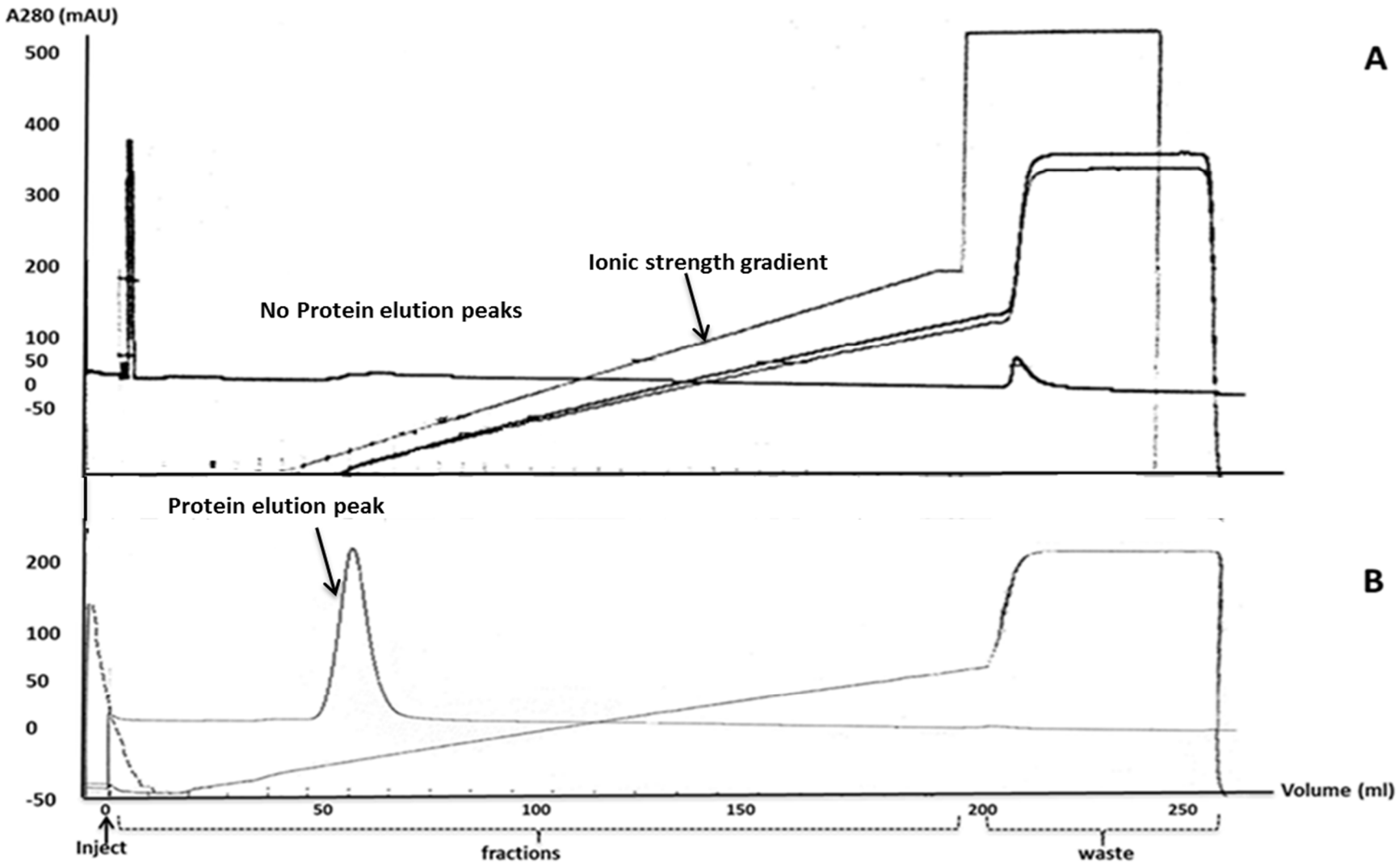


Figure 4-12 : Ion exchange chromatography for SN230G6 (IHB-Hu-033) monoclonal HLA-specific antibody (A – Anion exchange using Resource-Q and B – Cation exchange using SP-Sepharose)

3.1.1.2 *Purification of the WK1D12 monoclonal antibody (IHB-Hu-059)*

Culture supernatant (100 ml) containing WK1D12 was dialysed overnight against PBS (pH 7.4). This was followed by HLA-B7-Sepharose affinity chromatography (4 ml column), with protein eluted in 100 mM Glycine pH 11.0. The peak elution fractions were pooled together and neutralised with 1M TRIS and the solution was dialysed against 10 mM HEPES pH 8.0 overnight. Following this, anion exchange chromatography was utilised for purifying WK1D12 (Figure 4-13). Luminex assay was performed on the pre-, flow-through samples (material that did not bind to HLA-B7-Sepharose and Resource-Q columns) and peak elution samples. Both the flow-through and peak elution fractions from the HLA-B7-Sepharose affinity column were positive by Luminex assay (MFI > 15,000) for HLA-B7/B27-specific reactivity suggesting saturation of the smaller HLA-B7-Sepharose column in addition to pH-mediated elution. Flow-through fractions from the Resource-Q were negative by Luminex assay (MFI < 500), indicating that the HLA-specific antibodies were binding to this anion exchange column. Elution had two distinct peaks and the concentration was too low.

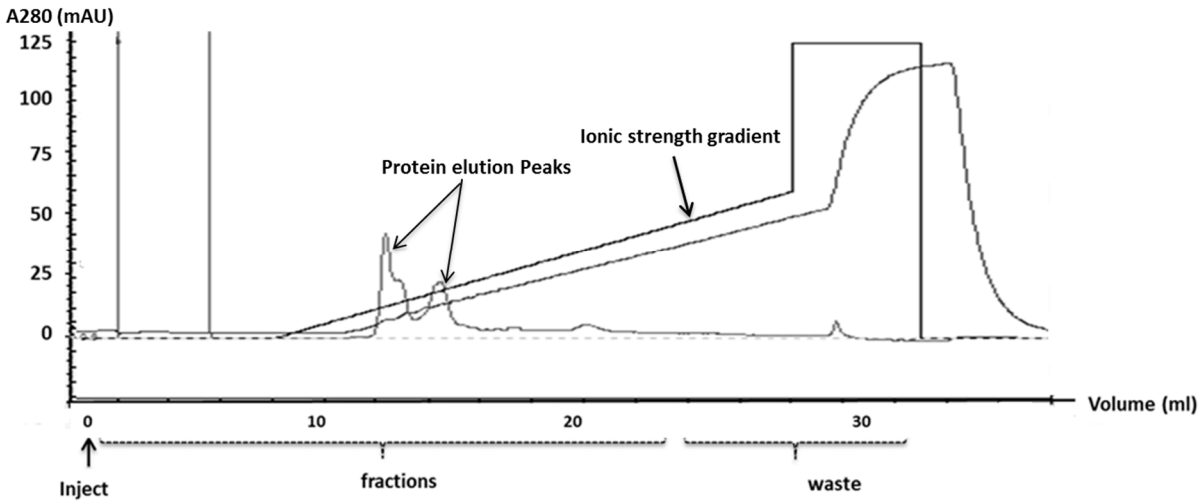
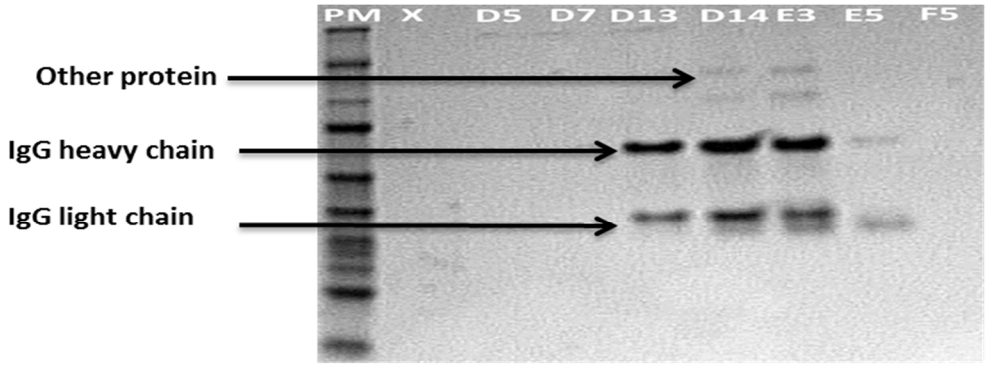


Figure 4-13 : Anion exchange chromatography (Resource-Q) for purification of WK1D12 (IHB-Hu-059) monoclonal HLA-B7/27 specific antibody.

A second batch (100 ml of culture supernatant) containing WK1D12 was purified using protocol B (Figure 4-8). The culture supernatant was dialysed against 10 mM sodium phosphate buffer pH 7.0 followed by affinity chromatography using 4 ml of Protein G-Sepharose. The peak elution was broad. Hence the elution fractions were pooled together and the solution was concentrated using spin concentrator (MWCO of 30,000) using PBS (pH 7.4) as exchange buffer. The material was concentrated to a volume of 2 ml and subjected to size exclusion chromatography (gel filtration) using a 30 cm Superose-12 column, at 1 ml per run in isocratic flow at a rate of 0.5 ml per minute. Figure 4-15 shows a large peak saturating the upper limit of detection by the A280 optical absorbance detector. Gel electrophoresis (Figure 4-14) and Luminex single antigen bead assay confirmed purity for IgG.



PM – Protein marker; X – Empty lane
D5-F5 – Fractions from size exclusion chromatography

Figure 4-14 : Gel electrophoresis of elution fractions following size exclusion chromatography of WK1D12 (IHB-Hu-059)

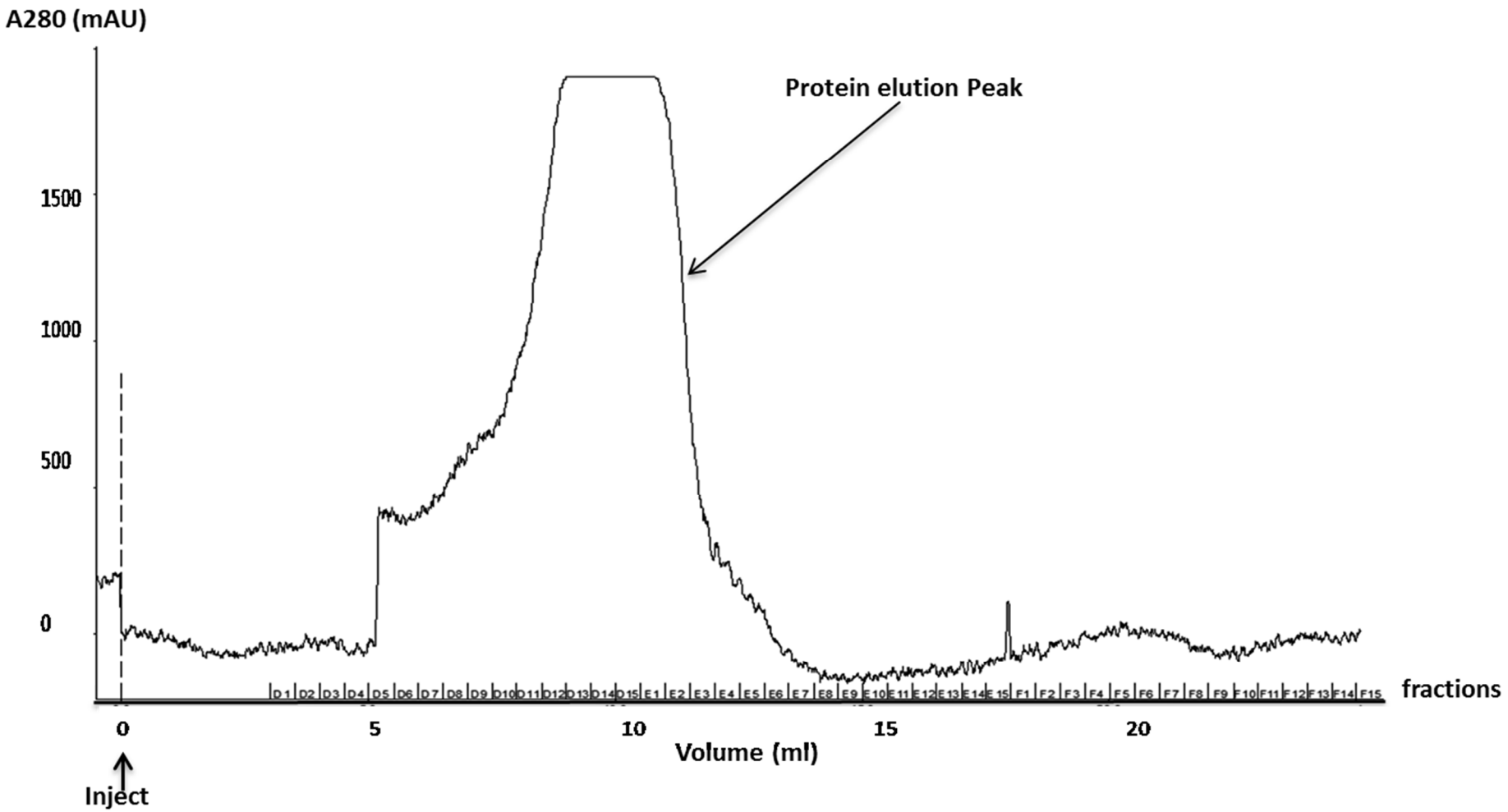
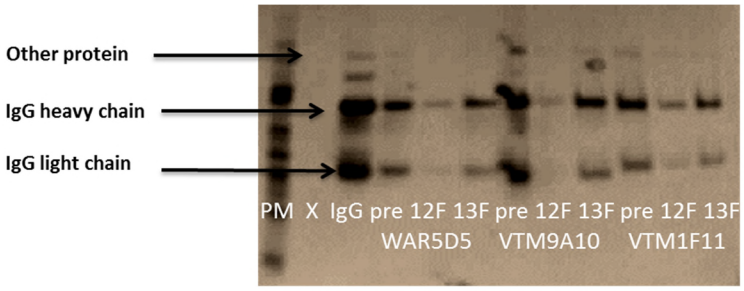


Figure 4-15 : Size exclusion chromatography using Superose-12 column showing elution peak of WK1D12 (D11-E2 fractions)

3.1.1.3 *Other monoclonal HLA-specific antibody purification*

Four other human monoclonal HLA-specific antibodies reacting against B7/B27 (WAR5D5, VTM1F11, VTM9A10 and OUW1F11) were purified as protocol-B (Figure 4-8). All of them underwent protein-G affinity purification followed by size exclusion chromatography (see Figure 4-17). Purity was checked by SDS PAGE gel electrophoresis (Figure 4-16) and Luminex single antigen bead analysis.



PM – Protein marker; X – Empty lane
IgG – Positive control
Pre – sample post protein-G affinity chromatography
12F & 13F – peak fractions on SEC

Figure 4-16 : SDS PAGE gel electrophoresis (reducing condition) showing bands of purification of monoclonal HLA-specific antibodies using protocol B.

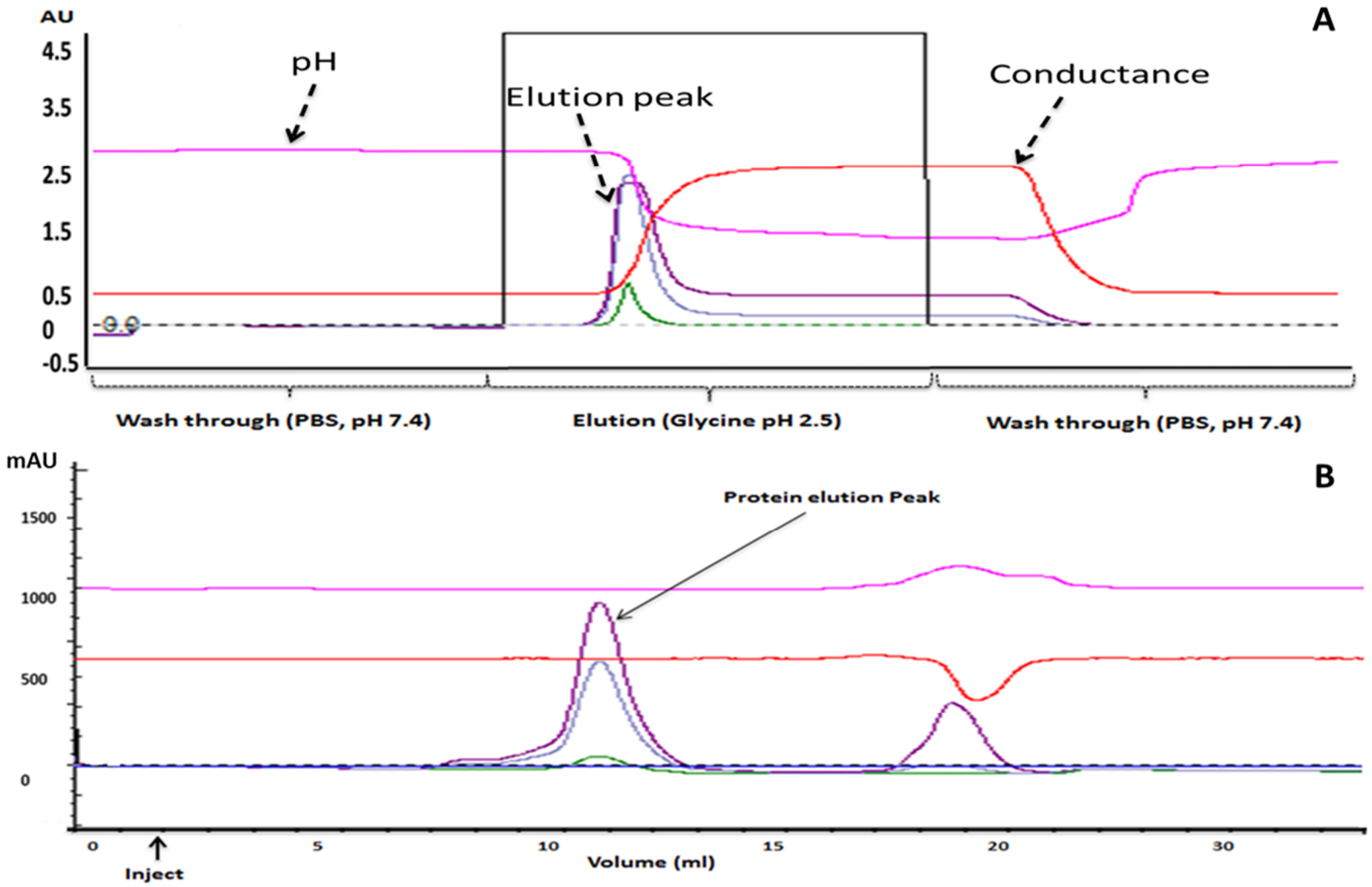


Figure 4-17 : Purification of monoclonal HLA-B7 specific antibody (VTM1F11) via protein-G affinity chromatography (A) followed by Size exclusion chromatography (B).

4.3.2 Analyte and ligand concentration measurement

Biotinylated HLA proteins kindly provided by Pure Protein LLC laboratories, Oklahoma, USA were quantified using both BCA protein assay and soluble HLA ELISA. Purified Monoclonal HLA-specific antibody (analyte) concentration was measured using Lowry and BCA protein assay as described in section 2.4.3. (See Table 4-1)

Biotinylated class I HLA proteins (described in chapter 2.1.2) were immobilised on neutravidin-coated NLC SPR biosensor chips. A 54 Angstrom linker was incorporated into the biotinylated HLA proteins in order to optimise and stabilise epitope availability in the SPR interaction experiments and reduce non-specific binding.

Fractions	Luminex (MFI values)	Concentration (mg/ml)
SN607D8 (IHB-Hu-081)		
AEC F-11/12	A2 – 23,000; A69 - 22,0000 & A68 – 23,000	1.06
AEC F-13		0.118
SN230G6 (IHB-Hu-033)		
SEC F-11	A2 – 22,000; B57 – 20,000 & B58 – 18,000	0.01
SEC F-12	A2 – 19,000; B57 – 18,000 & B58 – 17,000	0.118
WK1D12 (IHB-Hu-059)		
SEC 12 F	B7 – 16459; B27:05 – 15479; B40:01 – 13159; B40:02 – 13792	0.47
SEC 13F	B7 – 18087; B27:05 – 15722; B40:01 – 13494; B40:02 - 14179	0.44
WAR5D5 (IHB-Hu-074)		
SEC 12F	B7 – 18375; B27:05 – 18532	0.01
SEC 13F	B7 – 18042; B27:05 - 18178	0.31
VTM9A10 (IHB-Hu-093)		
SEC 12F	B7 – 17744; B27:05 – 16941	0.12
SEC 13F	B7 – 18083; B27:05 – 17012	0.60
VTM1F11 (IHB-Hu-045)		
SEC 12F	B7 – 17815; B27:05 – 14756; B40:01 – 12981; B40:02 – 13107	0.06
SEC 13F	B7 – 18801; B27:05 – 15558; B40:01 – 12699; B40:02 - 12321	0.23
OUW4F11 (IHB-Hu-041)		
SEC 12F	B7 – 8612; B27:05 – 4381; B40:01 – 8153; B40:02 – 6899	0.01
SEC 13F	B7 – 16231; B27:05 – 10099; B40:01 – 12792; B40:02 - 11478	0.23

Table 4-1 : Concentration & MFI levels of purified human monoclonal HLA-specific antibodies (AEC – Anion exchange chromatography & SEC – Size exclusion chromatography)

4.4 Optimising surface plasmon resonance assay condition

Purified monoclonal HLA-specific antibodies were used to optimise surface plasmon resonance assays for studying the binding kinetics of HLA-anti-HLA antibody interactions. Physicochemical properties such as temperature, pH and ionic strength for optimal HLA-antigen and antibody interactions were investigated and established.

4.4.1 Temperature settings for studying binding interactions

A higher temperature can increase the binding reaction rate for both association and dissociation phases. Data from Pure Protein LLC, suggests that soluble HLA-proteins are stable at 37^{o} Celsius up to one hour of exposure (Figure 2-2). Hence, we compared two temperatures to assess the effect in our SPR instrument: 22^{o} and 37^{o}C. It was noted that at 37^{o}C the binding reaction reached equilibrium earlier and the dissociation curve was slightly more pronounced compared with 22˚C, thus facilitating calculation of the dissociation rate (Figure 4-18). Additionally, 37^{o}C was considered more biological due to similar body temperature. The sensorgram data indicated that both the soluble HLA proteins immobilised on the chips and the antibodies flowed over in solution phase were stable at 37˚C.

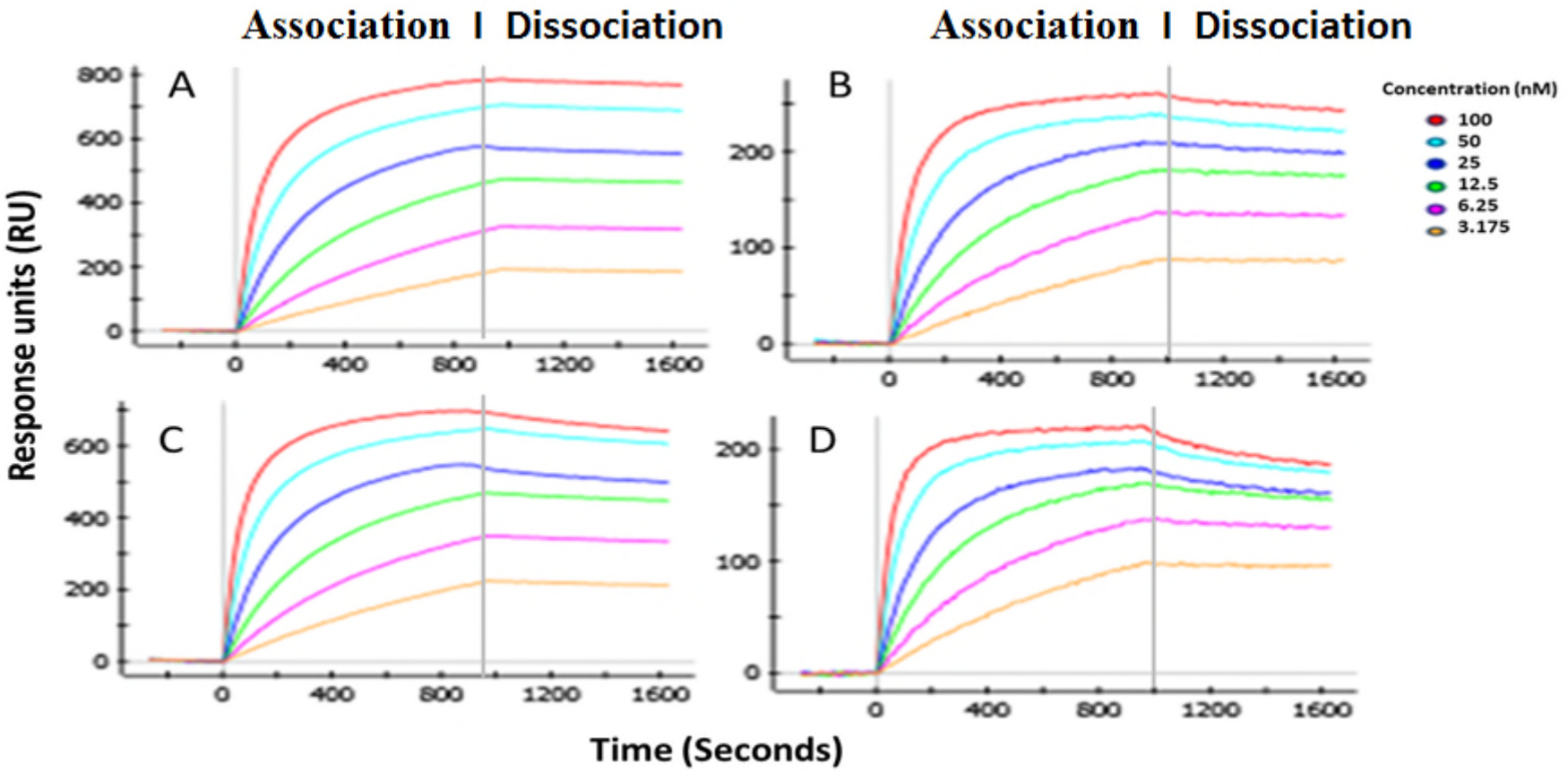


Figure 4-18 : Binding curves at 22° (A&B) and 37° (C&D) Celsius for a range of concentration of human monoclonal HLA-A2 specific antibody (SN607D8 (IHB-Hu-081)) studied at two different ligand density (A&C – 2.5 mcg/ml and B&D – 0.25mcg/ml).

4.4.2 Running buffer

PBS x 1 with pH 7.2-7.4 was chosen as the running buffer and detergent composition was tested to examine sensorgram fidelity. The use of detergents in SPR experiments is commonplace as a means of reducing non-specific binding artefacts during experiments and also preventing the formation of protein deposits in the instrument's microscale flow cells. Occasionally, and depending on the interacting system under investigation, higher quantities of detergent (>0.01%) are required for optimal results. Two different strengths of Tween-20 (0.05 and 0.005% v/v) were added to PBS pH 7.4. At the higher Tween-20 concentration of 0.05%, the HLA proteins showed lower reactivity to the monoclonal HLA-specific antibodies compared with 0.005% Tween-20, indicated by reduced Rmax levels at relative concentrations (Figure 4-19).

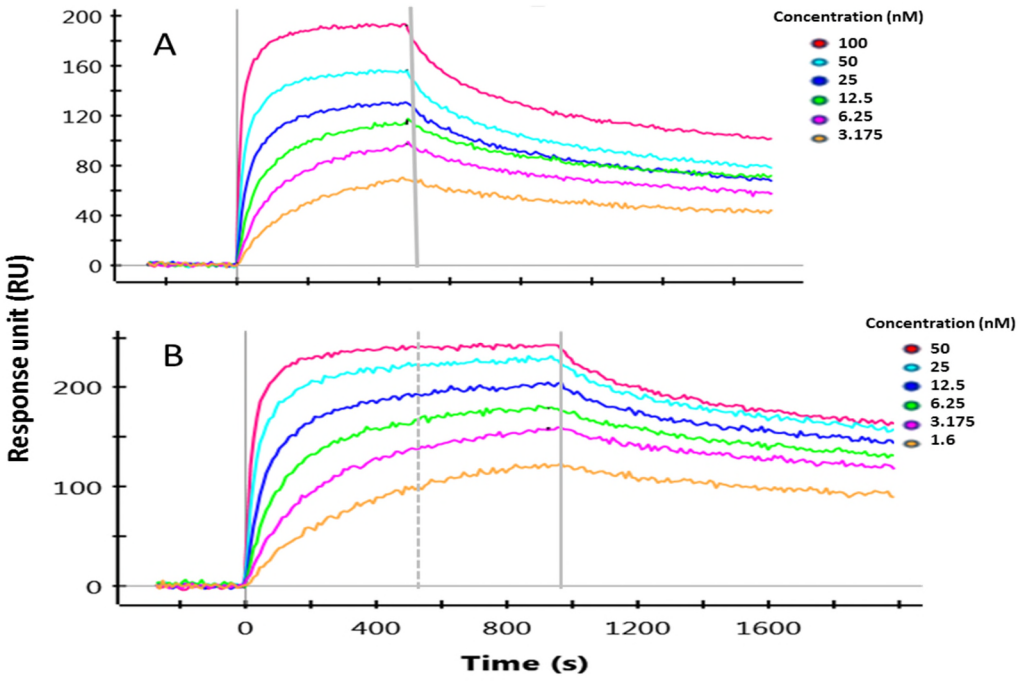


Figure 4-19 : HLA-A2 protein and HLA-specific antibody (IHB-HU-033 (SN230G6)) interaction at two different concentration of Tween 20 (A – 0.05% and B – 0.005%)

4.4.3 Regeneration buffer selection

Following establishment of the optimal conditions for studying the binding interactions of HLA-protein and HLA-specific antibodies, further experiments focused on identifying the most effective regeneration buffers. These buffers are similar to elution buffers that displace the antibody from antibody-antigen complexes without damaging or denaturing the antigen attached to the solid matrix (in the case of affinity chromatography, the folded protein covalently bound to the Sepharose resin). Ideally, this allows re-use of the sensor chip with coated HLA-proteins without compromising the signal. Regeneration steps are kept as short as possible to avoid damage to the HLA-protein; in the SPR assay we consistently used 18 seconds exposure time.

Initially, the elution conditions similar to HLA-column affinity chromatography were used for regeneration of sensor chips, i.e. 100 mM Glycine pH 11.0. However, this was not universally effective. As seen in the Figure 4-20, binding of monoclonal HLA-specific antibodies differed in regeneration; IHB-HU-033 (SN230G6) binding showed incomplete regeneration with 100 mM glycine whilst IHB-HU-081 (SN607D8) had complete regeneration. Hence other regeneration conditions were studied.

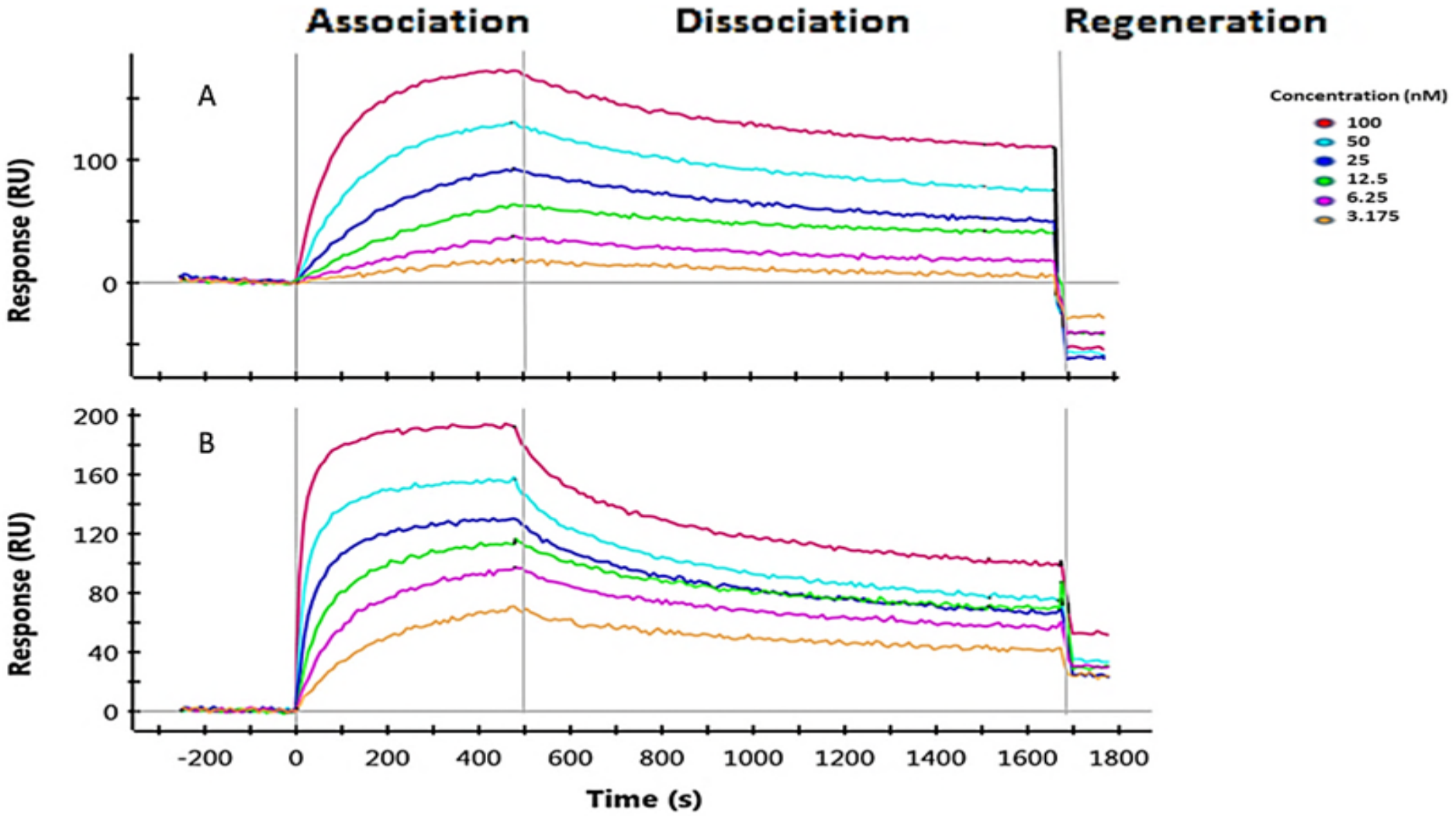


Figure 4-20 : Regeneration of HLA-protein coated sensor chip using Glycine (100nM, pH 11.0) A – IHB-HU-081 (SN607D8) showed complete regeneration B – IHB-HU-033 (SN230G6) – incomplete regeneration

3.1.1.4 *High pH regeneration - 10 mM sodium hydroxide pH 14*

The use of 10 mM sodium hydroxide pH 14 resulted in removal of antibody bound to the HLA-protein as shown in Figure 4-21. However, denaturation of HLA protein was evident as there was no binding of monoclonal HLA-specific antibody following treatment with sodium hydroxide (Figure 4-22).

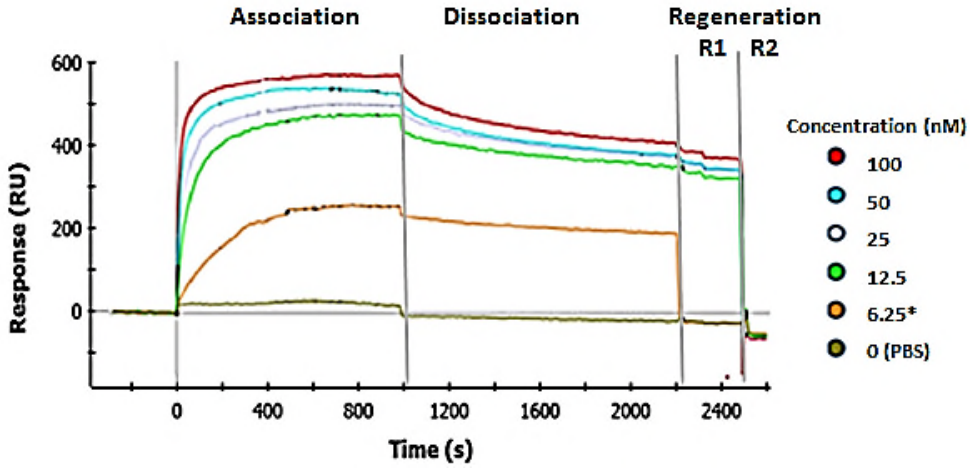


Figure 4-21 : Regeneration buffers R1 – Glycine 100mM pH 11.0 and R2 – NaOH; shows elution of IHB-HU-033 (SN230G6) with NaOH but not with glycine pH 11.0 (compared to IHB-HU-081 (SN607D8) – orange colour).

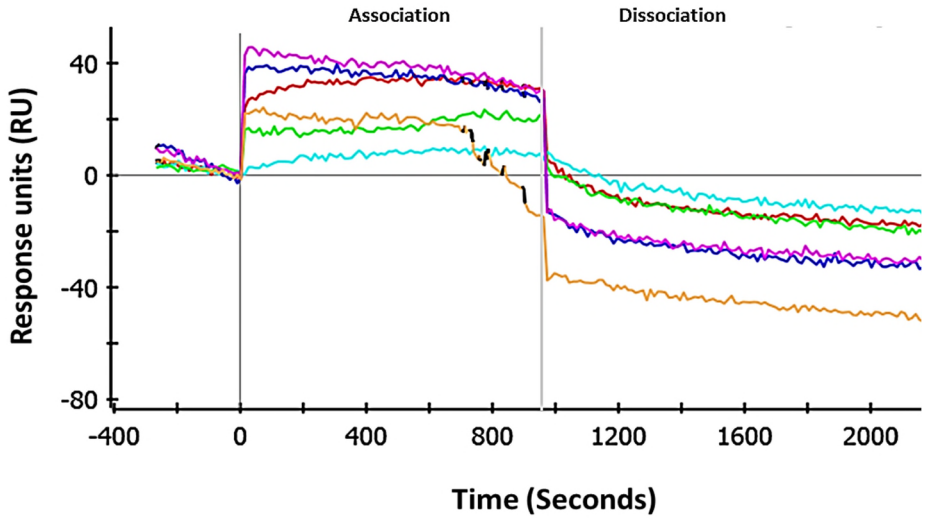


Figure 4-22 : Lack of binding following prior NaOH regeneration

3.1.1.5 *High ionic strength regeneration - 1 M NaCl and 1 M $MgCl_2$*

The 1M NaCl buffer alone did not have any regeneration effect on monoclonal antibody IHB-HU-081 (SN607D8) (Figure 4-23, regeneration R1). The combination of 1M NaCl and 100mM Glycine (pH 11.0) was attempted with the very tight IHB-HU-033 (SN230G6) monoclonal interactions with HLA-A2 protein, but the addition of 1 M NaCl did not result in any improvement in regeneration (see Figure 4-24).

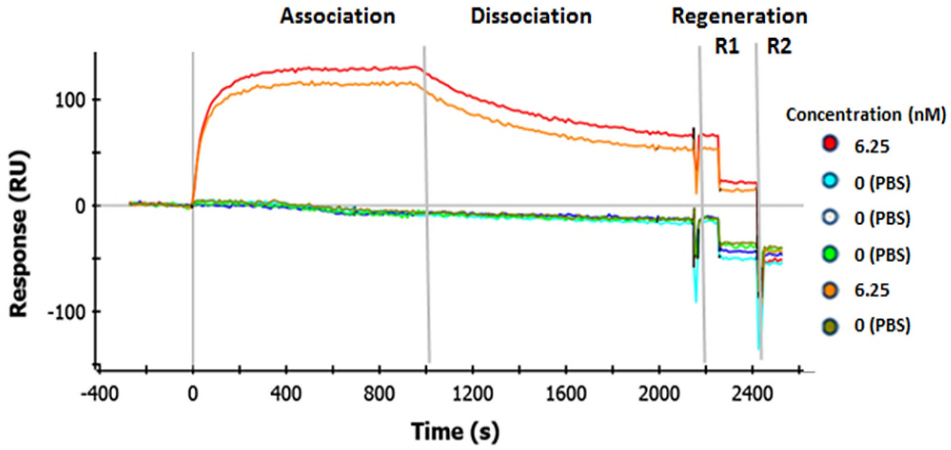


Figure 4-23 : Regeneration buffers R1 –1 M NaCl alone and R2 – 1M NaCl and Glycine 100mM pH 11.0; shows elution of IHB-HU-081 (SN607D8) with Glycine but not with NaCl

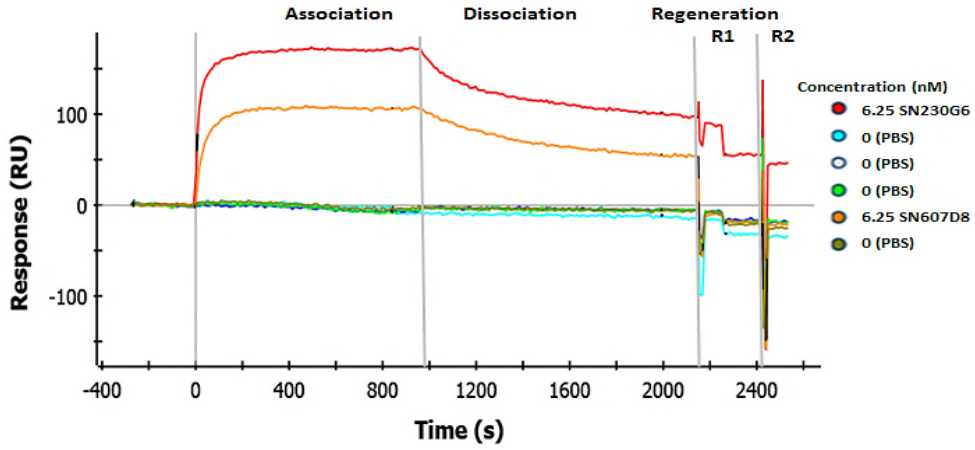


Figure 4-24 : Regeneration buffers; R1 - 1M NaCl and Glycine 100mM pH 11.0; shows release of IHB-HU-081 (SN607D8) but not with IHB-HU-033 (SN230G6); and R2 – 1 M $MgCl_2$ had no effect on regeneration of IHB-HU-033 (SN230G6)

3.1.1.6 *Other regeneration buffers – Acetate, CHAPS and chaotrope 4 M $MgCl_2$*

Additional regeneration buffers - 10mM sodium acetate pH 5.5, 20 mM CHAPS (a non-denaturing Zwitterionic detergent) and the very ionic strength chaotrope 4 M $MgCl_2$ were attempted. The sodium acetate and CHAPS had no regeneration effect and following 4 M $MgCl_2$, there was complete denaturation of the HLA-protein (Figure 4-25).

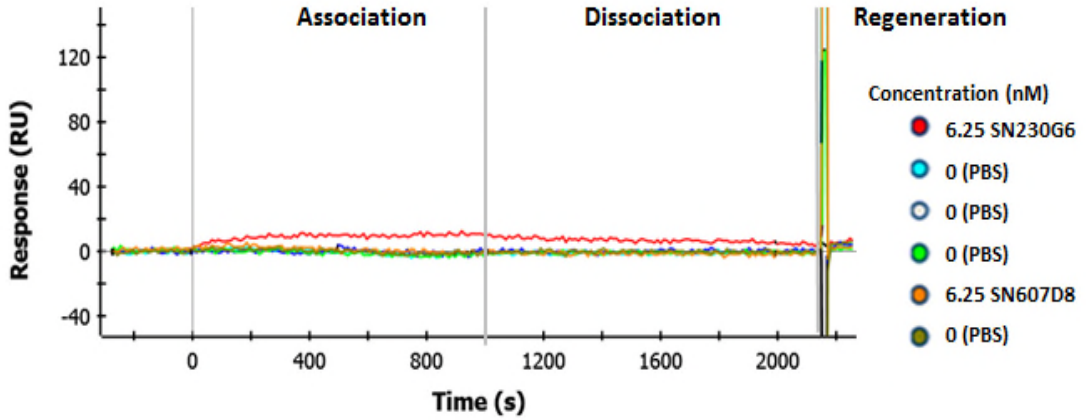


Figure 4-25 : HLA-protein denaturation following treatment with 4 M $MgCl_2$ step - subsequent run with two monoclonal HLA-specific antibody did not have any binding to HLA-A2 protein.

Vigorous regeneration conditions necessary for release of certain antibodies resulted in damage to HLA-protein and hence further attempts at regeneration in these cases were closed. 100mM Glycine pH 11.0 was used as the best general regeneration buffer and if no regeneration occurred in certain HLA protein/antibody binding experiments, then further experiments was carried out using an adjacent, uncoupled sensor channel or a fresh sensor chip.

4.5 Established protocol standards for real-time kinetic experiments

Step 1 – Preparation

Running Buffer - PBS-Tween 0.005% (0.05 ml of TWEEN-20 in 1L PBS, pH 7.4) – filtered (0.45 µm filter) and degassed prior to use.

Samples - centrifuged 13000 rpm for 10 minutes prior to running on SPR.

Step 2 – Ligand immobilisation

Biotinylated HLA protein used at 2.5 or 0.25 µg/ml is immobilised on chip with flow rate of ***25 µl/min*** over 300 seconds. A volume of 150 µl for each sample is required for this step and taking in to account of void volume intrinsic to the instrument, the total volume of ligand solution required is **250 µl**.

Step 3 - Association

The samples are pipetted in the sample rack (sampler temperature – 22^{o}C) and the machine aspirates and goes through the fluidics and finally over the chip at rate of ***25 µl/min*** for 960 seconds (at 37^{o}C). This will require 400 µl of sample for the association and taking in to account of void fill, one will require **600 µl** of sample per well.

Step 3 - Dissociation

The running buffer (PBS-T) is passed over for 960 seconds at a flow rate of 25 µl/min.

Step 4 - Regeneration

Regeneration buffer (100 mM Glycine pH 11.0) is passed through at rate of 100 µl/min for 18 seconds.

Step 5 – Data fitting and kinetic analysis

The Biorad XPR36 ProteOn Manager software was used to fit data to sets of established kinetic algorithms. Equilibrium analysis was used to calculate equilibrium dissociation ***constants*** (K_D) and kinetic analysis using bivalent analyte or heterogonous ligand/analyte model were used to obtain the kinetic association (k_a) and dissociation (k_d) ***rates*** that can also be used to calculate K_D via an alternative route using the simple equation - $K_D = k_d / k_a$.

Experiments were performed using two ligand (biotinylated HLA) densities (loading concentrations of 2.5 and 0.25 µg/ml yielding loading level Response Maxima (RU; Rmax) values of 1200 and 400 respectively) using the NLC sensor chip. Ranges of concentration of analyte (HLA-specific antibodies) were tested against the different ligand densities. The concentrations of monoclonal HLA-specific antibodies ranged from 1.6 to 100 nM.

4.6 Data analysis and estimation of affinity parameters

Data obtained from the experiments can be processed within the Proteon Manager Software suite and also exported into Microsoft Excel for potential analysis using a remote system.

4.6.1 Equilibrium analysis

A range of concentrations of HLA-specific antibody analyte is allowed to interact with constant amount of immobilised HLA-protein and until equilibrium or near-equilibrium is reached (see Figure 4-26 & 4-27). The Rmax (maximum RU at a particular concentration at a particular time point) is plotted against the concentration to obtain curve for equilibrium analysis and apparent K_D is obtained (see Chapter 1-9). A lower K_D indicates higher binding affinity.

Using this method, the apparent K_D value of HLA-A2 interaction with IHB-HU-033 (SN230G6) (2.39 E-09 M) is lower than IHB-HU-081 (SN607D8) (3.975 E-09 M) by a factor of 1.67. The data suggests IHB-HU-033 (SN230G6) has stronger affinity for HLA-A2 compared to IHB-HU-081 (SN607D8). The apparent K_D calculated from higher concentration of monoclonal antibodies may not be accurate as seen the values were spread out compared to lower concentrations for both antigen/ligand densities over the chip. This is secondary to steric hindrance at higher concentration of antibody competing for the same epitope over the antigen. However the estimation from lower concentration may be not accurate either as the reactions did not reach equilibrium.

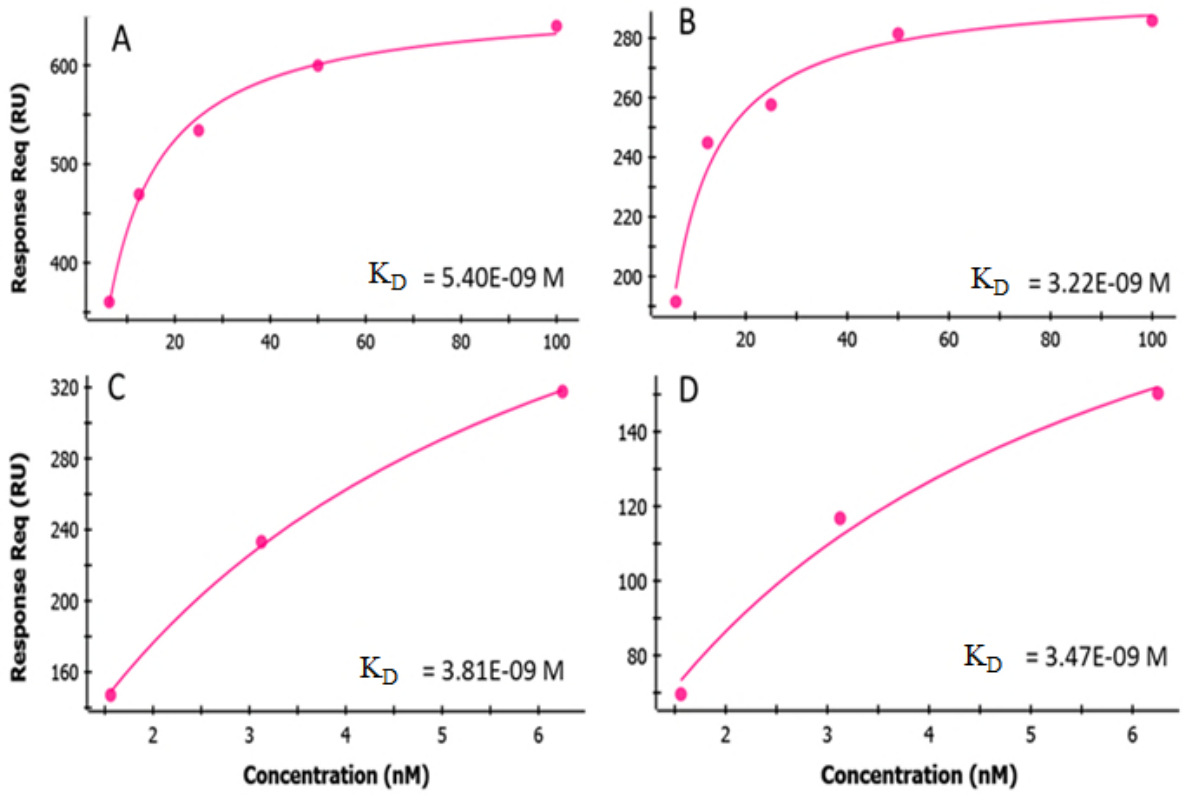


Figure 4-26 : Equilibrium analysis of SN607D8 (IHB-Hu-081) at different concentration against two different HLA protein densities (A&C – 2.5 µg/ml and B&D – 0.25 µg/ml).

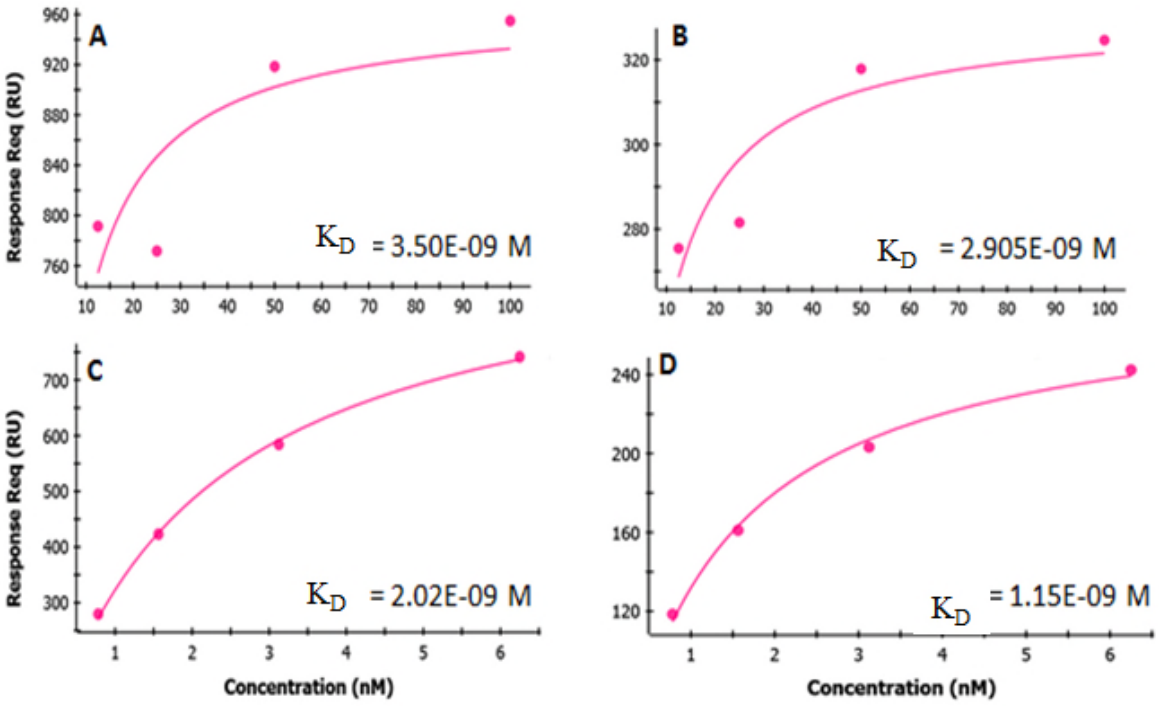


Figure 4-27 : Equilibrium analysis of SN230G6 (IHB-Hu-033) at different concentration against two different HLA-protein density (A&C – 2.5 mcg/ml and B&D – 0.25 mcg/ml).

4.6.2 Kinetic analysis

Langmuir with mass transport and bivalent analyte models was used for analysis of binding kinetics for monoclonal HLA-specific antibodies tested individually (Muller et al., 1998).

3.1.1.7 *Kinetic analysis of SN230G6 interactions with HLA-A2 (IHB-Hu-033)*

The experiments were performed at two ligand densities described in Section 3.3 and shown in Figures 4-28 and 4-30 respectively. Concentrations of IHB-HU-033 (SN230G6) used in these experiments ranged between 12.5 and 100 nM (A-B) and 1.6 to 12.5 nM (C-D). Data fitted using the bivalent analyte model (B and D) was better compared to the simpler Langmuir with

mass transport model (A and C) as seen by the better quality of residuals except the combination of lower ligand and lower concentration experiments from Figure 4-30;D. (Figure 4-29 and 4-31). Kinetic binding parameters obtained from the anlaysis are shown in Table 4-2.

Model	HLA-A2 Ligand density	Concentration range of IHB-HU-033 (SN230G6)	Kinetic rate	K_D
Langmuir with Mass transport	2.5 µg/ml	100 to 12.5 nM	k_a : 6.3E+05 1/Ms K_d : 1.55E-04 1/s	2.44E-10 M
		12.5 to 1.6 nM	k_a : 1.07E+06 1/Ms K_d : 1.74E-04 1/s	1.63E-10 M
	0.25 µg/ml	100 to 12.5 nM	k_a : 1.07E+06 1/Ms K_d : 2.70E-04 1/s	2.52E-10 M
		12.5 to 1.6 nM	k_a : 4.16E+05 1/Ms K_d : 2.49E-03 1/s	5.98E-09 M
Bivalent analyte	2.5 µg/ml	100 to 12.5 nM	k_a : 2.87E+05 1/Ms K_d : 7.25E-04 1/s	2.53E-09 M
		12.5 to 1.6 nM	k_a : 6.95E+05 1/Ms K_d : 5.01E-05 1/s	7.21E-11 M
	0.25 µg/ml	100 to 12.5 nM	k_a : 2.13E+06 1/Ms K_d: 1.54E-04 1/s	7.25E-11 M
		12.5 to 1.6 nM	k_a : 1.12E+06 1/Ms K_d : 1.18E-04 1/s	1.05E-10 M

Table 4-2: Binding kinetics of IHB-HU-033 (SN230G6) using different kinetic models

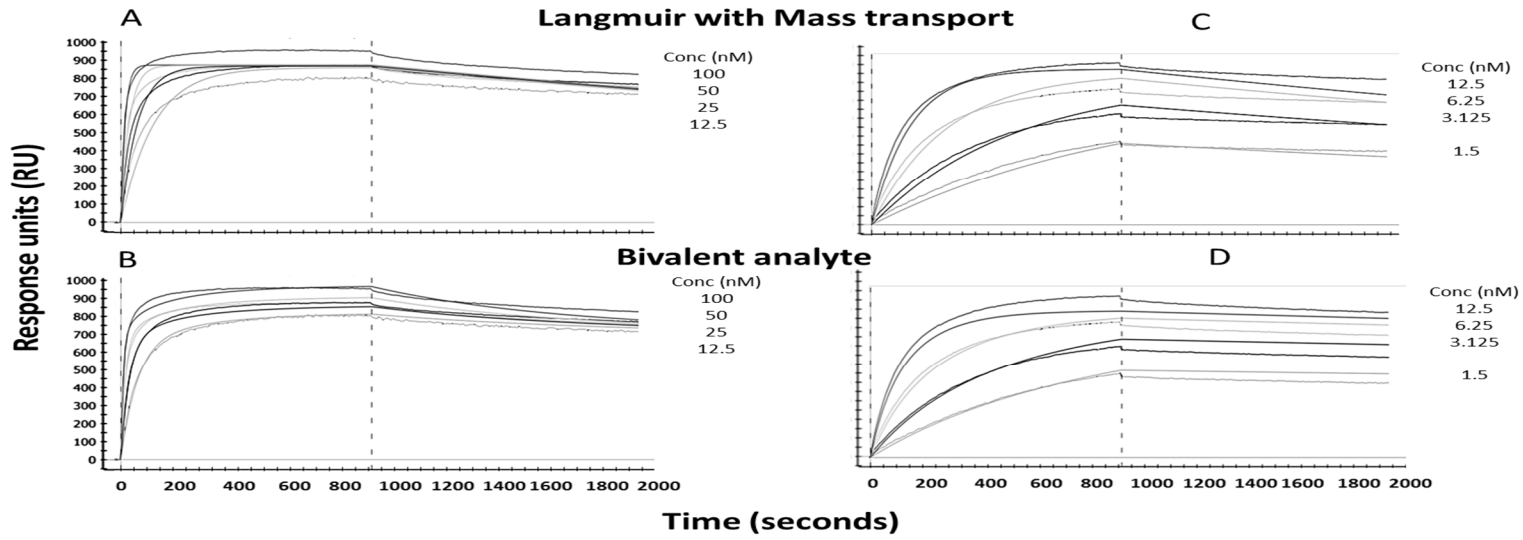


Figure 4-28 : Langmuir with mass transport (A and C) and Bivalent analyte (B and D) model fitted to the kinetic data obtained from IgG monoclonal HLA-A2 specific antibody (IHB-HU-033 (SN230G6)) at higher ligand concentration (HLA-A2 = 2.5 µg/ml).

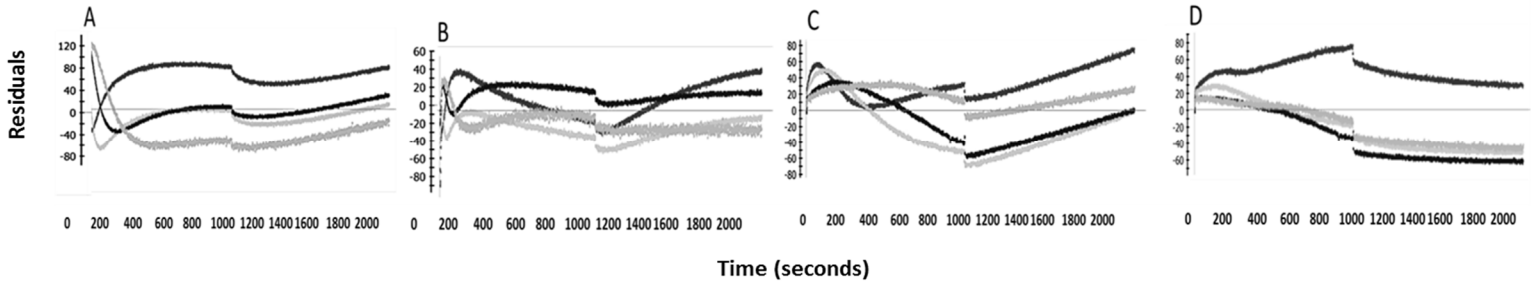


Figure 4-29 : Residual plots for kinetic model fittings corresponding to the Figure 4-28.

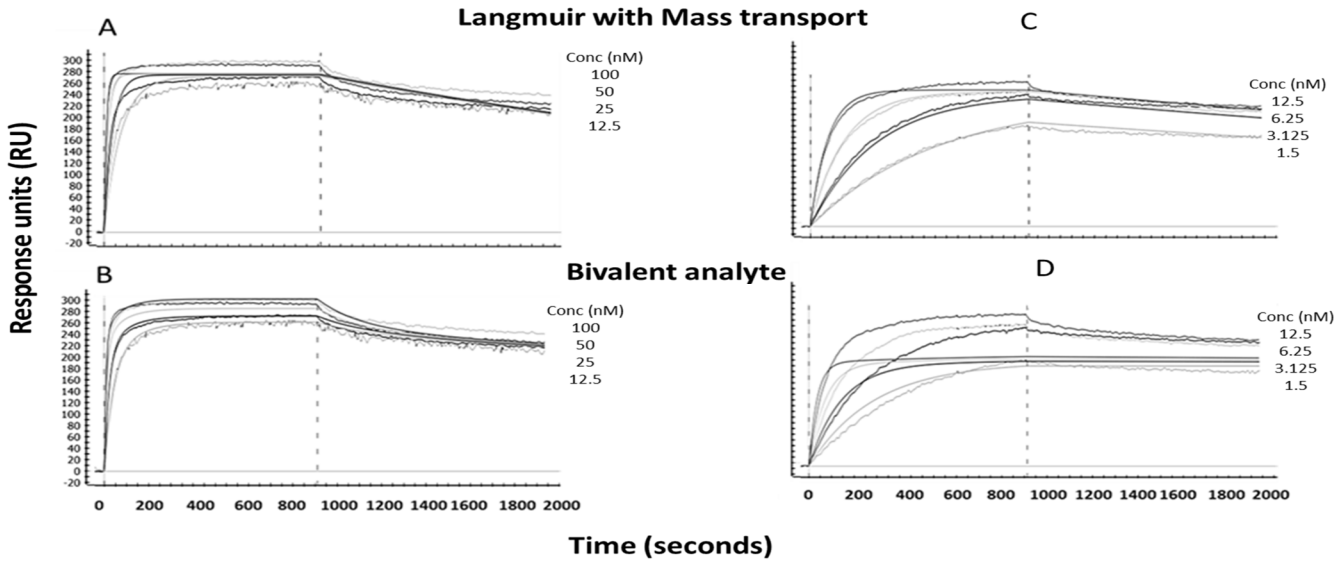


Figure 4-30 : Langmuir with mass transport (A and C) and Bivalent analyte (B and D) model fitted to the kinetic data obtained from IgG monoclonal HLA-A2 specific antibody (IHB-HU-033 (SN230G6)) at lower ligand concentration (HLA-A2 = 0.25 µg/ml).

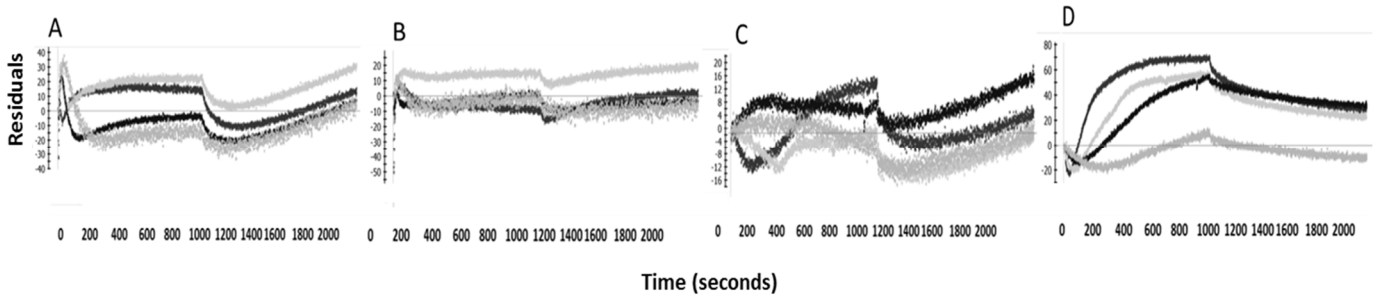


Figure 4-31 : Residual plots for kinetic model fittings corresponding to the Figure 4-30

3.1.1.8 *Kinetic analysis of SN607D8 interactions with HLA-A2 (IHB-Hu-081)*

Similar to previous sets of experiments, binding kinetics of HLA-A2 specific monoclonal antibody SN607D8 were studied at two different concentration of antigen density and at antibody concentration range of 100 to 6.25 nM (A-B) and 6.25 to 1.6 nM (C-D) (Figures 4-32 and 4-34 respectively). Data fitting using Bivalent analyte model (B&D) was better compared to Langmuir with mass transport model. Kinetic binding values obtained from the analysis are shown in the Table 4-3.

Model	HLA-A2 Ligand density	Concentration range of SN607D8 (IHB-Hu-081)	Kinetic rate	K_D
Langmuir with Mass transport	2.5 µg/ml	100 to 6.25 nM	k_a : 2.76E+05 1/Ms k_d : 2.61E-04 1/s	9.44E-10 M
		6.25 to 1.6 nM	k_a : 5.52E+05 1/Ms k_d : 1.58E-04 1/s	2.86E-10 M
	0.25 µg/ml	100 to 6.25 nM	k_a : 4.46E+05 1/Ms k_d : 2.83E-04 1/s	6.34E-10 M
		6.25 to 1.6 nM	k_a : 6.59E+05 1/Ms k_d : 1.15E-04 1/s	1.75E-10 M
Bivalent analyte	2.5 µg/ml	100 to 6.25 nM	k_a : 1.43E+05 1/Ms k_d : 6.62E-04 1/s	4.63E-09 M
		6.25 to 1.6 nM	k_a : 2.69E+05 1/Ms k_d : 1.89E-04 1/s	7.01E-10 M
	0.25 µg/ml	100 to 6.25 nM	k_a : 1.38E+05 1/Ms k_d : 2.454E-03 1/s	1.78E-08 M
		6.25 to 1.6 nM	k_a : 2.29E+05 1/Ms k_d : 3.13E-04 1/s	1.36E-09 M

Table 4-3 : Binding kinetics of SN607D8 (IHB-HU-081) using different kinetic models

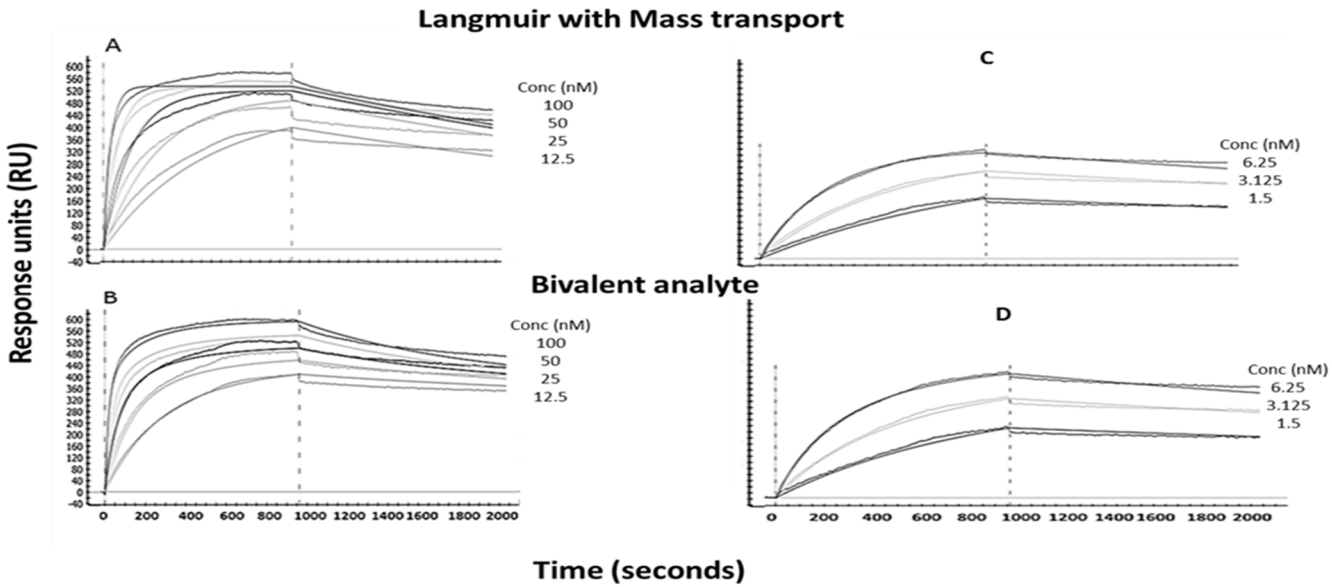


Figure 4-32 : Langmuir with mass transport (A and C) and Bivalent analyte (B and D) model fitted to the kinetic data obtained from IgG monoclonal HLA-A2 specific antibody (IHB-HU-081 (SN607D8)) at higher concentration (HLA-A2 = 2.5 µg/ml).

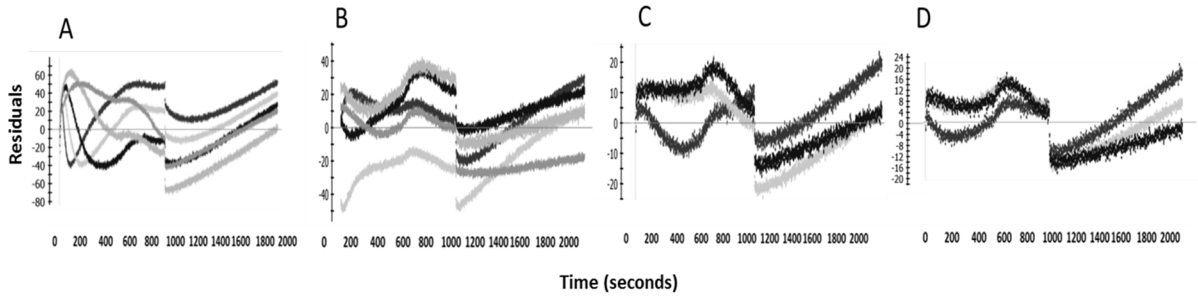


Figure 4-33 : Residual plots for kinetic model fitting corresponding to the Figure 4-32

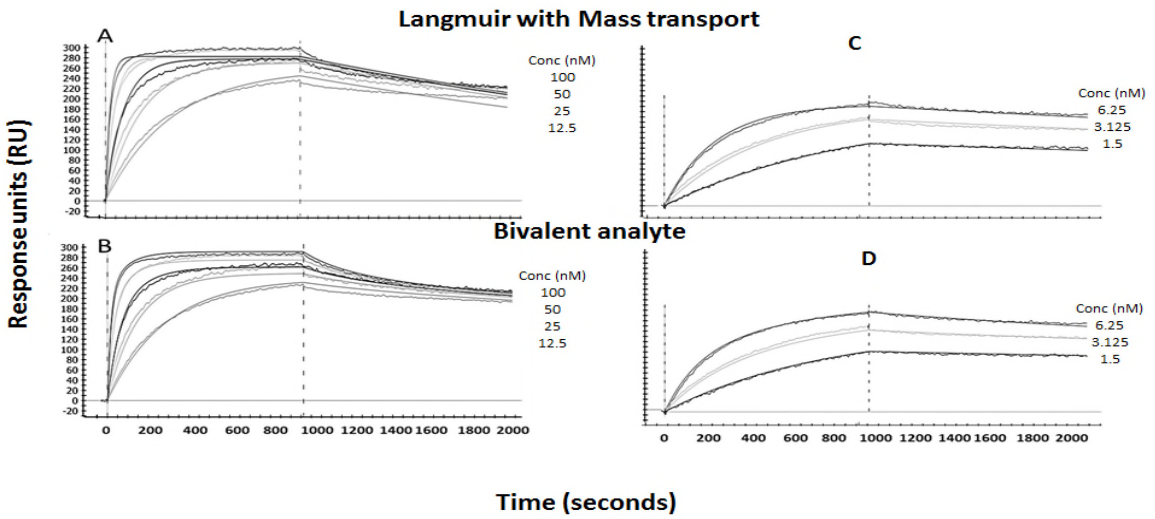


Figure 4-34 : Langmuir with mass transport (A and C) and Bivalent analyte (B and D) model fitted to the kinetic data obtained from IgG monoclonal HLA-A2 specific antibody (IHB-HU-081 (SN607D8)) at lower concentration (HLA-A2 = 0.25 µg/ml).

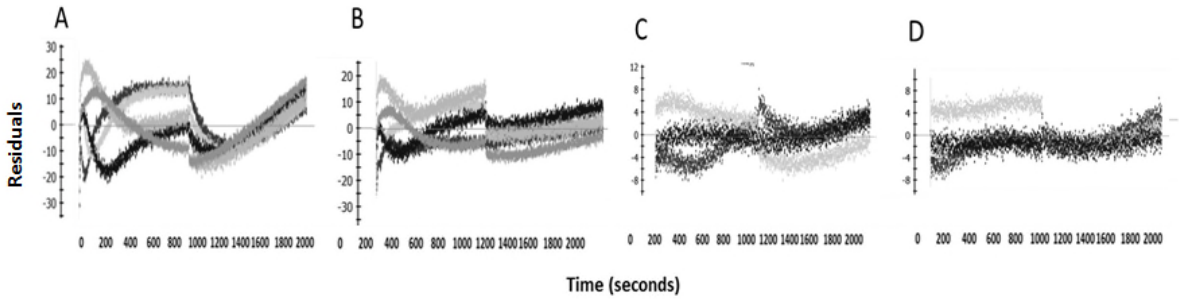


Figure 4-35 : Residual plots for kinetic model fitting corresponding for the Figure 4-3.

Comparison of average apparent K_D from the kinetic analysis shows the IHB-HU-033 (SN230G6) monoclonal antibody (K_D = 2.77 nM) is 2.5 times stronger than IHB-HU-081 (SN607D8) (7.08 nM). This is secondary to faster association rate of IHB-HU-033 (SN230G6) compared to IHB-HU-081 (SN607D8) antibody.

4.6.3 EC_{50} estimation using ELISA

Sandwich ELISA described in chapter 2 was used to calculate effective concentration of antibody that gave fifty percent of maximal response (EC_{50}). In equilibrium, the concentration corresponding to the EC_{50} is equal to apparent K_D. Both the HLA-A2 specific monoclonal antibodies were tested in triplicates to derive the titration curve (Figure 4-36).

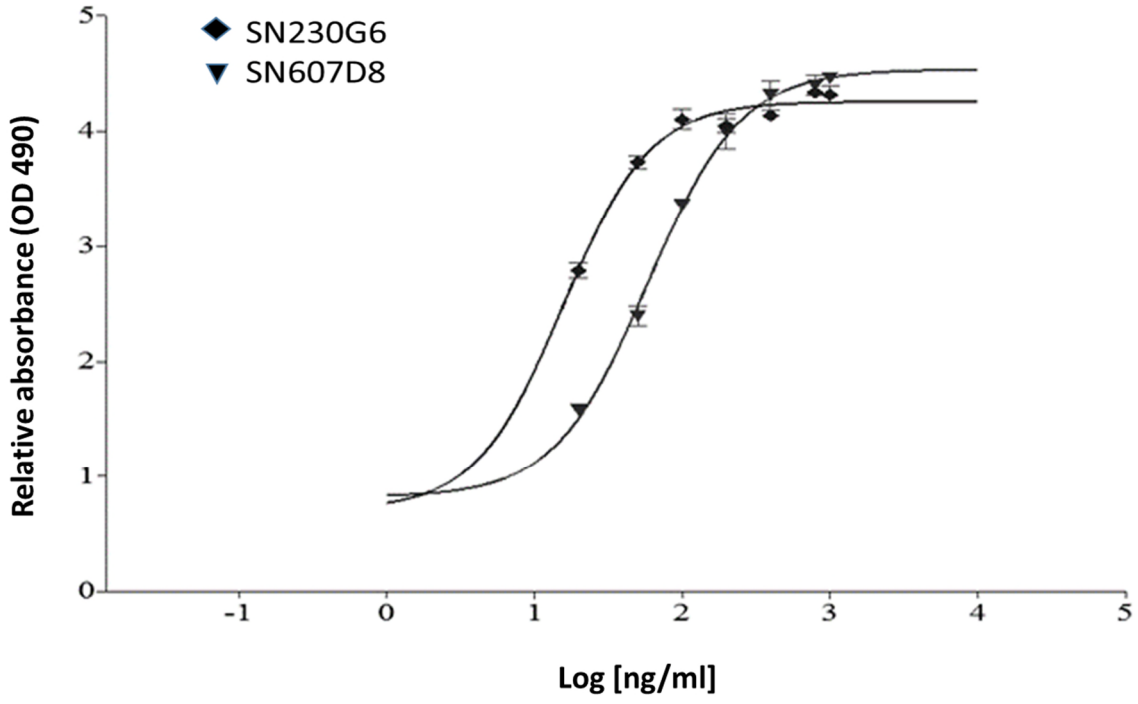


Figure 4-36 : Titration curve showing relationship between the concentration and relative absorbance measured in ELLISA assay for HLA-A2 specific monoclonal antibodies

Comparison of EC_{50} of the two monoclonal antibodies show the IHB-HU-033 (SN230G6) is 3.7 times stronger than IHB-HU-081 (SN607D8). EC_{50} values were 15.68 and 58 for SN230G6 and SN607D8 respectively.

4.7 Sensitivity, specificity and Reproducibility of assay

4.7.1 Sensitivity

Sensitivity depends to some extent upon the ligand density on the sensor chip. A lower ligand density has added advantages to signal quality as discussed in Chapter 2.7. Monoclonal HLA-specific antibodies were diluted down to 1.6 nM and tested against ligand density of 0.25 µg/ml. (Figure 4-37).

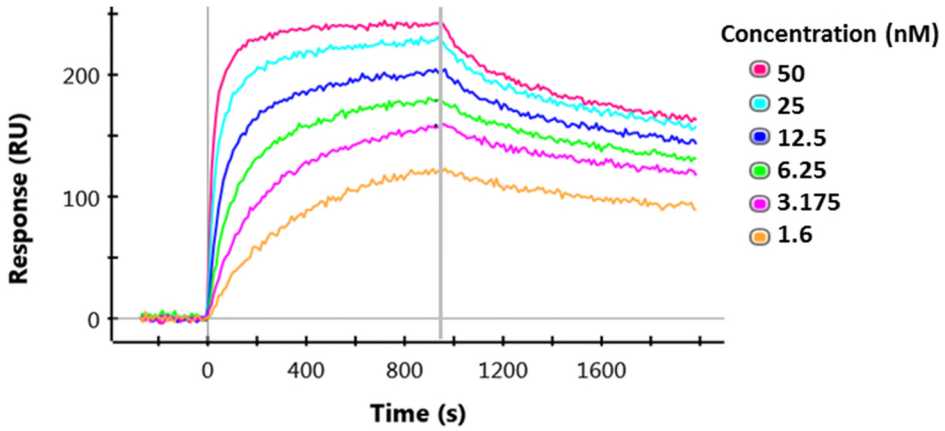


Figure 4-37 : Sensitivity of SPR assay – distinct signal with lowering concentration of monoclonal HLA-specific antibody

4.7.2 Upper limit of detection

Higher concentration of monoclonal HLA-specific antibodies resulted in blunting of maximal response of antigen-antibody binding. Both saturation and hook effects were observed. In hook effect, higher concentration of analyte results in reduction or inhibition of binding responses. As seen in Figure 4-38 (A), a concentration of 150 nM of monoclonal HLA-specific antibodies resulted in hooking effect for interaction with HLA-A2 protein. However this depends on the HLA-proteins such that despite same epitope interactions, there was increment effect on interaction with HLA-A69 protein (Figure 4-38 (B)).

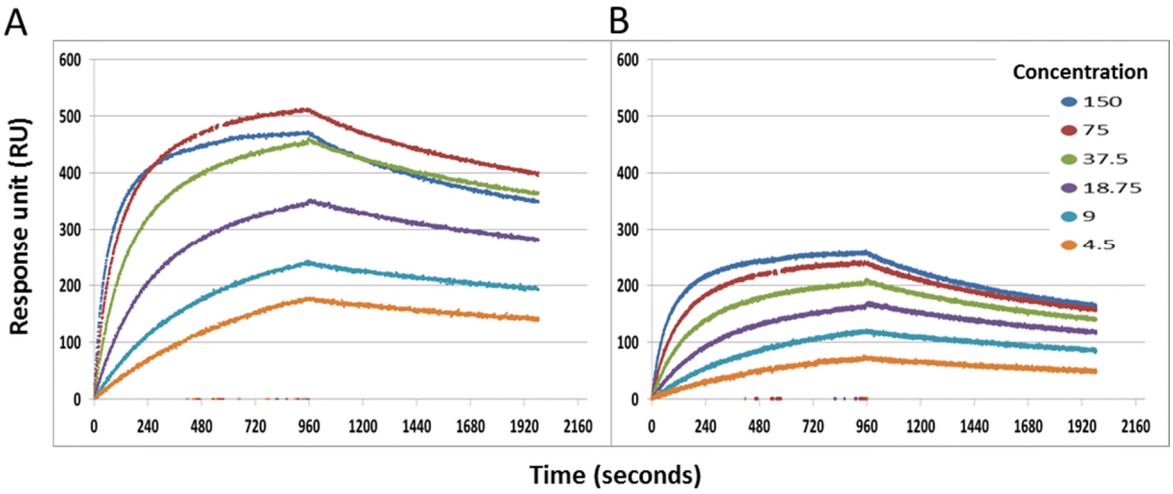


Figure 4-38 : Binding responses to higher concentration of monoclonal HLA-specific antibodies to HLA-A2 (A) and HLA-A69 (B) proteins

4.7.3 Specificity

Two monoclonal human HLA-specific antibodies with known reactivity to specific HLA proteins on Luminex and CDC assay were tested on surface plasmon resonance assay for specificity. As seen in Figure 4-39, SN607D8 (IHB-Hu-081) had binding to A2, A68 & A69 but not to A1 and B57 proteins, and similarly SN230G6 (IHB-Hu_033) bound to A2 and B57 but not to A1, A68 or A69. The binding pattern was consistent with the reactivity on Luminex and CDC assays, and also with the epitope distribution associated with the alleles used in the experiments.

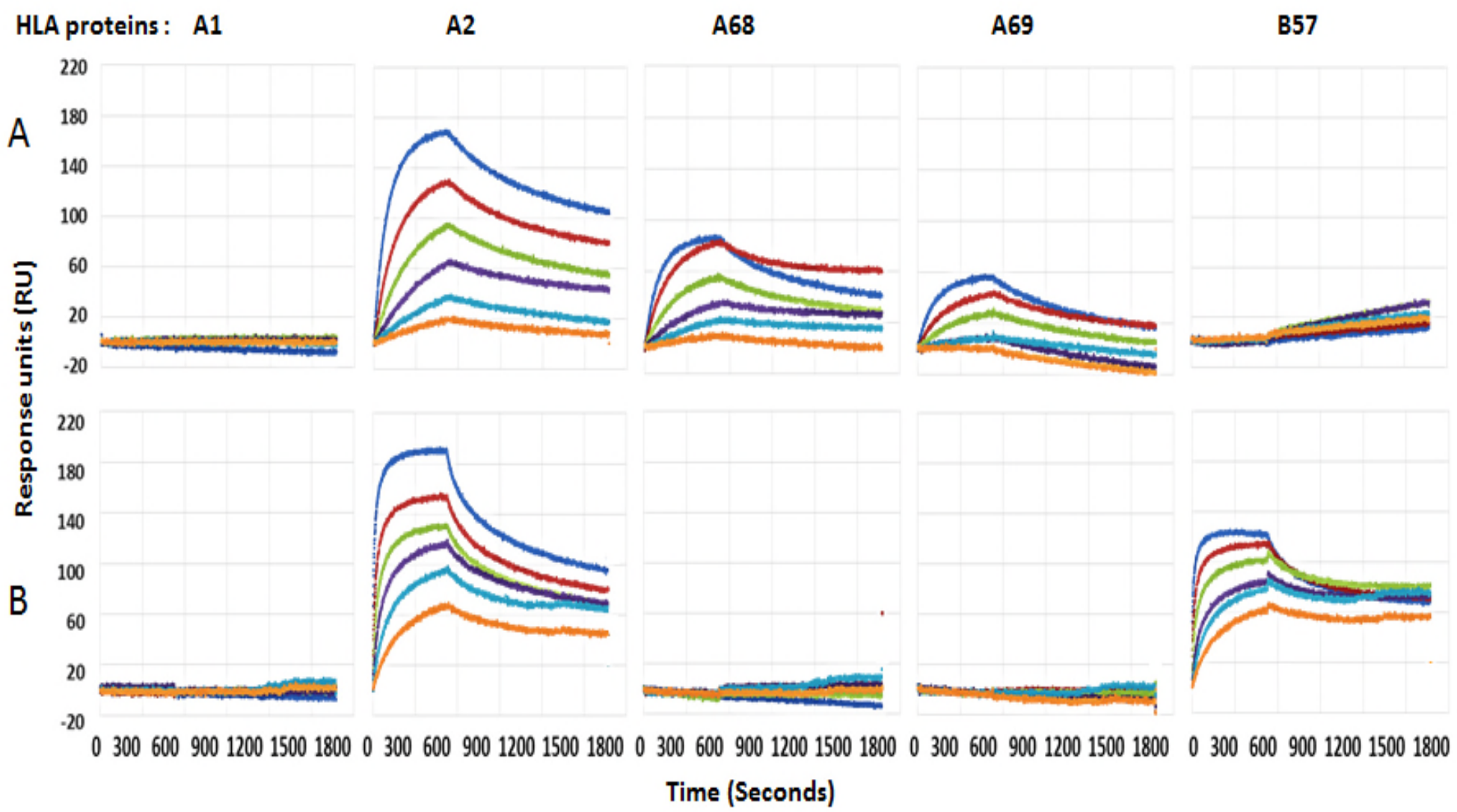


Figure 4-39 : Binding reactivity of two human monoclonal HLA-specific antibodies against different HLA proteins

4.7.4 Reproducibility

Binding experiments were done in duplicates for both the human monoclonal anti-HLA-A2 antibodies and kinetic values were calculated using Langmuir with mass transport and bivalent analyte models. Differences in K_D and standard deviation between experiments are shown in Table 3-4 and Table 3-5.

Model	HLA-A2 density	Concentration range (nM)	K_{D1} (nM)	K_{D2} (nM)	AVG K_D (nM)	StdDev
SN230G6 (IHB-Hu-033)						
Langmuir with Mass transport	2.5 µg/ml	100 to 12.5	2.66E-10	2.44E-10	2.55E-10	1.56E-11
		12.5 to 1.6		1.63E-10	1.63E-10	
	0.25 µg/ml	100 to 12.5	2.52E-10	2.76E-10	2.64E-10	1.70E-11
		12.5 to 1.6	1.33E-10	0.725E-10	1.03E-10	4.28E-11
Bivalent analyte	2.5 µg/ml	100 to 12.5	1.74E-09	2.53E-09	2.14E-09	5.59E-10
		12.5 to 1.6	6.50E-11	7.21E-11	6.86E-11	5.02E-12
	0.25 µg/ml	100 to 12.5	5.98E-09	3.77E-09	4.88E-09	1.56E-09
		12.5 to 1.6	1.10E-10	1.05E-10	1.08E-10	3.54E-12

Table 4-4: Binding kinetics from two separate experiments for assessment of reproducibility (SN230G6)

Model	HLA-A2 density	Concentration range (nM)	K_{D1} (nM)	K_{D2} (nM)	AVG K_D (nM)	StdDev
SN607D8 (IHB-Hu-081)						
Langmuir with Mass transport	2.5 µg/ml	100 to 6.25	9.44E-10	7.27E-10	8.36E-10	1.53E-10
		6.25 to 1.6	1.33E-10	2.86E-10	2.10E-10	1.08E-10
	0.25 µg/ml	100 to 6.25	6.34E-10	5.23E-10	5.79E-10	7.85-11
		6.25 to 1.6	1.75E-10	9.30E-11	1.34E-10	5.80E-11
Bivalent analyte	2.5 µg/ml	100 to 6.25	1.46E-09	4.63E-09	3.05E-09	2.24E-09
		6.25 to 1.6	5.63E-10	7.01E-10	6.32E-10	9.76E-11
	0.25 µg/ml	100 to 6.25	1.78E-08	1.31E-08	1.55E-08	3.32E-09
		6.25 to 1.6	1.36E-09	1.09E-09	1.23E-09	1.91E-10

Table 4-5: Binding kinetics from two separate experiments for assessment of reproducibility (SN607D8)

4.8 Discussions

Biosensor based assay can be successfully applied to measure binding kinetics and binding strength in real time. Using two HLA-A2 specific monoclonal antibodies, the experiments described in this chapter established optimal conditions to study the binding kinetics. Sensitivity of SPR is quoted in pico-molar range for kinetic analysis (Bravman et al., 2008) but for purpose of affinity determination 1 to 100 nM is sufficient. Specificity of assay is consistent with quality of HLA-protein used and shows clear epitope fashion binding (Figure 4-39). Upper limit of detection of the assay is dependent on the ligand density and is limited by steric hindrance at higher concentration (Figure 4-38). The experiments also demonstrated importance of purification of monoclonal HLA-specific antibodies. Following establishment of protocol to study binding kinetics, demonstrated the assay was sensitive down to 1.6 nM concentrations of antibodies (with the antigen density at 0.25 mcg/ml) and was specific to intended HLA-proteins. The difference in apparent K_D calculated using equilibrium and kinetic analysis was similar to obtained from sandwich ELISA.

Purification of monoclonal HLA-specific antibodies provided generously in culture supernatant by University of Leiden required a two-step protocol (affinity purification followed by either ion exchange or size exclusion chromatography). Single step using HLA-specific affinity chromatography had non-specific impurities that resulted in non-specific binding on the sensor chips and inaccurate calculation of antibody concentration using protein assays. It was interesting to observe the difference in requirements for purification of different monoclonal HLA-specific antibodies despite all been IgG1 isotype. The difference in charges of monoclonal antibodies attributed to different ion exchange chromatography

technique. IHB-HU-081 (SN607D8) bound to anion exchange resin compared to IHB-HU-033 (SN230G6) bound to cation exchange resin. The only structural difference between them was difference of light chains; IHB-HU-081 (SN607D8) had κ-light chain compared to IHB-HU-033 (SN230G6) which has λ-light chains. The binding of IHB-HU-033 (SN230G6) to HLA-proteins on sensor chip could not be disrupted using 100mM Glycine at pH 11.0. And finally the apparent K_D was lower for IHB-HU-033 secondary to faster association rate. The third monoclonal HLA-specific antibody IHB-Hu-059 (WK1D12) had two peaks with anion exchange chromatography and hence size exclusion chromatography was used. This suggests the charge and binding strength of monoclonal HLA-specific antibodies dictates the purification protocols. However, both of these monoclonal antibodies interacted with different epitopes on HLA-A2 proteins and the difference could just be due the difference in structural epitope itself.

Optimal conditions for binding studies were established and the data from forced degradation study by Pure Protein LLC at Oklahoma, USA (Chapter -2) were a useful guide. Temperature of 37^o C was chosen as it was physiological temperature. Both association and dissociation rates increased with increase in temperature from 22 to 37 ^{o}C. This may suggest that the increase in temperature facilitated more hydrophilic interactions. Also at 22 ^{o}C there was minimal dissociation for monoclonal antibodies which would make calculation of dissociation rate difficult in the set time of 960 seconds. Thus for the above reasons further experiments were carried out at 37 ^{o}C. Higher Tween-20 concentration resulted in reduction of specific binding and hence a concentration of 0.005% was chosen for the running buffer. The most difficult part was regeneration condition and this was limited by damage of HLA-proteins in

harsh conditions. Increase in ionic concentration (1 M) did not help to disrupt the binding of IHB-HU-033 to HLA-A2 proteins whilst other conditions such as sodium bicarbonate or higher ionic concentration (2-4 M) resulted in damage to HLA-proteins with subsequent reduction or abolishment of binding response. This resulted in continuing use of 100 mM Glycine at pH 11.0 irrespective if it allowed disruption of binding or not. Where the regeneration buffer was not effective the sensor chip was not re-used.

Both the monoclonal HLA-A2 specific antibodies were studied using equilibrium, kinetic analysis using data from surface plasmon resonance and dose response curves using sandwich ELISA. All three analyses showed that IHB-HU-033 (SN230G6) monoclonal antibodies against HLA-A2 (epitope 62G) were stronger with higher affinity compared to IB-Hu-081 (SN607D8, epitope 142T). The absolute values obtained differed due to different assumptions used. For the equilibrium analysis ideally the experiments are required to be conducted until all the samples with range of concentration reaches equilibrium and then the values obtained using such a state should be used to derive dissociation constant. This depends on the association rate and for the antibodies with faster association rate, this can be achieved easily. The duration of association will be prolonged due to flow component of the biosensor assay used here. This is limited due to scare availability of the purified material. Hence the values obtained using kinetic analyses are more reliable. However, the challenge with kinetic analysis is the appropriate and precise mathematical model used to fit the data. As shown in these experiments Langmuir model based on 1:1 binding assumptions does not give better fit and the bivalent analyte model improves these fits.

Although the apparent K_D values obtained have lesser standard deviation, coefficient of variation is high at 160 to 190 %. This is due to comparison of K_D obtained from different combination of ligand and analyte concentration. At higher concentration of analyte, steric hindrance blunt the binding responses such that the maximal response units are similar for higher dilution of the antibodies. The coefficient of variance was better for lower concentration of analyte (39 to 50 %) compared to higher antibody concentrations (64 to 84 %). There was no difference on comparing between low and high density ligand lanes. Hence a lower density lane with lower concentration is better conditions to study monoclonal HLA-specific antibodies due to lesser steric hindrance. This will improve the mathematical model fitting and increase in accuracy of the estimates. The data obtained through these experiments are used to develop newer mathematical models in collaboration with school of engineering (Evans et al., 2013).

4.9 **Conclusion:**

Surface plasmon resonance assay can be optimised to study interaction of HLA-protein and HLA-specific antibodies. The binding kinetics and dissociation constant (K_D) were quantified with mathematical model such as bivalent analyte model.

5 Binding kinetics of epitope-paratope interactions

Recent studies have indicated that HLA-specific antibody reactivity (antigenicity) can differ between different HLA alleles that possess the same defined epitopes and thus support ostensibly the same epitope-paratope interactions (Duquesnoy et al., 2013). Using HLA class I specific monoclonal antibodies (mainly of the IgM class), the study concluded that the binding strength of epitope-paratope interactions may differ between different HLA proteins and that this binding strength may influence the subsequent activities of these antibodies within C1q-binding and CDC assays. Duquesnoy et al., proposed the concept of functional and structural epitopes (chapter 1) and according to this the specific binding of paratope (antibody) is with three amino acid residues on functional epitope but there is also non-specific binding between other amino acid residues around the functional epitopes with the CDR loops on the variable portion of the antigen constituting the structural epitope. As the stability of binding depends on the best fit and the overall surface area interacting, the structural epitope determines the binding strength / affinity. The study speculated that there is higher energy released during the binding is proportional to the binding strength of the interactions and this released free energy brings conformational change in the Fc portion of the antibody molecule to enhance the binding of complement.

The real-time biosensor assay based on the surface plasmon resonance principle, described in Chapter 4, provides an ideal opportunity to study and explore whether binding kinetics differ between different HLA protein-HLA-specific antibody pairings that possess the same epitope-paratope combinations. If possible, this would be a valuable tool to assess the reactivity of HLA-specific antibodies to a particular HLA-antigen and potentially predict its effector function activity. Simultaneously, we aim to determine whether the intrinsic binding

kinetics and affinities of different IgG human monoclonal HLA-specific antibodies can be distinguished quantitatively. Heterogeneity in these parameters could pioneer efforts to correlate specific allele- and epitope-binding patterns with affinity and clinical outcome following kidney transplantation. Additionally, we aim to investigate whether the SPR biosensor platform can be used to study competitive binding *in real time* of separate antibody clones with reactivity towards the same HLA protein. This is particularly helpful to assess the relative affinities of HLA-specific antibodies in the context of their epitope specificities.

5.1 **Aim**

1. To determine the binding kinetics and affinities of IgG human monoclonal HLA-specific antibodies using a real time assay platform.

2. To establish whether binding kinetics of the same epitope-paratope interactions differ between different HLA haplotypes.

3. To explore whether dual and/or competitive binding of HLA-specific antibodies can be studied using surface plasmon resonance and to study the effects on overall affinity by mixing two different monoclonal antibodies with the same HLA haplotype specificity.

5.2 Method and materials:

The mouse IgG anti-human HLA Class I monoclonal antibody W6/32) was tested against range of class-I HLA proteins. Similarly, five human IgG HLA Class I-specific antibodies were kindly obtained from the University of Leiden (F Claas and A Mulder). See Table 5-1. These antibodies were produced by hybridoma derived from B-cell that produced antibodies following exposure of foetal HLA-types; described as immunising antigens.

Clone (ID)	Immunising Ag	HLA-specificity defined by CDC	Epitope	HLA proteins tested
SN203G6 (IHB-Hu-033)	A2/B57	A2/B17 (B57,58)	62G	A2 & B57
SN607D8 (IHB-Hu-081)	A2/B57	A2/A28 (A68,69)	142T --H	A2, A68 & A69
WK1D12 (IHB-Hu-059)	B27	B27,7,60	163EW	B7 & B27:05
VTM1F11 (IHB-Hu-045)	B7	B27,7,60	163E	B7, B27:05 & B40:01
WAR5D5 (IHB-Hu-074)	B27	B7,27,42,55	62RN-QIY and 62R—QIC	B7 & B27:05

Table 5-1 : Human monoclonal HLA-specific antibodies used for studying difference in binding kinetics between different alleles.

The immobilisation densities of all the HLA proteins on the sensor chips were kept constant at a response unit level of 400 during the experiments. The human monoclonal HLA-specific antibodies were used over the concentration range of 1.6 – 100 nM. Experiments and analysis of binding data followed the protocols described in Chapter 4.

5.3 Results:

5.3.1 Binding kinetics of IgG monoclonal HLA-specific antibodies and interactions with different HLA proteins

5.3.1.1 *Mouse anti-human class-I HLA specific monoclonal antibody (W6/32)*

The W6/32 antibody binds to a conformational epitope formed by residue 3 arginine amino acid of β2 microglobulin and residue 121 on alpha-1 of the heavy chain of Class I HLA molecule (Ladasky et al., 1999). Interactions of W6/32 were studied against HLA proteins (Figure 5-1). The apparent K_D values calculated using bivalent analyte model and equilibrium analysis are shown in Table 5-2. Interesting, the highest affinity (i.e. lowest K_D value) is observed for HLA-B57 (up to 3-fold lower K_D) and a component of this appears to be attributable to both a faster association rate and slower dissociation rate compared with the interactions with other HLA haplotypes.

W6/32 – HLA protein	Bivalent analyte Kinetics	Bivalent analyte K_D	Equilibrium K_D
HLA-A2	k_a = 1.14E+05 1/Ms k_d = 5.48E-04 1/s	4.80E-09 M	1.31E-08 M
HLA-A69	k_a = 1.12E+05 1/Ms k_d = 6.64E-04 1/s	5.96E-09 M	8.75E-09 M
HLA-B57	k_a = 2.07E+05 1/Ms k_d = 4.13E-04 1/s	2.00E-09 M	6.10E-09 M
HLA-A1	k_a = 1.72E+05 1/Ms k_d = 4.79E-04 1/s	2.78E-09 M	1.65E-08 M

Table 5-2: Binding parameters of interactions of W6/32 with respective HLA proteins

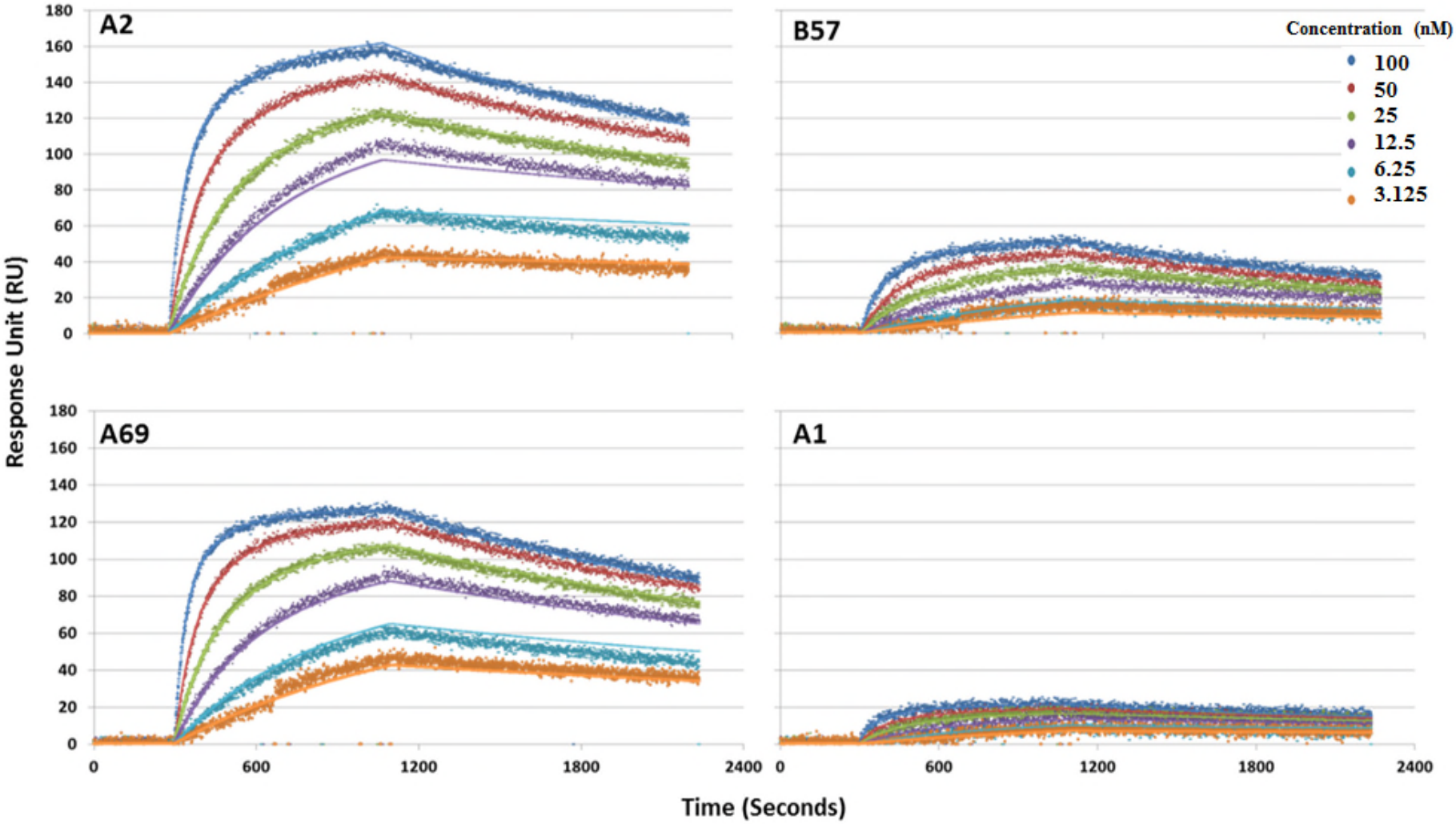


Figure 5-1 : W6/32 binding to HLA proteins (bivalent analyte model fits are shown as solid lines)

5.3.1.2 *Human monoclonal HLA-specific antibodies interactions with HLA-class I proteins*

6. SN607D8 (IHB-Hu-081)

SN607D8 is an IgG1 human monoclonal HLA-specific antibody with reactivity against the A2, A68 & A69 haplotypes. The principal immunising antigen is HLA-A2, but SN607D8 has been shown to bear specificity for the 142T epitope that is shared between HLA-A2,-68 & -69 but not present on –B57 (Figure 4-33). Figure 5-2 shows reactivity to the respective proteins.

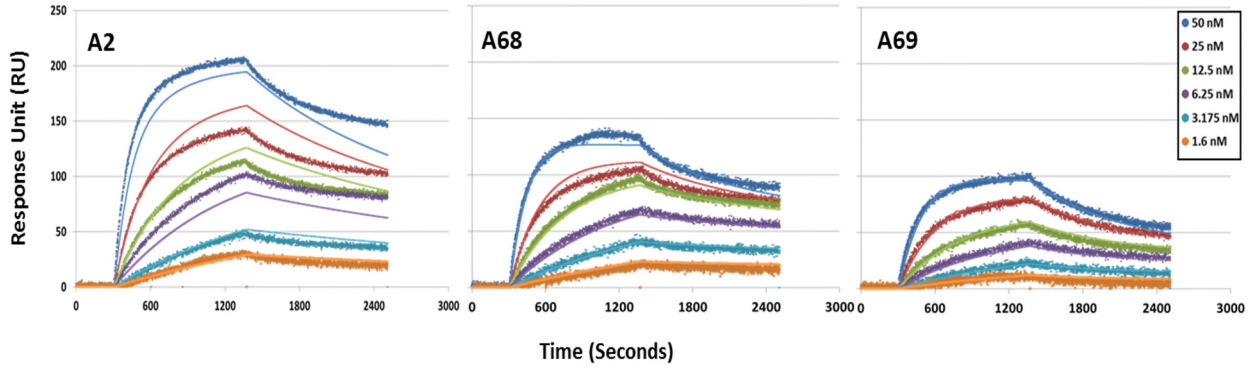


Figure 5-2 : SN607D8 binding to HLA proteins with data fitting using the bivalent analyte model indicated as solid lines.

SN607D8 – HLA protein	Bivalent analyte Kinetics	Bivalent analyte K_D	Equilibrium K_D
HLA-A2	k_a = 4.53E+04 1/Ms k_d = 5.05E-04 1/s	1.12E-08 M	2.65E-08 M
HLA-A68	k_a = 4.46E+04 1/Ms k_d = 1.07E-03 1/s	2.41E-08 M	1.87E-08 M
HLA-A69	k_a = 5.43E+04 1/Ms k_d = 4.94E-04 1/s	9.11E-09 M	2.63E-08 M

Table 5-3: Binding parameters of interaction of SN607D8 to HLA proteins

Binding kinetics revealed that affinity values were similar for HLA-A2 and -69, and two-fold higher compared to HLA-A68. The dissociation constant obtained from equilibrium analysis showed similar findings. The association rate (k_a) was highest for interactions with HLA-A69 and the dissociation rate was slower for interactions with HLA-A2 & A69 compared to HLA-A68 (Table 5-3).

7. SN230G6 (IHB-Hu-033)

SN230G6 is an IgG1 human monoclonal HLA-specific antibody produced from the same patient as for SN607D8 but interacts with A2, B57 & A58 class I HLA proteins. The immunising antigens were HLA-A2 and –B57 and the epitope specificity is for 62G (which is absent on HLA-A68 & -69) (Figure 4-33). Figure 5-3 shows binding data with respect to these proteins.

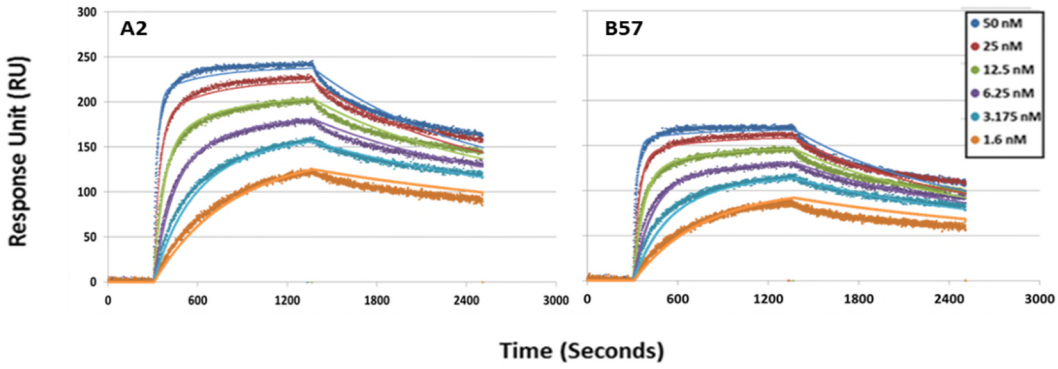


Figure 5-3. SN203G6 binding to HLA proteins with data fitting via the bivalent analyte model shown as solid lines.

SN230G6 – HLA protein	Bivalent analyte Kinetics	Bivalent analyte K_D	Equilibrium K_D
HLA-A2	k_a = 3.66E+05 1/Ms k_d = 6.48E-04 1/s	1.77E-09 M	2.72E-09
HLA-B57	k_a = 4.27E+05 1/Ms k_d = 6.92E-04 1/s	1.62E-09 M	2.43E-09

Table 5-4 : Binding parameters of interaction of SN230G6 to the respected HLA-proteins

Binding data for SN230G6 were shown to be very similar between HLA-A2 & -B57, with closely matching association and dissociation rate constants (Table 5-4). The dissociation constants were similar for interactions with HLA-A2 and HLA-B57 measured by both bivalent analyte model and equilibrium analysis.

8. WK1D12 (IHB-Hu-059)

WK1D12 is an IgG1 human monoclonal HLA-specific antibody that is directed against the HLA-B7, B27 and B60 proteins. The immunising antigen is HLA-B27 and the specific epitope is 163EW. Figure 5-4 shows reactivity to HLA-B7 and –B27. The K_D values calculated using the bivalent analyte mathematical model and equilibrium analysis are shown in Table 5-5.

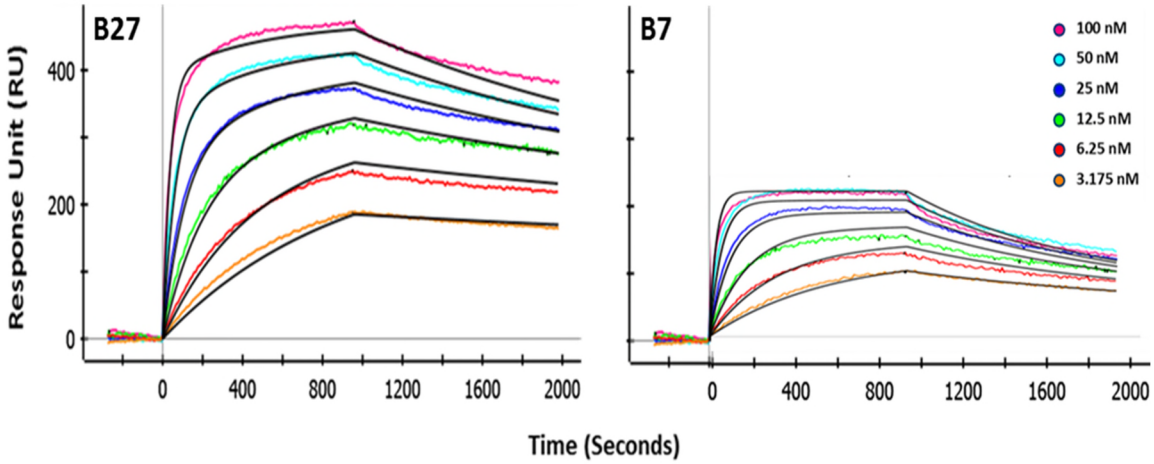


Figure 5-4 : WK1D12 binding to HLA-protein- B7 &-B27 (with data fitting via bivalent analyte model in solid lines)

WK1D12 – HLA protein	Bivalent analyte Kinetics	Bivalent analyte K_D	Equilibrium K_D
HLA-B27	k_a = 9.69E+04 1/Ms k_d = 3.34E-04 1/s	3.45E-09 M	1.00E-08
HLA-B7	k_a = 1.43E+05 1/Ms k_d = 8.14E-04 1/s	5.89E-09 M	8.28E-09

Table 5-5 : Kinetic (Bivalent analyte) analysis of interaction of WK1D12 to the respected HLA-proteins

The binding strength is 1.7 times higher for interactions with the immunising antigen HLA-B27 compared to HLA-B7. The key contributing component causing the difference is the slower dissociation rate (k_d) for the interaction with HLA-B27 (Table 5-5).

9. VTM1F11 (IHB-Hu-045)

VTM1F11 is an IgG1 human monoclonal HLA-specific antibody directed against the B7, B27 and B40:01 HLA Class I proteins. The immunising antigen is HLA-B7 and the specific epitope is 163E. Figure 5-5 shows the interactions of VTM1F11 with these proteins. The kinetic values calculated using the bivalent analyte model and equilibrium analysis are shown in Table 5-6.

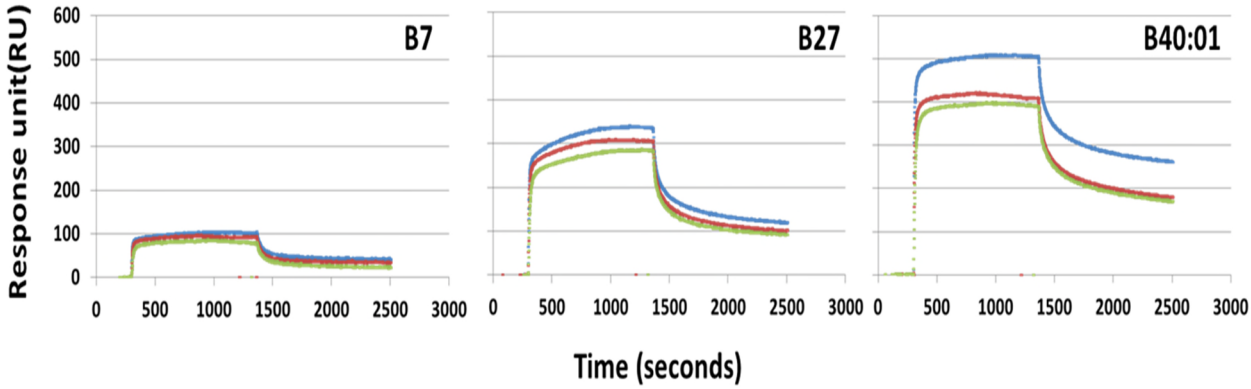


Figure 5-5 : Binding kinetics of VTM1F11 MAb against the HLA proteins B7, B27 & B40:01. (Lines corresponds to concentration of the monoclonal antibodies – 100nM, 50 nM and 25 nM from top to bottom respectively).

VTM1F11 – HLA protein	Bivalent analyte Kinetics	Bivalent analyte K_D	Equilibrium K_D
HLA-B7	k_a = 4.43E+07 1/Ms k_d = 1.78E-02 1/s	4.01E-10 M	2.53E-09 M
HLA-B27:05	k_a = 2.46E+06 1/Ms k_d = 9.40E-03 1/s	3.81E-09 M	6.87E-09 M
HLA-B40:01	k_a = 1.54E+06 1/Ms k_d = 6.14E-03 1/s	3.99E-09 M	1.08E-09 M

Table 5-6 : Kinetic data for the interaction of VTM1F11 with the cognate HLA proteins

The binding strength calculated using bivalent analyte kinetic model appears to be 10-fold higher for VTM1F11 interactions with immunising antigen HLA-B7 compared to HLA-B27 and –B40:01. This appears to be due to a faster association rate (k_a) for interaction with HLA-B7 compared to other two.

10. WAR5D5 (IHB-Hu-074)

WAR5D5 is a human IgG1 monoclonal HLA-specific antibody directed against B7 and B27 class I HLA proteins. The immunising antigen is HLA-B27 and the specific epitope is 62RN. Figure 5-6 shows its interactions with the respective proteins. The K_D values have been calculated using the bivalent analyte model and equilibrium analysis are summarised in Table 5-7.

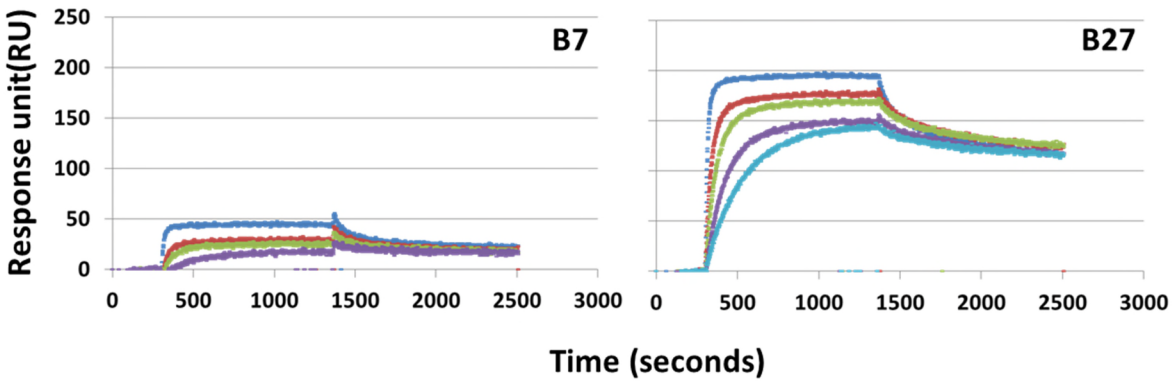


Figure 5-6 : Binding curves of WAR5D5 Mab against HLA-proteins B7 & B27. (lines corresponds to concentration of the monoclonal antibodies – 100nM, 50 nM and 25 nM from top to bottom respectively).

WAR5D5 – HLA protein	Bivalent analyte Kinetics	Bivalent analyte K_D	Equilibrium K_D
HLA-B27	k_a = 3.10E+05 1/Ms k_d = 4.72E-03 1/s	1.52E-08 M	1.72E-09 M
HLA-B7	k_a = 1.51E+05 1/Ms k_d = 1.94E-03 1/s	1.29E-08 M	1.11E-08 M

Table 5-7 : Kinetic data of interactions of WAR5D5 with HLA proteins

The apparent binding affinity is slightly higher for interactions with immunising antigen HLA-B27 and this is due to faster association rate which is almost double compared to the interactions with HLA-B7.

5.3.2 EC_{50} analysis

The monoclonal HLA-B7 specific antibodies were analysed using sandwich ELISA for the difference in relative binding strength using EC_{50} measurements. The EC50 analysis comparing HLA-A2 specific monoclonal is shown in chapter 4 (Figure 4-30). Experiments were carried out in triplicates and dose response curve was analysed as shown in Figure 5-7.

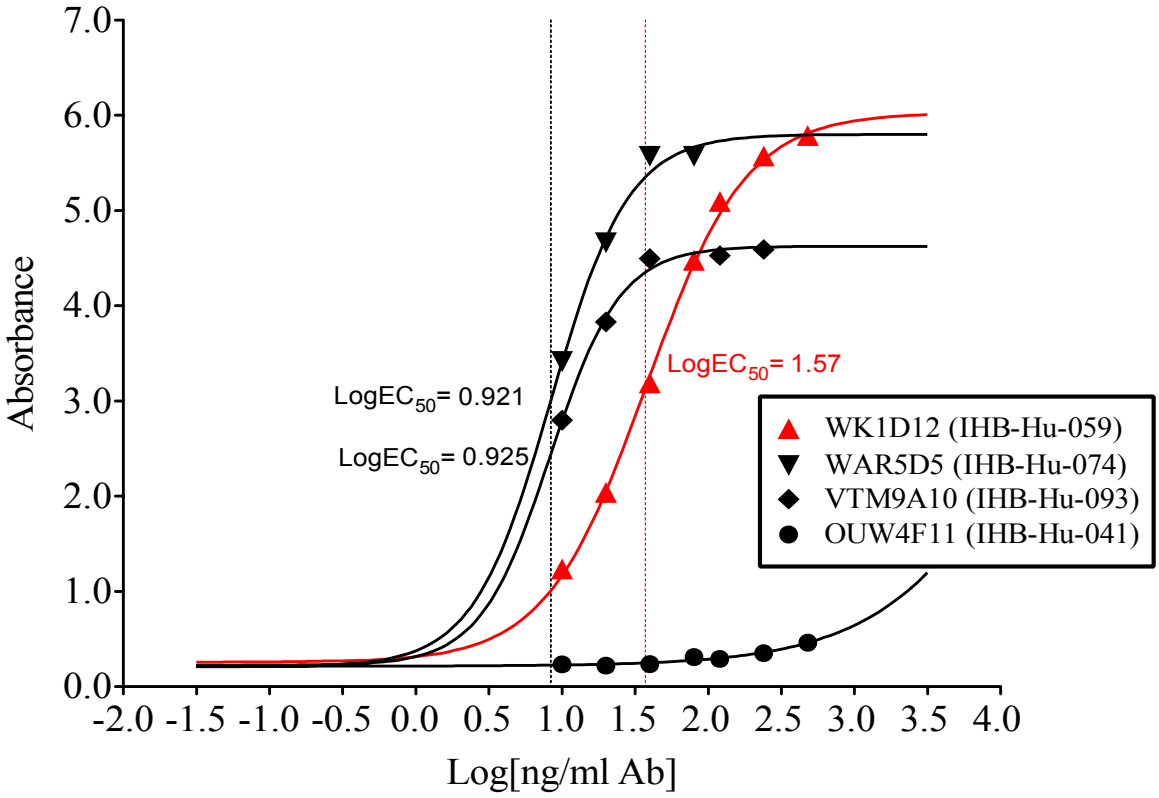


Figure 5-7 : Sandwich ELISA experiments for measurement of relative strength of monoclonal HLA-specific antibodies (EC_{50})

The EC_{50} value for interaction between WK1D12 and HLA-B7 was 37.4 compared to 8.41 for WAR5D5 monoclonal HLA-B7 specific antibody. Thus WAR5D5 is 4.5 times stronger than WK1D12. Monoclonal OUW4F11 had very minimal binding suggesting weaker affinity compared to other monoclonal HLA-B7 specific antibodies.

5.3.3 Measurement of the thermodynamics parameters of antibody binding

The thermodynamics of protein-protein interactions provide vital information relating to the binding energies involved and yield quantitative data pertaining to the energetic favourability of a particular interaction. This information has not been obtained previously for HLA/HLA-specific antibody interactions using real-time and quantitative techniques and thus warrants exploration. A valuable feature of the XPR36 biosensor instrument is its ability to measure binding kinetics over a range of temperatures, from which thermodynamic parameters (such as enthalpy and entropy) can be determined via algorithms such as Van't Hoff analysis. It has therefore been possible to perform preliminary experiments aimed at probing not only the kinetic but also the thermodynamic properties of HLA-specific antibodies.

For this sets of experiments human monoclonal HLA-A2 specific antibody (SN607D8 (IHB-Hu-081)) was used and interactions were studied with HLA-A2 protein at a range of temperatures (15 to 40 ^{o}C). The binding data obtained were analysed bivalent analyte model (Figure 5-8). As expected, the dissociation rates (k_d) and association rates (k_a) increased with increasing the temperature (Table 5-8 and Figure 5-9). The dissociation rates increase more markedly as a function of temperature compared with the association rates, hence K_D values increase with increasing temperature, and overall affinity decreases. However, interactions with HLA-A68 did not show a steady trend and this may reflect effects of temperature on certain epitope-antibody interactions (Johnstone et al., 1990), or the need for optimisation. Interestingly, the fidelity of fitting the data using the bivalent analyte model varied with temperature such that better fits were observed below 30˚C. As seen in Figure 5-8, residuals were less pronounced when fitting the data for lower temperatures.

This offers some support to hypotheses that epitope and possibly even paratope exposure and behaviour could be influenced by temperature. HLA proteins are acknowledged as highly flexible molecules wherein complex changes in vibrational and conformational stability could affect their ability to bind to specific antibodies (Mian et al., 1991).

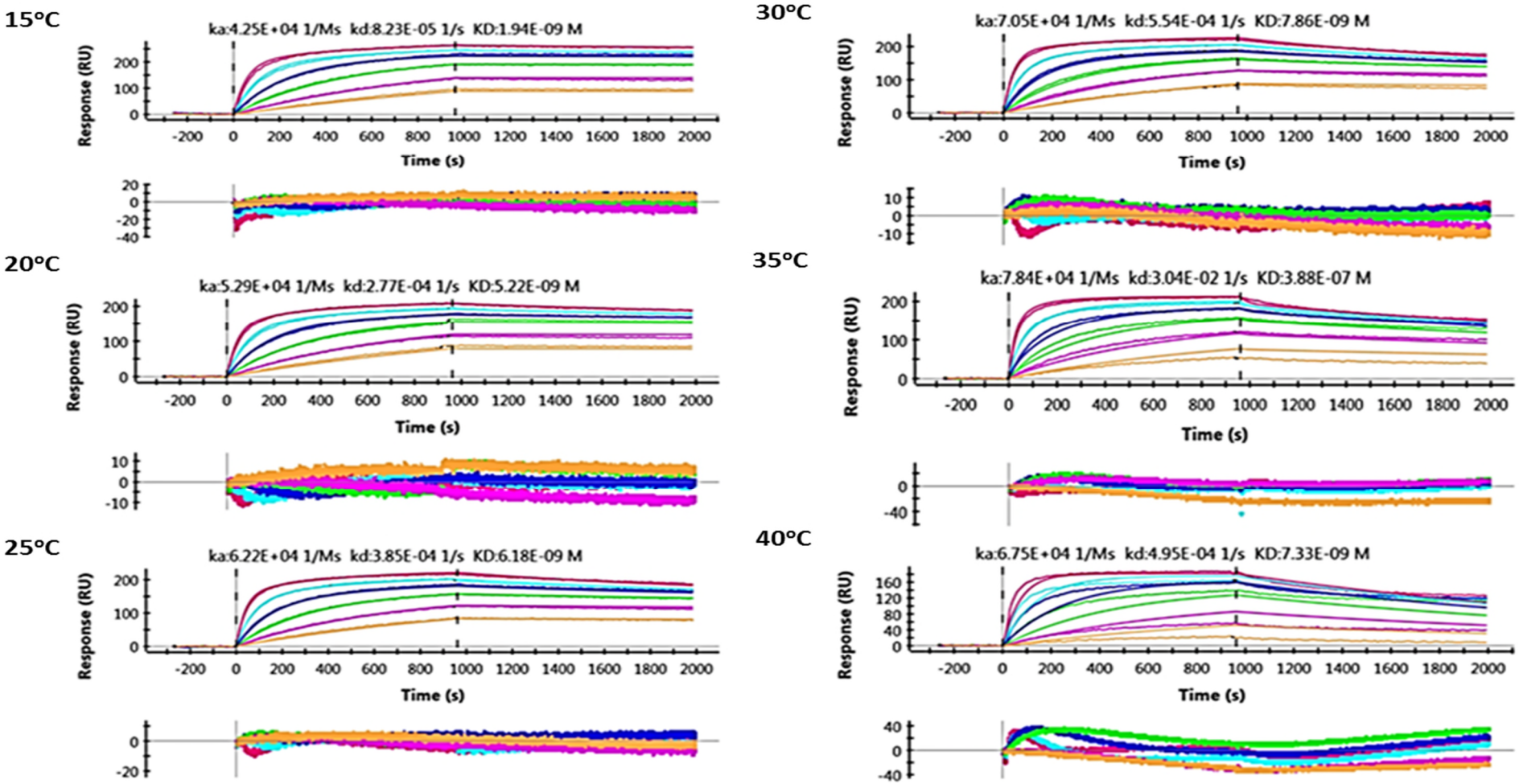


Figure 5-8 : Thermodynamic analysis of interactions of HLA-A2 and SN607D8 (IHB-Hu-081) at a range of temperatures (15 to 40 °C)

Shows binding sensorgram and residual plots at different temperatures. The binding curves from top to bottom corresponds to concentration dilution (100nM to 3.175nM).

	A2			A68			A69		
Temp (Celsius)	k_a 1/Ms	k_d 1/s	K_D M	k_a 1/Ms	k_d 1/s	K_D M	k_a 1/Ms	k_d 1/s	K_D M
15	4.25E+04	8.23E-05	1.94E-09	4.67E+04	1.13E-04	2.43E-09	4.75E+04	1.09E-04	2.30E-09
20	5.29E+04	2.77E-04	5.22E-09	4.97E+04	2.94E-04	5.91E-09	5.43E+04	3.36E-04	6.18E-09
25	6.22E+04	3.85E-04	6.18E-09	1.01E+05	1.52E-04	1.50E-09	6.63E+04	2.91E-04	4.39E-09
30	7.05E+04	5.54E-04	7.86E-09	1.07E+05	2.27E-04	2.12E-09	7.46E+04	4.24E-04	5.68E-09
35	7.84E+04	3.04E-02	3.88E-07	7.42E+04	2.66E-02	3.58E-07	7.84E+04	5.51E-04	7.02E-09
40	6.75E+04	4.95E-04	7.33E-09	6.29E+04	4.38E-04	6.97E-09	5.81E+04	8.84E-04	1.52E-08

Table 5-8 : Kinetic parameter of binding interaction of HLA proteins – A2, -A68 & -69 to SN607D8 at different temperatures

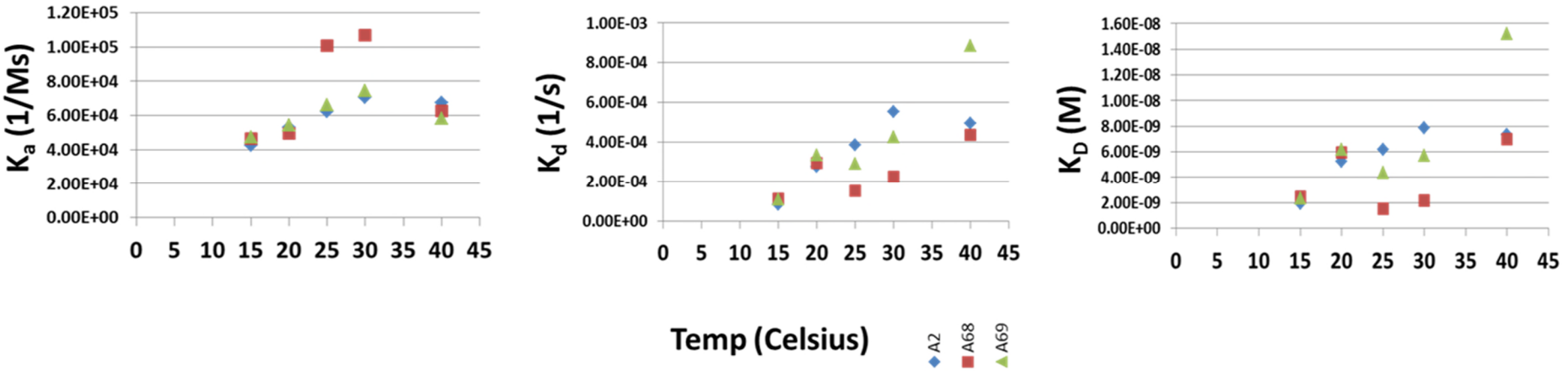


Figure 5-9 : Kinetics of binding interactions between HLA-proteins (-A2,-A68 & -69) and SN607D8.

5.3.3.1 *Van't Hoff analysis:*

Thermodynamic parameters such as Gibbs free energy (ΔG), Enthalpy (ΔH) and Entropy (ΔS) can be calculated using Van't Hoff plot. Enthalpy is the amount of heat absorbed or released during a chemical reaction. The value will be positive for endothermic reactions and negative for exothermic reactions. Entropy is measure of increase in disorder or dispersal of the energy; a positive value suggests increase in disorder. For an interactions to happen it needs to overcome activation energy barrier and this reveals energy required for binding and dissociations. Thermodynamic measurement using ProteOn BioRad platform has been described and the current experiment follows same methodology (Bravman et al., 2008).

Gibbs free energy is described by following equations

$$\text{Gibbs free energy } (\Delta G) = \Delta H - T\Delta S \text{ and}$$

$$(\Delta G) = RT \log (K_D)$$

$$\text{Thus, } \log (K_D) = \Delta H/RT - \Delta S/R$$

Van't Hoff plot is derived by plotting Inverse of temperature (in Kelvin) and $\log K_D$. The assumption is both enthalpy and entropy are constant with temperature changes. From this curve, slope is equal to ΔH/RT and intercept is equal to ΔS/R. As seen in the Figure 4-6, the relationship between temperature and $\log K_D$ was linear for HLA-A2 and HLA-A69 but inconsistent for HLA-A68. Binding enthalpy (ΔH) is calculated by determining the slope of the curve obtained via the linear equation shown in the Figure 5-10. ΔH is calculated by multiplying the coefficient of the slope with Universal Gas Constant (R = 8.314472 J/K.M) and ΔS is calculated by multiplying the intercept of the slope with the Universal Gas constant (R).

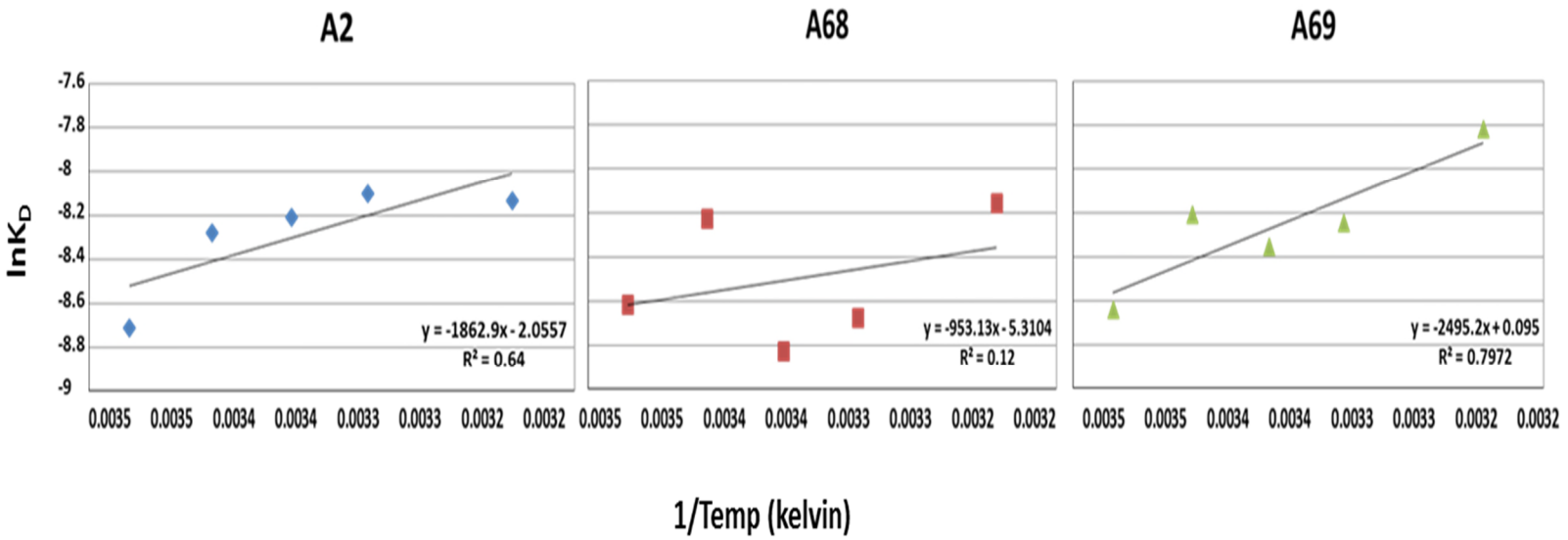


Figure 5-10 : Vant Hoff's analysis for HLA-protein and SN607D8 Mab interactions

SN607D8 interaction with HLA-protein	ΔH KJ mol^{-1} K^{-1}	ΔS J mol^{-1} K^{-1}	ΔG at temp = 310 K (37 °C) KJ mol^{-1} K^{-1}
A2	-15.49	-17.5	-10
A68	-7.925	-44.15	5.76
A69	-20.75	0.79	-20.9

Table 5-9 : Thermodynamic parameters for interactions of HLA proteins and SN607D8

Table 5-9 shows calculated thermodynamic parameter using van't Hoff analysis. These data suggests that binding enthalpy is most favourable for interactions with HLA-A69 compared with HLA-A2 and HLA-A68. However, the limitation of Van't Hoff analysis is the assumption of linear relationship and as it can be seen the straight line obtained by linear regression gave R2 value 0.6 for HLA-A2 and 0.8 for HLA-A69 interactions whilst it was very poor for interactions with HLA-A68. This may be due to altered thermodynamics due to temperature dependant process. Higher negative entropy value suggests the interaction resulted in lesser randomness for HLA-A68 interactions. Equally the relationship of $\log K_D$ and 1/T was very poor and even a non-linear fitting would struggle to fit the data.

From the equation of Gibbs free energy, reaction is spontaneous at all temperature if ΔG and ΔH are negative and ΔS is positive, like observed for interactions with HLA-A69. A reaction can be spontaneous even when ΔS is negative but the preference is at low temperature, such as observed with HLA-A2. If ΔS and ΔH are positive then the reaction is usually spontaneous at higher temperature. However if ΔG is positive the reactions are usually non-spontaneous such as observed with HLA-A68. Although the estimates are not highly accurate, the Van't Hoff plot analysis provides useful insight in to the thermodynamic and underlying chemical interactions of HLA-protein and HLA-specific antibodies. This type of analysis can help understand the predominant bonds involved in the interactions; such as if the reaction is better at higher temperature that suggests involvement of hydrophobic bonds as their strengths increases with increase in temperature. Whilst the interactions due to hydrogen bonds are more stable at low temperature as they are exothermic reactions (Reverberi and Reverberi, 2007). The

importance of insights in to thermodynamic of an interaction may allow manipulation of conditions to disrupt the bond between the antigen-antibody interactions.

5.3.4 Competitive and additive binding of IgG human monoclonal HLA-specific antibodies to selected HLA proteins

Biosensor platform provides an ideal opportunity to study interactions of different HLA-specific antibodies with HLA-protein. Thus one could test the hypothesis of conformational change following an earlier binding with the HLA protein, which may potentiate or inhibit subsequent binding of second antibody to the same HLA-proteins. Similarly it could allow testing competitive binding between two antibodies for the same epitope on the HLA-protein thus confirming the epitope definition of the antibodies.

Due to steric hindrance at higher concentrations of antibodies binding to the HLA-proteins (see Figure 4-32); the experiments were performed in sequential cross over fashion. Thus the initial binding reaction was allowed between the maximal concentrations of the antibodies on separate lanes followed by cross-over of the antibodies at same concentration in stepwise fashion. This allowed binding to all the available binding sites (epitopes) during the first phase of association and immediately after the initial association, the further binding in second association phase was studied. Experiments below demonstrates additive binding by using two human monoclonal HLA-specific antibodies that had specificities for different epitopes that were located on different alpha chain of class I HLA molecules and the distance between them was more than 30 Angstrom (Figure 5-11). For competitive binding, human monoclonal

HLA-specific antibodies that had specificities against same epitopes or closely located epitopes (with in 20 Angstrom) were selected (Figure 5-13). The distance was estimated using Cn3D software (Madej et al., 2012).

5.3.4.1 *Human monoclonal HLA-A2-specific antibodies: additive effect*

Two separate IgG human monoclonal HLA-specific antibodies against HLA-A2 were studied with known positional information for their respective target epitopes on the HLA-A2 molecule (Figure 5-11). These two monoclonal HLA-A2 specific antibodies (SN607D8 & SN230G6) were investigated in such a fashion that 1) the first antibody was allowed to associate with low-density immobilised HLA-A2 protein on the SPR channel; 2) Regeneration at high pH was NOT performed, such that a proportion of antibody remained bound to the channel; 3) the second antibody was flowed over the same channel in a second "association phase". This procedure was reciprocated and data are shown in Figure 5-12). In both cases, the binding of the antibodies in the second association phase was not influenced by presence of pre-bound antibodies as the distance between epitopes was more than 30 Angstrom. This would suggest additive binding and the accommodation of two separate antibodies.

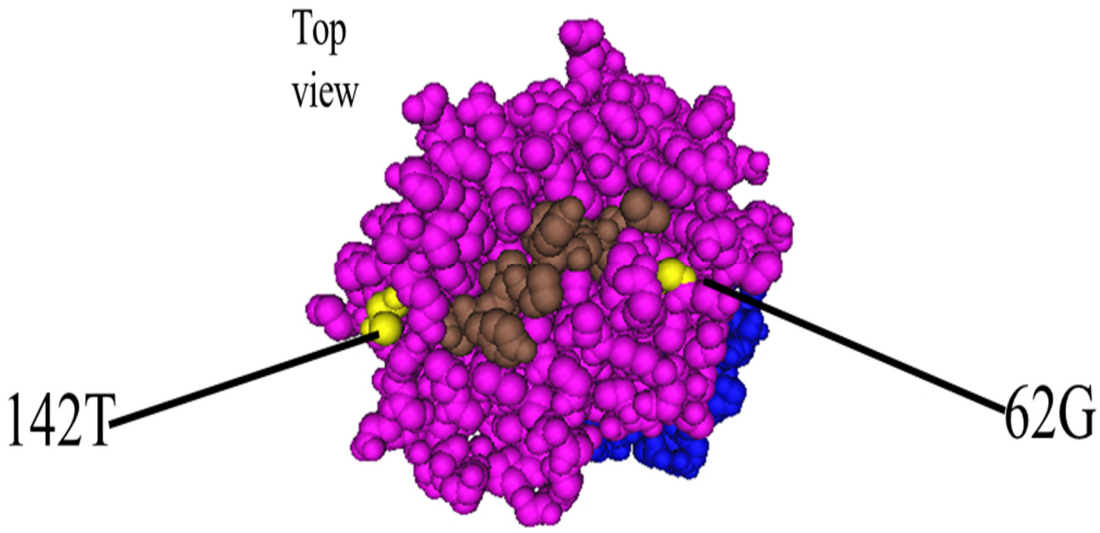


Figure 5-11 : HLA-A2 protein structure showing epitope 62G & 142T

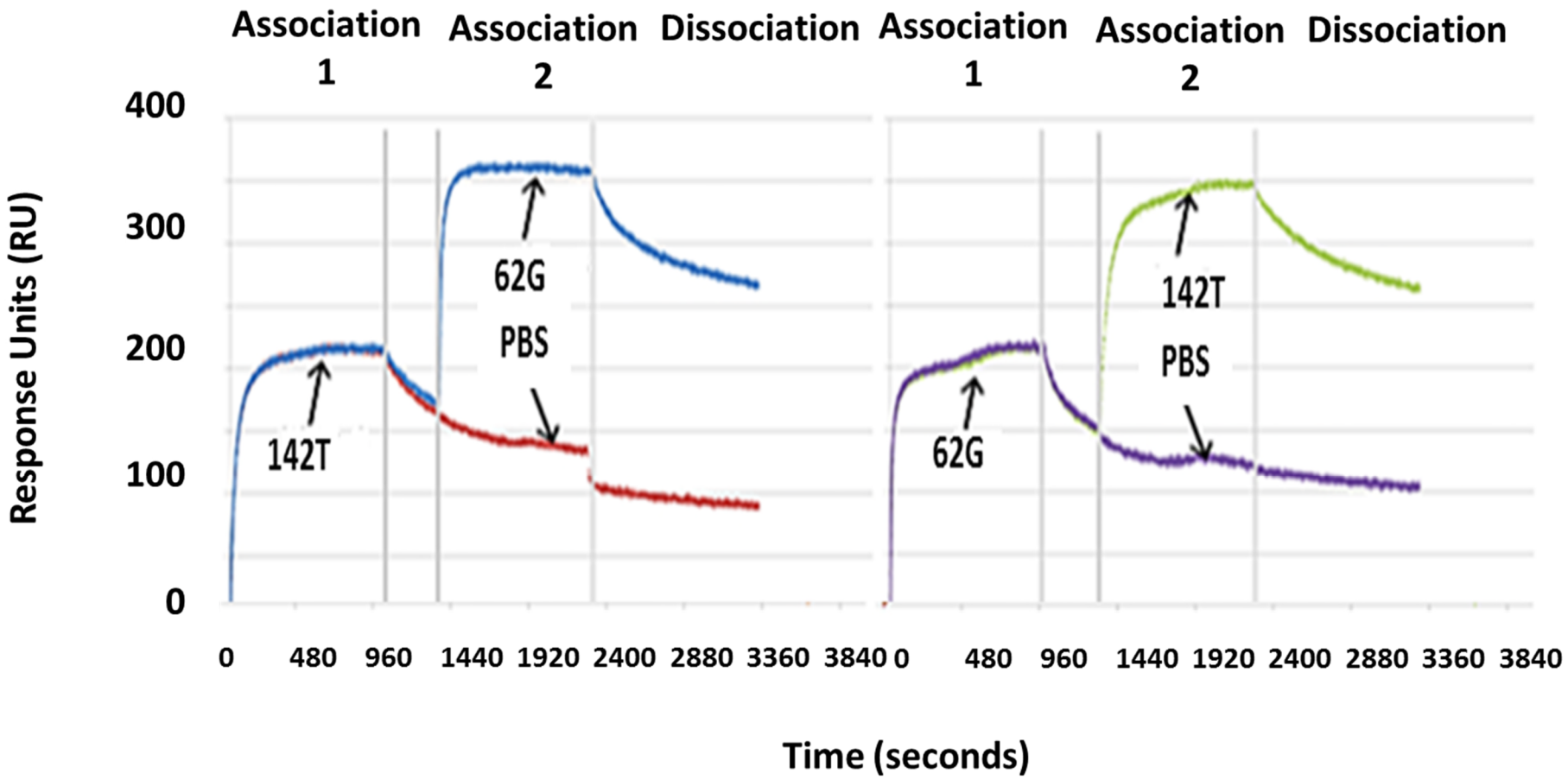


Figure 5-12 : Sequential interactions of HLA-A2 protein and monoclonal antibodies against epitopes 142T (SN607D8) and 62G (SN230G6). Clear evidence of accommodation with the appearance of concatenated sensorgrams in a "stacking" appearance.

5.3.4.2 *Human monoclonal HLA-B antibodies: competitive inhibition*

Three human IgG monoclonal HLA-specific antibodies were available with the spatial position of their epitopes known and mapped on their cognate HLA molecules (Figure 5-13). These were used and tested in pairs (Figure 5-14 & 5-15). The epitope for WK1D12 and VTM1F11 was same 163E shared between B7, B27 and B40:01. Whereas second pair, WK1D12 and OUW4F11 the epitopes were different but the distance between them was less than 20 Angstrom. The OUW4F11 was specific for epitope 80I which is shared between HLA-B7 and HLA-B40:01 but not HLA-B27. As seen in Figure 5-14, WK1D12 and VTM1F11 were studied in sequential fashion (as described above), and evidence of blunting of the binding response involving antibodies was seen in the "second association" phase. Similar observations were noted involving interactions between WK1D12 and OUW1F11 (Figure 5-15). This suggests that the monoclonal HLA-specific antibodies are competing for the same epitopes unlike the accommodation phenomenon shown in Figure 5-12.

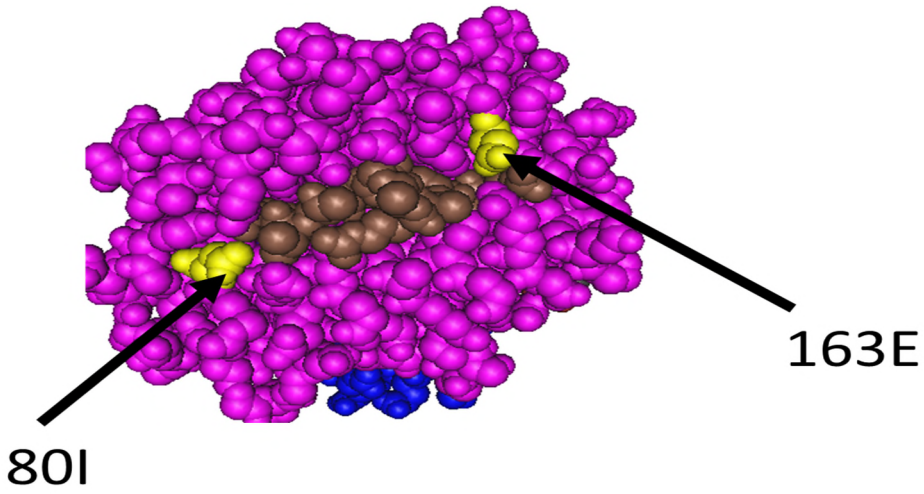


Figure 5-13 : Structure of HLA-B7 indicating location of epitopes 80I and 163E

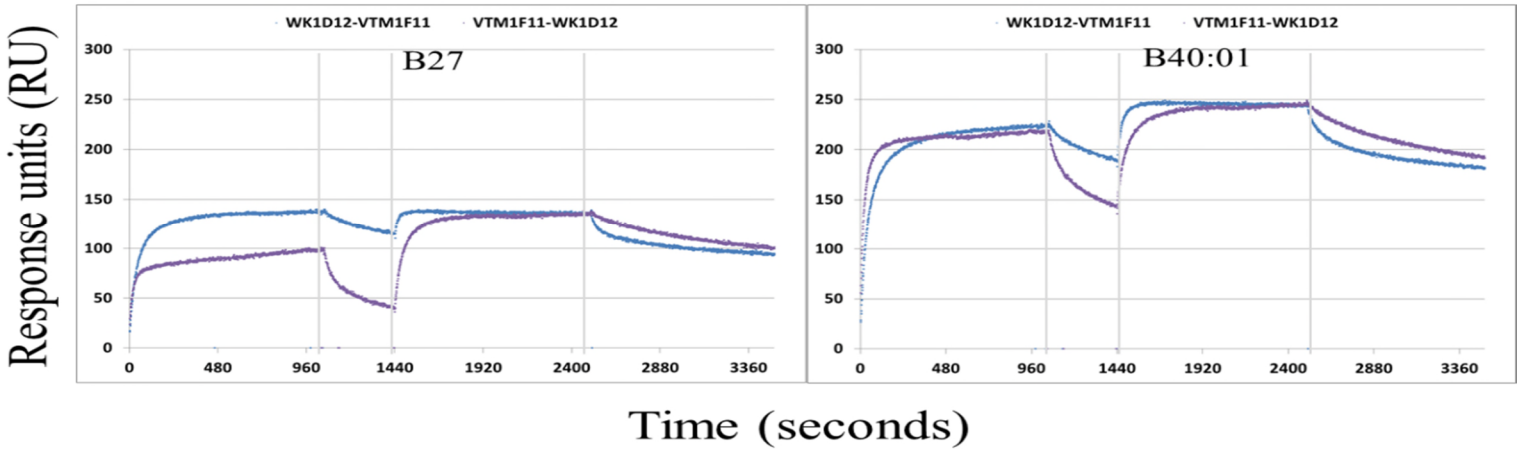


Figure 5-14 : Sequential binding experiments showing evidence of competitive inhibition / blunting and poor accommodation of binding response to a second monoclonal antibody

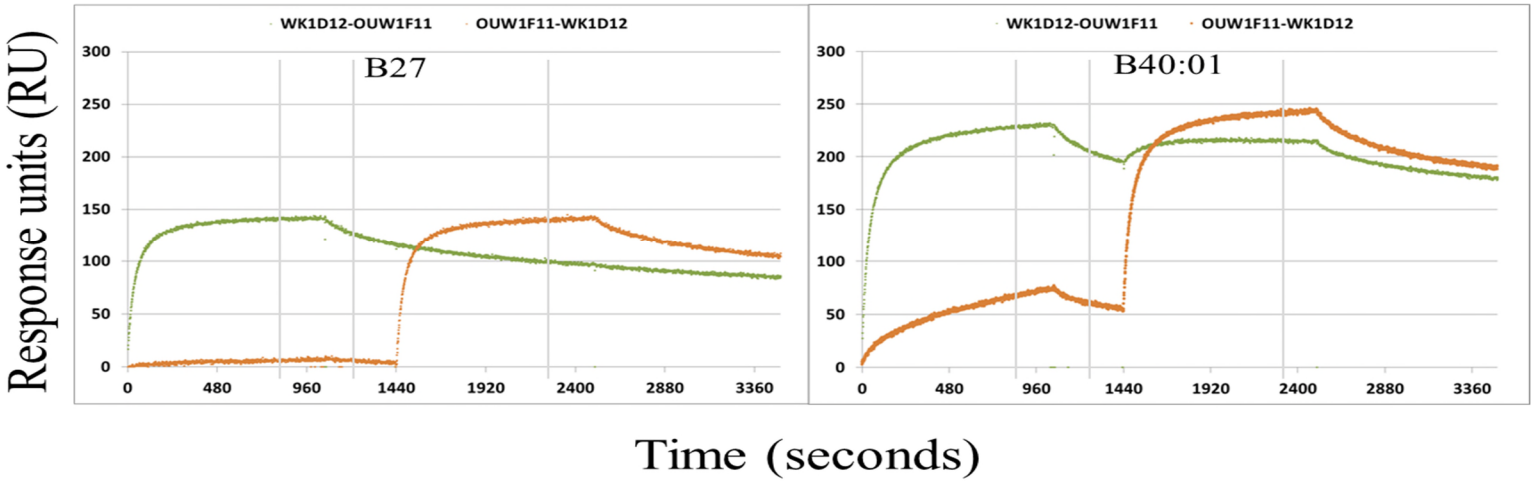


Figure 5-15 : Sequential binding experiments showing binding interactions of WK1D12 and OUWF11

Thus biosensor platform could be used to study effects of antibodies binding to epitopes on same HLA-proteins. Above experiments demonstrate use of human monoclonal HLA-specific antibodies, but these can be replicated for purified polyclonal HLA-specific antibodies from patient material. For patient material the epitope specific purification can be done using sequential HLA-protein affinity chromatography. We have demonstrated use of this principle and tested CDC reactivity in presence of two epitope specific polyclonal HLA-specific antibodies (Lowe et al., 2013b).

The pairs used here demonstrated additive (non-overlapping) and competitive (overlapping) effects, but equally an allosteric effect can be studied using biosensor platform.

5.3.5 Effect on overall affinity by mixing two IgG monoclonal HLA-specific antibodies displaying accommodative binding

Human IgG monoclonal HLA-specific antibodies with known accommodative binding as demonstrated Figure 5-12 (SN607D8 and SN203G6) were mixed in five different proportions (0, 25, 50, 75 & 100% relative to SN607D8) in PBS prior to running kinetic binding experiments in the established fashion. The effect of two monoclonal epitope specific antibodies was assessed on overall binding response and calculated affinity. As both the monoclonal antibodies bind to different epitopes located at distal regions of the HLA molecule target region, the overall binding signal response (Rmax) was higher when mixed in all proportions compared with one antibody applied to the chip exclusively (Figure 5-16). To calculate overall apparent K_D for the mixed solution, heterogeneous analyte model was used for fitting the data for calculating the binding kinetic parameters and for individual monoclonal HLA-specific antibodies, the bivalent analyte model was used (Table 5-10).

SN607D8:SN230G6	Kinetics	K_D
N: 0	k_a = 4.53E+04 1/Ms k_d = 5.05E-04 1/s	1.12E-08 M
75:25	k_a = 5.05E+04 1/Ms k_d = 3.82E-04 1/s	7.56E-09 M
50:50	k_a = 5.40E+04 1/Ms k_d = 3.46E-04 1/s	6.42E-09 M
25:75	k_a = 4.77E+04 1/Ms k_d = 3.28E-04	6.88E-09 M
0:N	k_a = 3.66E+05 1/Ms k_d = 6.48E-04 1/s	1.77E-09 M

Table 5-10 : Binding Kinetics of mixed monoclonal HLA-specific antibody solution

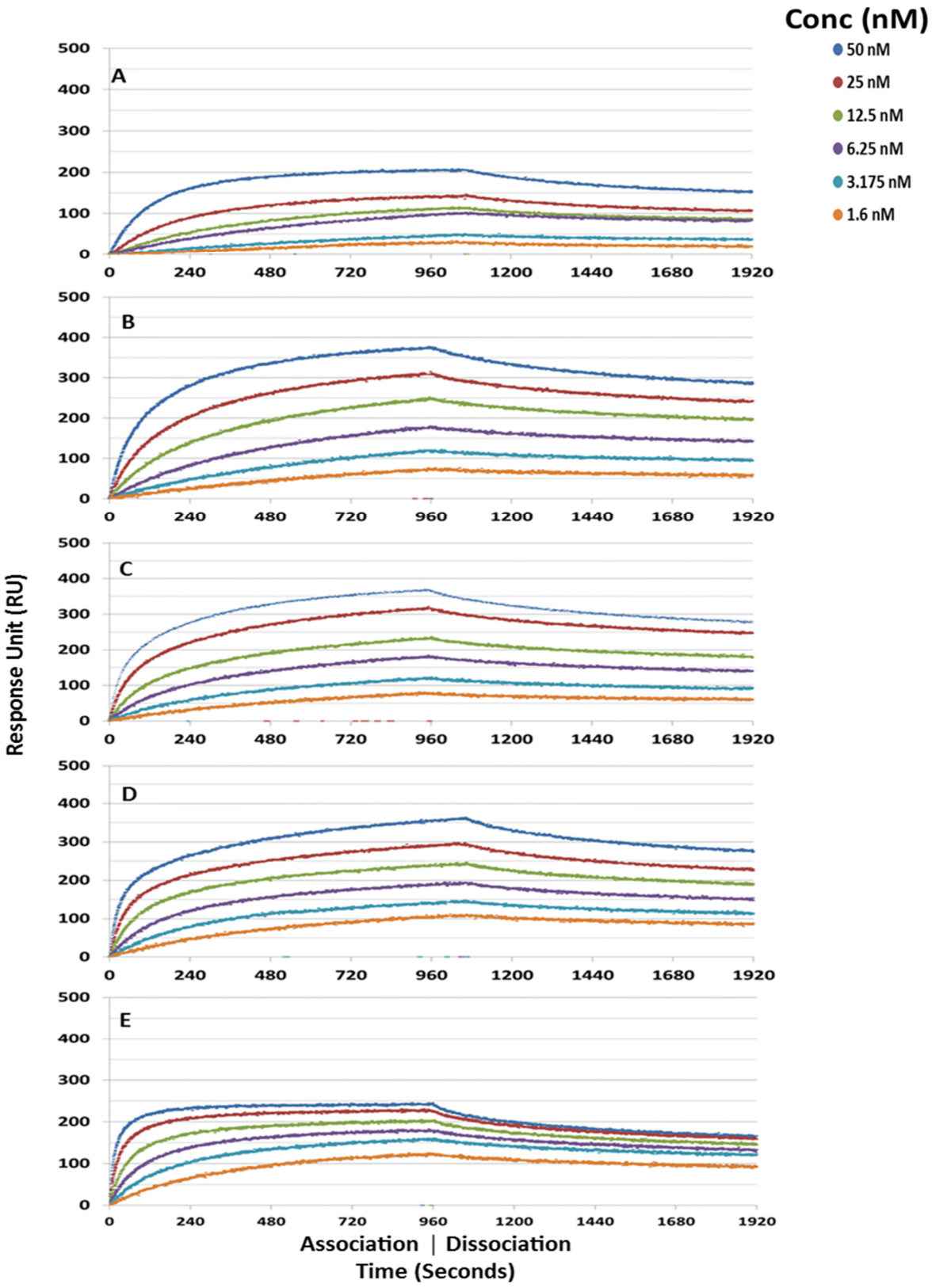


Figure 5-16 : Binding kinetics of solution containing two human monoclonal HLA-A2 specific antibodies (SN607D8 & SN230G6) mixed in different proportions (A - SN607D8 100% (50nM); B – D8 75% (37.5 nM) & G6 25% (12.5nM); C – D8 & G6 50% (25nM each); D = D8 25% (12.5nM) & G6 75% (37.5nM) and E – SN230G6 100%)

Experiments using mixture of monoclonal HLA-specific antibodies demonstrated the binding strength of the mixed solution is the average of individual binding strength and that even a small concentration of high affinity antibody effects overall apparent binding strength. Compared to the high affinity monoclonal HLA-specific antibodies (SN230G6) the binding kinetics of mixed solution demonstrated slower k_d and k_a values.

5.4 **Discussion**

Binding kinetic parameters, including dissociation constants can be measured in real time using surface plasmon resonance instruments such as the ProteOn XPR36. This has made possible the measurement of simultaneous, multiple interactions across range of concentrations. Binding assays of this kind possess added advantage over current assays since they capture a broader, real-time picture of the binding events and levels, compared with simpler end-point assays such as Luminex bead assay, ELISA and flow cytometry. In addition to being semi-quantitative rather than fully-quantitative, these latter assays may also be heavily influenced by protocol wash steps that are not easy to monitor precisely.

Wash steps applied in current Luminex and flow cytometry assays, whilst similar in some aspects, are not the same as the dissociation phases recorded in a SPR biosensor assay. In the former cases, wash steps are designed to remove non-specific, non-HLA-specific IgG and other irrelevant serum proteins from the beads or cells. The washing times and mechanisms are arrived at and settled upon in protocols largely due to satisfying a detection level arbitrarily. Subsequently, the end point measured could very easily be affected by faster

dissociation rates of certain studied antibodies. As seen in the Figure 5-17, the association rates for monoclonal HLA-specific antibodies (SN230G6, VTM1F11 & WAR5D5) binding to their cognate HLA proteins are fast, but the dissociation rates vary relative to Rmax, with faster dissociation for VTM1F11 compared to SN230G6 and WAR5D5. Conversely, the dissociation rate is slower for WK1D12 & SN607D8 compared to VTM1F11 and OUW4F11. Thus WK1D12 and SN607D8 antibody appears to “stick” better.

Depending upon where the cut-off point of the wash (dissociation) phase, the relative signal intensities can potentially vary substantially – a quality that would be missed in an end-point assay (such as Luminex or ELISA). The discrepancy between dissociation constant derived from EC_{50} calculation and kinetic analysis may be due to different equilibrium time for ELISA and SPR but equally been end-point assay it may wash-out lower affinity binding such as observed for OUW4F11 monoclonal antibody. Hence real time observations provide deeper qualitative and quantitative information capable of highlighting biophysical differences between discrete antibodies.

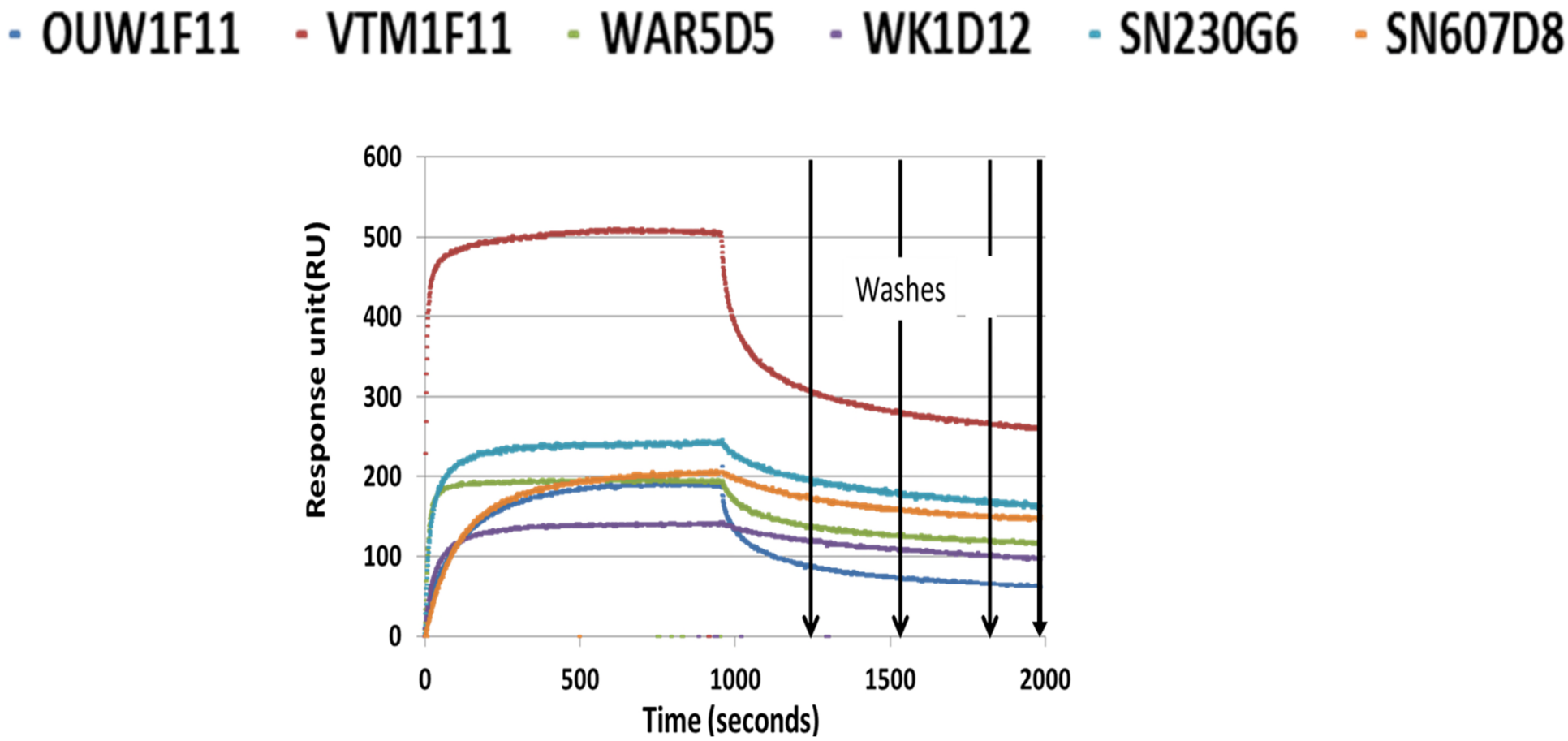


Figure 5-17 : Binding curves for different HLA-class I specific antibodies observed real time using biosensor assay

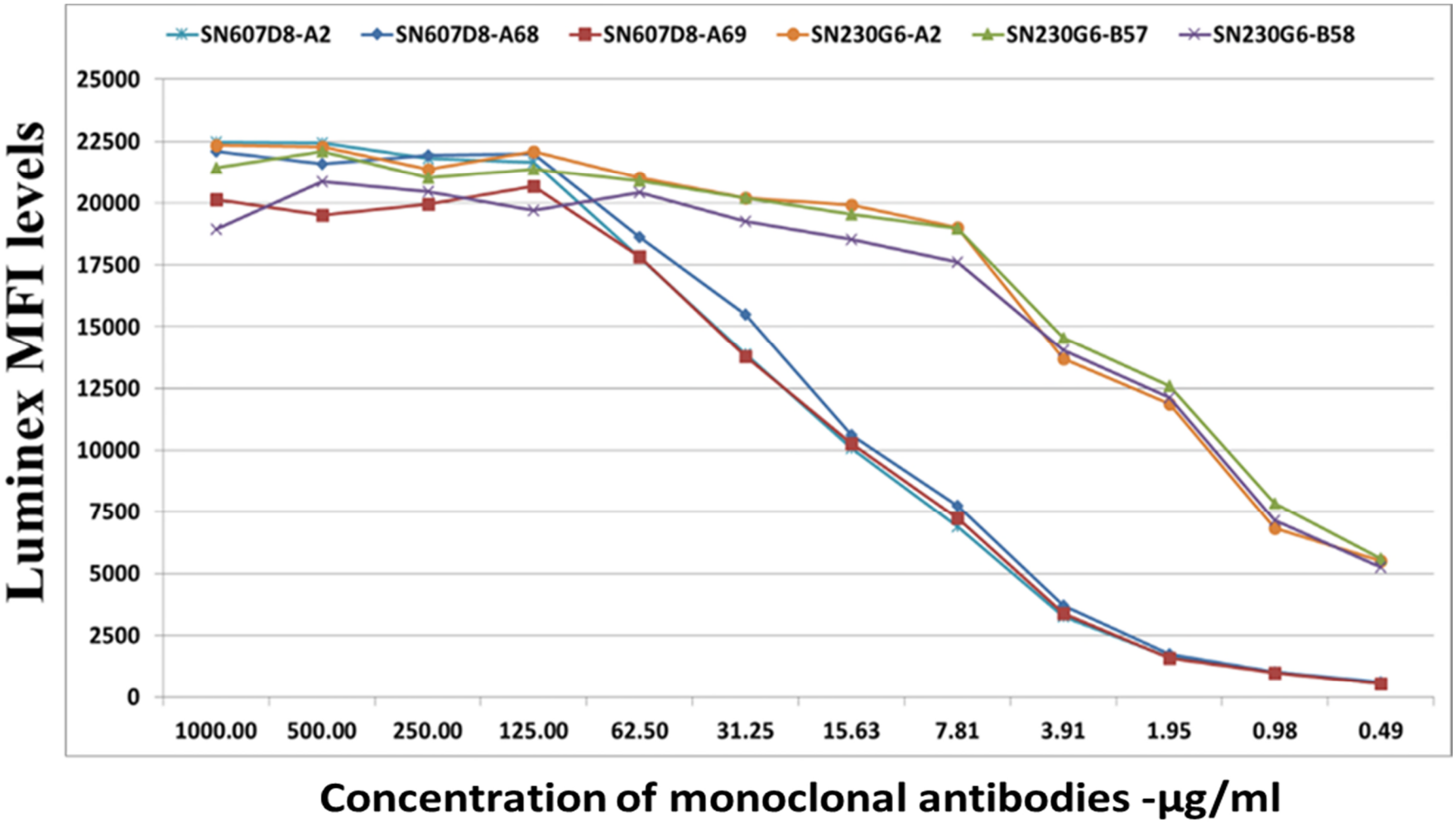


Figure 5-18 : Dose response curves of monoclonal HLA-specific antibodies (SN607D8 & SN230G6) on Luminex assay

In addition, Luminex assay is not able to evaluate the affinity of the same antibodies to different HLA-proteins that share the same epitopes. In fact, using Luminex assay the reactivity yields data that are virtually indistinguishable when showing binding to epitopes on different HLA proteins. As seen in Figure 5-18, the MFI values derived are extremely similar for binding responses of purified SN607D8 to HLA-A2,-A68 & -A69 proteins on the beads, and also for SN230G6 binding to HLA-A2, HLA-B57 & HLA-B58 proteins. Luminex assay is optimised for studying antibody binding at 22^{o}C whilst biosensor assay can be studied at varying temperatures and at physiological temperature of 37^{o}C.

The ability now to purify sufficient HLA-specific antibodies to a very high standard and perform real-time kinetic analysis generates additional antibody "metrics" such as k_a, k_d and K_D that could deepen and improve the ability to resolve relationships between HLA-specific antibodies, their immunological potency and, ultimately, their potential to affect risk of rejection. Another exciting opportunity lies in the ability of SPR experiments to obtain thermodynamic data for these interactions, yielding further parameters of potential physiological value plus deepening our understanding of antibody function and biophysics. This is especially interesting here in the context of HLA-specific antibodies binding to different HLA proteins that present the same functional epitope(s). The binding affinity is greatest for interactions between antibody and the original immunising HLA protein. The difference in dissociation constant measured ranged between 0.5 to 10 folds. Although the significant cut-off difference is not established for antibody-antigen interactions, it is clear from the above experiments that even a smaller difference can be due to different kinetic behaviours. Hence

when evaluating differences between the interactions the full picture should be taken in to account.

A recent study (Duquesnoy et al., 2013) suggested that reactivity on C1q-binding and CDC is highest for interactions of monoclonal HLA-specific antibodies against the immunising HLA protein and described a subjective and purely qualitative one/two/three-star property of "binding energy". The work described here goes much further in at least two capacities – 1) the majority of antibodies used in the Duquesnoy *et al*., study were of the IgM class, in contrast to the study in this thesis that has used IgG class HLA-specific antibodies. -2) The thermodynamics of epitope-paratope interactions were calculated. Although not fully optimised and precise, these experiments represent significant first steps towards understanding new territories in the field of HLA-specific antibodies. It is acknowledged that the best technique for measuring thermodynamic data in protein-protein interactions is isothermal titration calorimetry. However, this technique requires very large amounts of each purified component of the binding partnership – typically in the milligram range. This required quantity of antibody and HLA protein is prohibitive, in addition to raising the risk of experimental setbacks such as protein precipitation/solubility issues at high concentration (Bravman et al., 2008).

In addition to providing a deeper and broader range of quantitative measurements, the real-time SPR approach can also visualise competitive and accommodative binding between monoclonal HLA-specific antibodies and the same HLA protein molecule. Interactions

between SN230G6 and SN607D8 monoclonal HLA-specific antibodies against HLA-A2 protein performed in sequence showed accommodative/additive binding. Also, when tested using mixtures of these antibodies, the amplitudes of the concentration-rationalised responses were higher than single species of antibody alone, in agreement with the notion of the greater mass of individual monoclonal HLA-specific antibodies bound to the same immobilised HLA protein. This accommodative phenomenon could reflect the physiological process of epitope spreading following immunological exposure of non-self HLA alleles (N.B. both SN607D8 and SN203G3 were derived from the same multiparous woman that had been exposed to foetal HLA-A2 and HLA-B57) and the resultant rise in MFI and lytic activity when analysed by Luminex and CDC assays respectively (Mulder et al., 2003, Marrari et al., 2010, Duquesnoy, 2014b). The binding strength (Table 5-10) is constant for different proportion of monoclonal HLA-A2 specific antibodies and is roughly the average of affinities of individual antibodies. The maximal response is similarly constant across the different proportions but is higher than the individual maximal responses. Therefore, the overall apparent affinity from a polyclonal, clinical sample could very well reflect the contribution of higher affinity antibodies and thus could prove to be clinically applicable. Additionally, competitive binding experiments on this assay could be used to validate and evaluate the key epitopes recognised by antibodies within a polyclonal sample by competing with known concentrations of monoclonal antibodies of defined epitope specificity.

Clinical cases usually display polyclonal HLA-specific antibody responses with antibodies directed against different epitopes present on the same HLA-proteins. These observations can vary with time and the appearance of newer antibodies may alter HLA binding responses as

measured by changes in MFI levels or CDC positivity. A study looking at CDC-positive responses have shown that the presence of two or more monoclonal antibody clones augments CDC reactivity when compared to a single monoclonal antibody (Kushihata et al., 2004) and this effect was observed if the antibodies reacted to different epitopes located at critical distance. These overall antibody binding characteristics could be useful in pre-transplantation settings such as early prediction of acute adverse clinical events (antibody mediated rejection) and also in post-transplantation settings where longitudinal follow-up could track changes in affinity predicting chronic rejection and its prognosis. Another important application could be the ability to define non-detrimental donor HLA-specific antibodies in highly sensitised cases, and thus make feasible transplants that would otherwise be denied.

6 Purification and quantification of polyclonal HLA-specific antibodies and characterisation of affinity

6.1 Introduction

Using human monoclonal HLA-specific antibodies as described in Chapter 4, we have validated the SPR biosensor platform for studying the binding kinetics and affinity of their interactions with HLA proteins. Using the experience from these experiments and methods, clinical samples can now be approached with a view to initiate description of the distribution of affinity parameters across clinical sample cohorts. When serum samples were used as neat or in a 1:50 dilution, two major problems emerged instantly. Firstly, there was massive non-specific binding to the intrinsic matrix of biosensor chips and secondly, accurate quantification of antibody concentration – a vital parameter required for calculating the binding to the specific HLA-proteins quantitatively and rationally was not possible.

To be able to study clinical samples, various methods for purifying immunoglobulin G (IgG) were tested for their efficacy and selectivity similar to protocols described for purification of monoclonal human HLA-specific antibodies described in Chapter 4. Two additional techniques were used for purification of polyclonal IgG – sequential precipitation from whole serum (using octanoic acid and ammonium sulphate) and size exclusion chromatography (gel filtration). Ion exchange chromatography techniques that were used in the purification of monoclonal HLA-specific antibodies were not considered, since it was anticipated that polyclonal antibodies possess a range of isoelectric points and would very likely give multiple peaks contaminated with other serum proteins. A combination of affinity chromatography and size exclusion chromatography yielded purified HLA-specific antibodies but overall recovery was low due to the multiple protein dialysis and concentration steps required in

between the two separate chromatography steps. Whilst the combination of sequential precipitation and size exclusion chromatography was not specific for HLA, it is specific for IgG enrichment and did not require multiple protein concentrating steps. The experiments below show that commercial polyclonal IgG (i.e. not HLA-sensitised and extracted from pooled normal human serum) did not cause any non-specific binding to SPR chips and thus the strategy of enriching HLA-specific IgG antibodies alongside bystander, non-HLA-specific IgG can resolve the HLA-specific binding response on SPR sensor chips and ultimately allow for the study of relevant binding kinetics.

Methods to quantify HLA-specific antibodies were studied simultaneously with the purification protocols. Protein quantification methods such as Nano drop, Lowry's and BCA assays were used for estimating and determining concentrations of the monoclonal HLA-specific antibodies described previously, as they were available in large quantities and were comparatively easy to purify using HLA-protein affinity chromatography and ion-exchange chromatography. Experiments with polyclonal antibodies demonstrated that the concentration levels of antibodies recovered from a patient sample were typically too low for accurate detection using Nano drop, and gave inconsistent data when applied to eluted fractions following affinity chromatography. Similarly, Lowry's assay and BCA assay couldn't quantify polyclonal HLA-specific antibodies (in nanogram concentration) accurately due to their being below the lower concentration threshold following affinity chromatography. Hence, more sensitive quantification assays using standard curves calibrated with human monoclonal HLA-specific antibodies were used.

Here, three different quantification assays were approached to determine the relative concentrations of polyclonal HLA-specific antibodies. Luminex assays can be used for this purpose but they are limited by very high cost. The basic protocol of a sandwich ELISA assay first developed by PTS, Oklahoma (R Buchli) was used with to probe HLA-A2- and HLA-B7-specific antibodies, determined by the availability of these soluble HLA proteins for coating the ELISA plates. In addition SPR experiments possess a unique faculty using binding data obtained during first 30 seconds of analyte-ligand contact in isocratic flow (Lynch et al., 2014, Karlsson et al., 1991). Advantages of this latter approach include its label free quantification and potentially broader HLA haplotype range, since calibration uses monoclonal antibodies with multiple haplotype specificity (e.g. SN607D8 to A2, A68 & A69; SN230G6 to A2, B57 & B58 and WK1D12 to B7 & B27).

6.2 Material and methods - overview

Human sera and plasma effluent were collected from patients undergoing HLA-incompatible kidney transplantation at University Hospital, Coventry UK, as discussed in Chapter 2.

Purification protocols were used as described in chapter-2 and various combinations and chronologies were applied. SPR experiments followed tailored protocols established for monoclonal antibodies (See Chapter 4). Preliminary kinetic data were calculated using the heterogeneous analyte model (Gomes et al., 1999, Bowles et al., 1997).

6.3 Purification of polyclonal HLA-specific antibodies

6.3.1 Non-specific binding related signal from plasma effluent and serum samples

Both serum and plasma effluent were tested in neat and dilution after centrifugation at 13,500 rpm for 15 minutes. This was sufficient to obtain distinct specific signal via Luminex assay. However, there no specific signal in SPR experiments due to a very high level of noise, attributable to non-specific or gross protein/serum component binding (Figure 6-1).

Binding experiments using serum from **case LT65** (Anti-HLA-A2 MFI values of 7011) against immobilised HLA-A2 protein showed non-specific binding (NSB) on both the test (A & B) and blank reference channels (C). Remarkably, non-specific signal on the reference channel was higher compared to the test channels and thus reference subtractions resulted in negative binding curves such that affinity parameters could not be calculated in any way (see Figure 6-2). Importantly, the level non-specific binding was present even at lower dilution levels of serum sample for all channels.

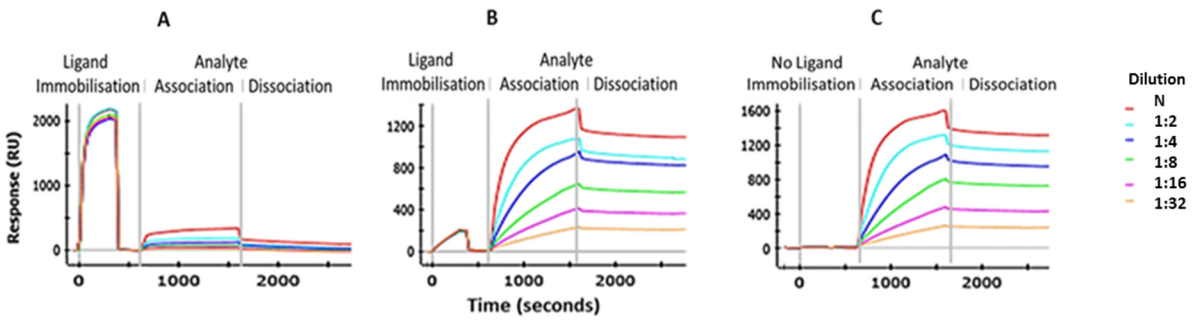


Figure 6-1 : Non-specific/undesirable binding to the matrix of the sensor chip with serum from the LT65 case. Higher NSB to blank lane (C) compared to low density ligand lane (B) and high density ligand lane (A)

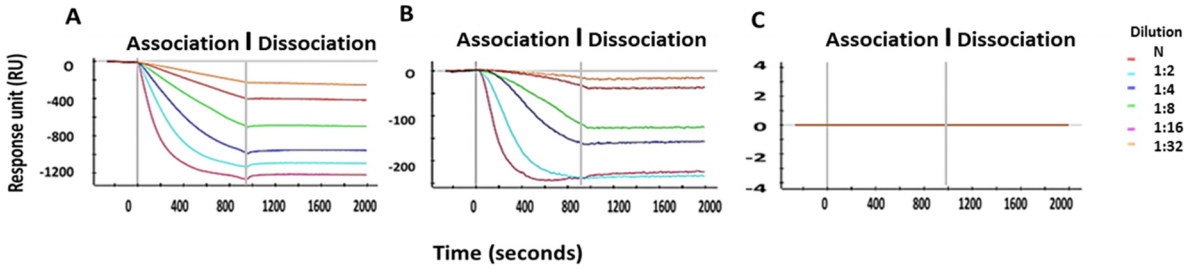


Figure 6-2 : Effect of non-specific binding on data distortion following reference subtraction against the blank channel (C); Low density ligand lane (B) and high density ligand lane (A)

The presence of 0.005% Tween-20, and the addition of 1 M NaCl to the running buffer did not reduce the non-specific binding, as seen in Figure 6-3.

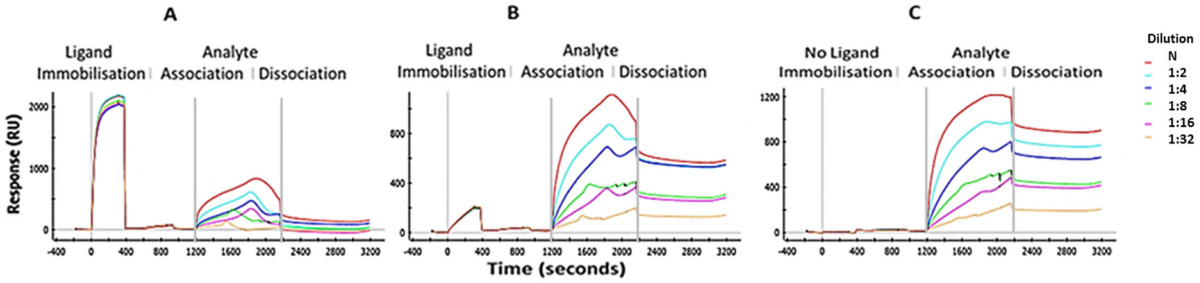


Figure 6-3 : Persistence of NSB with PBS-TS running buffer and case LT65 interaction with HLA-A2 protein and biosensor chip . Higher NSB to blank lane (C) compared to low density ligand lane (B) and high density ligand lane (A)

Monoclonal HLA-A2 specific antibody in negative serum sample:

Aliquots (900 µl) of negative (control) human serum (used in Luminex assay, NHSBT) was spiked with 100 µl of SN607D8 (MFI – 20,000) from a 1 mg/ml stock to assess whether the lack of signal be secondary to lower concentration of HLA-specific polyclonal antibodies in above experiments. This gave a final concentration of HLA-specific antibody of 0.1 mg/ml, which is very high and very likely supra-physiological.

This experiment demonstrated persistence of signal noise, but after reference subtractions the test lanes had distinguishable signals as seen in Figure 6-4 and Figure 6-5. This demonstrated that the concentrations of polyclonal HLA-specific antibodies were lower, as expected and removal of noise through improvement of sample quality would be required.

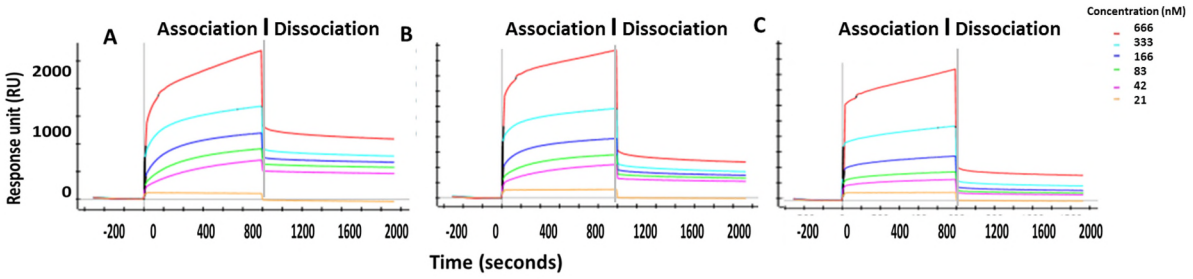


Figure 6-4 : Mab spiked in human serum (NHSBT negative control). Blank reference lane (C), Low density ligand lane (B) and High density ligand lane (A)

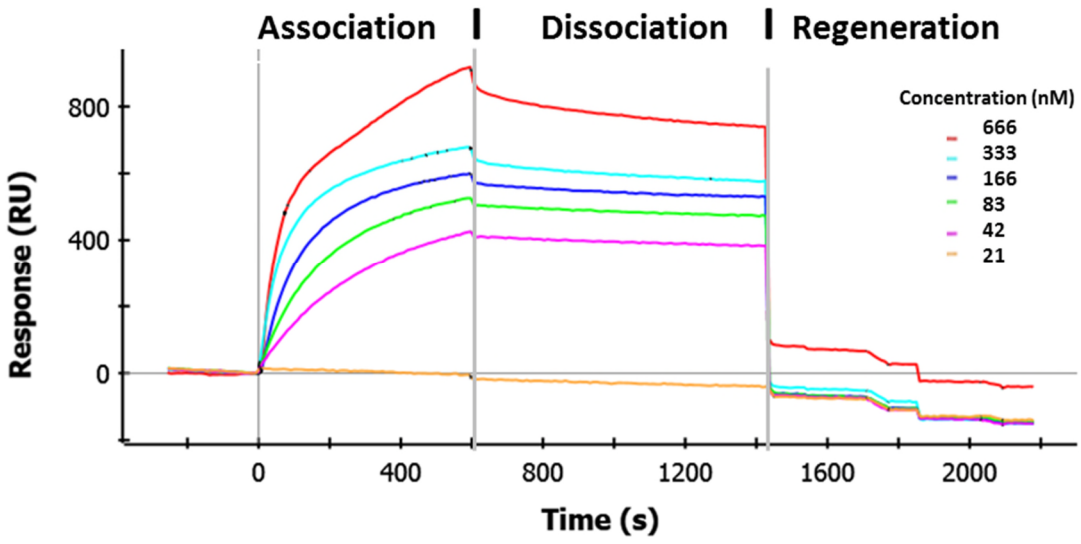


Figure 6-5 Binding curve of monoclonal anti-HLA-A2 diluted with negative control human serum (after ref subtraction)

6.3.2 Protocols for enriching HLA-specific antibodies from large volumes of plasma effluent

Several purification techniques were considered as described in Chapter 2. Initial experiments were done to determine what may be causing the non-specific binding. Human serum albumin (Sigma) and commercial polyclonal IgG (Pierce) were tested in the SPR system. Commercial polyclonal IgG was purified from human donor serum by ammonium sulphate precipitation and ion exchange chromatography and was in 1% PBS buffer, according to the supplier. Figure 6-6 shows relatively low non-specific binding and only the bulk mass effect (positive for albumin (A) and negative for polyclonal IgG (B)) compared to running buffer.

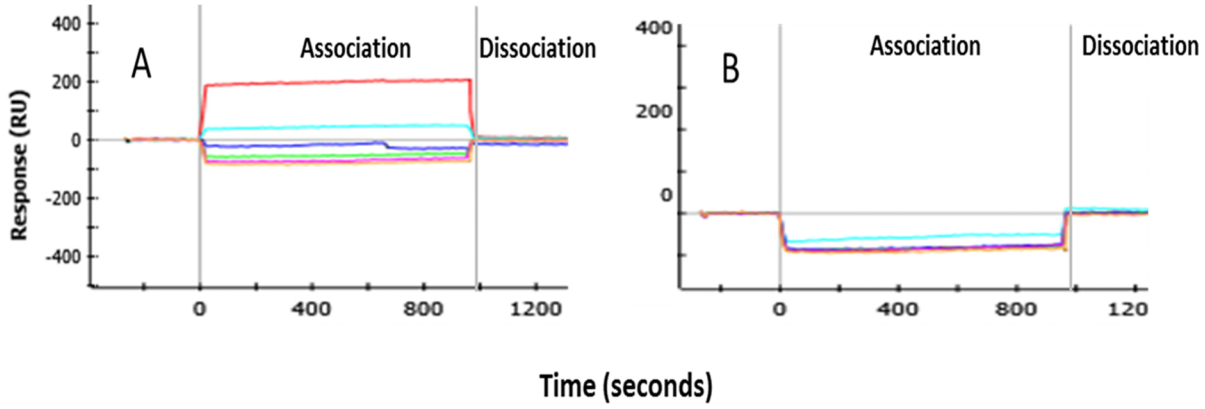


Figure 6-6 : Absence of non-specific binding signal with purified human serum albumin (A) and polyclonal IgG (B)

These data show that neither human albumin nor human polyclonal IgG were significant contributors to the noisy non-specific binding, implicating to other, unknown impurities present in serum and effluent.

6.3.2.1 *HLA affinity chromatography alone as downstream purification*

As described in Chapter 2, soluble HLA proteins immobilised on Sepharose were used for specific capture of HLA-specific antibodies. Plasma effluent preparations, recovered from three clinical cases (LT66, LT65 and LT45) were used.

Case LT66

Plasma effluent from case LT66 (250 ml) was centrifuged (13, 000 RPM for 15 minutes) and affinity chromatography was performed using the 60 ml HLA-A2 column. The captured antibodies were eluted, followed by spin concentration and buffer exchange into PBS. The MFI of this preparation for A2 was determined as 19709 and the concentration of protein measured by Lowry's assay was measured as 0.72 mg/ml. Figure 6-7 shows the persistence of non-specific binding to reference channel (B) and on test channel (A) despite antibody enrichment. Similar to previous observations, the amplitude of the non-specific binding signal was actually higher on empty reference channel, suggesting that unknown, non-IgG serum components could be interacting preferentially with the SPR sensor chip alginate matrix.

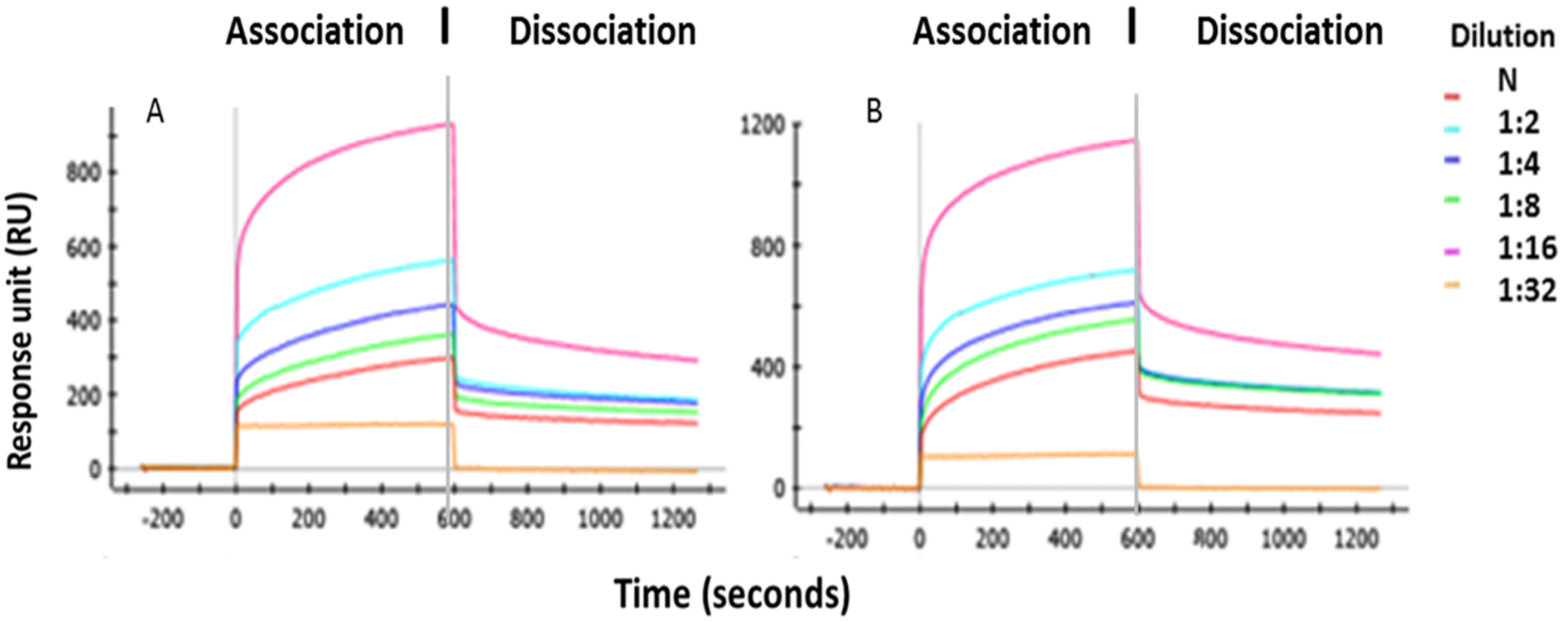


Figure 6-7 : Binding curves obtained from interaction of enriched plasma effluent sample (LT66) and HLA-A2 protein (A) and reference channel (B)

Cases LT65 and LT45

Similar results were obtained using IgG-enriched plasma effluent from cases LT65 and LT45 despite the recovered antibody-rich eluates possessing high MFI values for A2 of 12624 and 10,000 respectively. The concentration of protein measured by Lowry's assay was 1.51 and 0.64 mg/ml for enriched sample from LT65 and LT45 respectively.

Thus effluent centrifugation and HLA protein affinity chromatography did not result in samples of sufficient quality to yield distinct binding signals via SPR. These enriched materials were further purified and following this gave distinct signal (described in section 6.3.1.4.2).

6.3.2.2 *Sequential precipitation followed by HLA-protein affinity chromatography*

As antibody enrichment using HLA-protein affinity chromatography alone did not result in specific binding on biosensor chips either at all or of sufficient quality, further enrichment and purification techniques were applied. Sequential protein precipitation using octanoic acid followed by ammonium sulphate has been shown to enrich IgG selectively (Perosa et al., 1990). This precipitation protocol is also efficient at separating IgG from lipids, large lipoproteins and adhesive molecules such as fibronectin within human serum and similar, protein-dense preparations such as plasma effluent. It does not work well in preparations such as tissue culture supernatants.

Case LT77 Plasma effluent:

Plasma effluent (400 ml) from case LT77 was processed using the sequential precipitation method described in Chapter 2. The level of purity was assessed qualitatively by SDS-PAGE. Figure 6-8 shows evidence of high molecular weight protein removal. The resulting precipitate was dissolved in 1% PBS (10 ml) and passed through a guard column of uncoupled Sepharose (4 ml) to allow further removal of unwanted adhesive proteins ahead of HLA affinity chromatography. The flow-through from the guard column was then applied to 4 ml of HLA-A2-Sepharose. The material was eluted in 100 mM glycine pH 11.0 and dialysed into 1% PBS (2 ml) and protein quantified using nano drop (0.27 mg/ml) and Lowry's assay (0.38 mg/ml).

Figure 6-8 shows further reduction in high molecular weight and albumin bands. MFI values of the eluate measured by Luminex SAB assay are shown in Table 6-1.

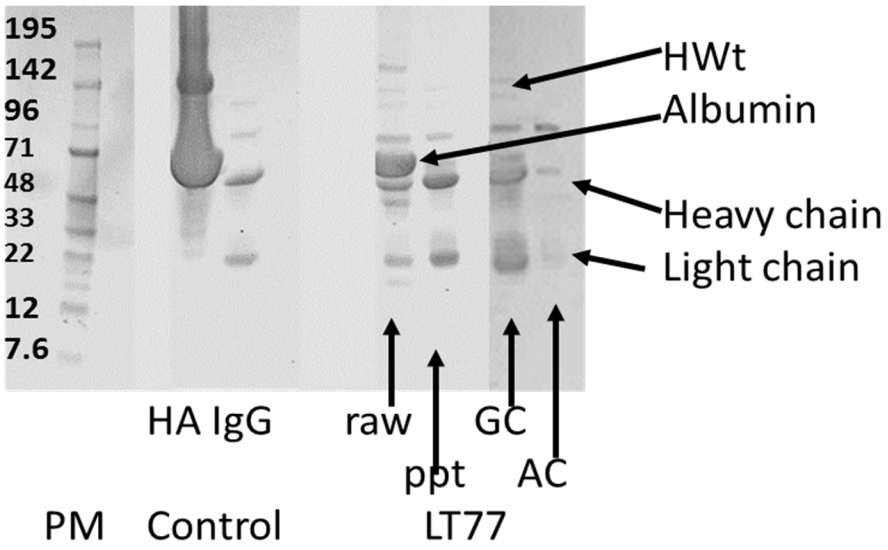


Figure 6-8 : SDS Page Gel electrophoresis (reducing gel) showing reduction in impurities by sequential precipitation

(PM – Protein marker; HA – Human albumin; IgG – Commercial Polyclonal IgG; LT77 – test sample code; ppt – post sequential precipitation; GC – post guard column; AC – post HLA affinity column; HWt – Higher molecular weight protein band)

Based on measurements by Lowry's assay, binding SPR experiments were performed using immobilised HLA-A2 with LT77 at an apparent concentration of 100 nM, plus the positive control antibody SN607D8 at 6.25 nM. Figure 6-9 shows lack of specific signal from LT77 and in fact, the persistence of non-specific binding despite the modification of purification protocols. The control monoclonal HLA-A2 specific antibody gave a clean, specific signal as expected (Figure 6-10, C). The non-specific binding is pronounced in reference channel (figure 6-10, B). A repeat experiment using higher concentrations of this LT77 sample increased the non-specific binding signal.

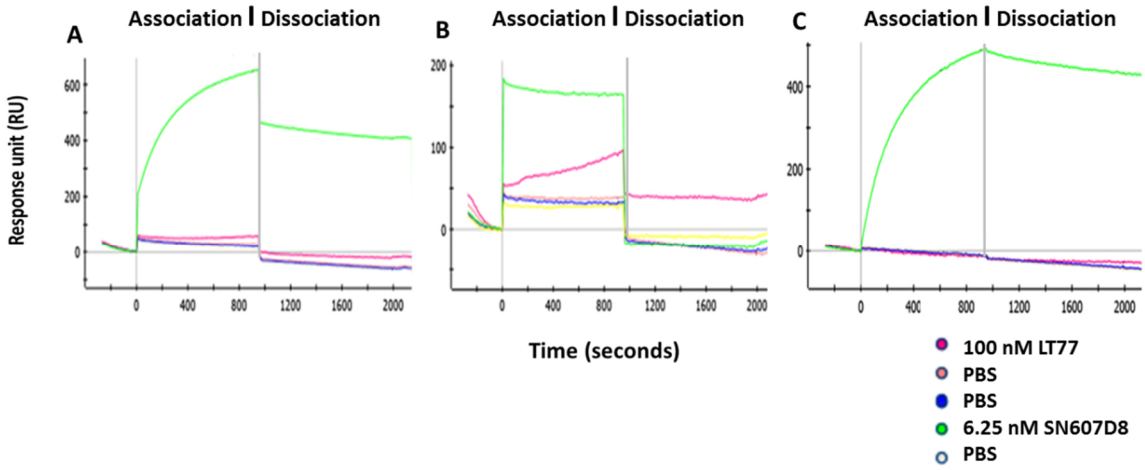


Figure 6-9 : Binding experiments of case LT77 plasma effluent enriched by sequential precipitation and affinity chromatography. High density ligand lane (A) & Blank reference lane (B) and signal on high density lane after reference subtraction (C).

Case LT77	HLA-A2	HLA-B57	HLA-A69
Plasma effluent	5948	3084	2813
Post Sequential precipitation	5057	2456	1460
Post Affinity Chromatography	16158	17496	1861
POST SEC	10703	10647	612

Table 6-1 : MFI values on plasma effluent from case LT77 processed using various enrichments

6.3.2.3 *Sequential precipitation followed by HLA-protein affinity and size exclusion chromatography*

The above enriched sample from LT77 plasma effluent was analysed using mass spectrometry to identify molecules that could be targeted for removal in advanced purification processes. Bands from SDS PAGE gels of LT77 were analysed using tandem mass spectrometry at the in-house Facility at Warwick. This successfully identified a panel of proteins summarised in Figure 6-11. No proteins were identified with confidence from band 1 when peptide data were used to search stock proteome databases such as MASCOT – probably due to profound post-translational modification and sub-optimal tryptic digestion. The mass spectrometry analysis did however confirm the enrichment of IgG via our protocol but it also showed the presence of albumin, IgM, IgA, a species called C9JA05 and CD5-like antigen.

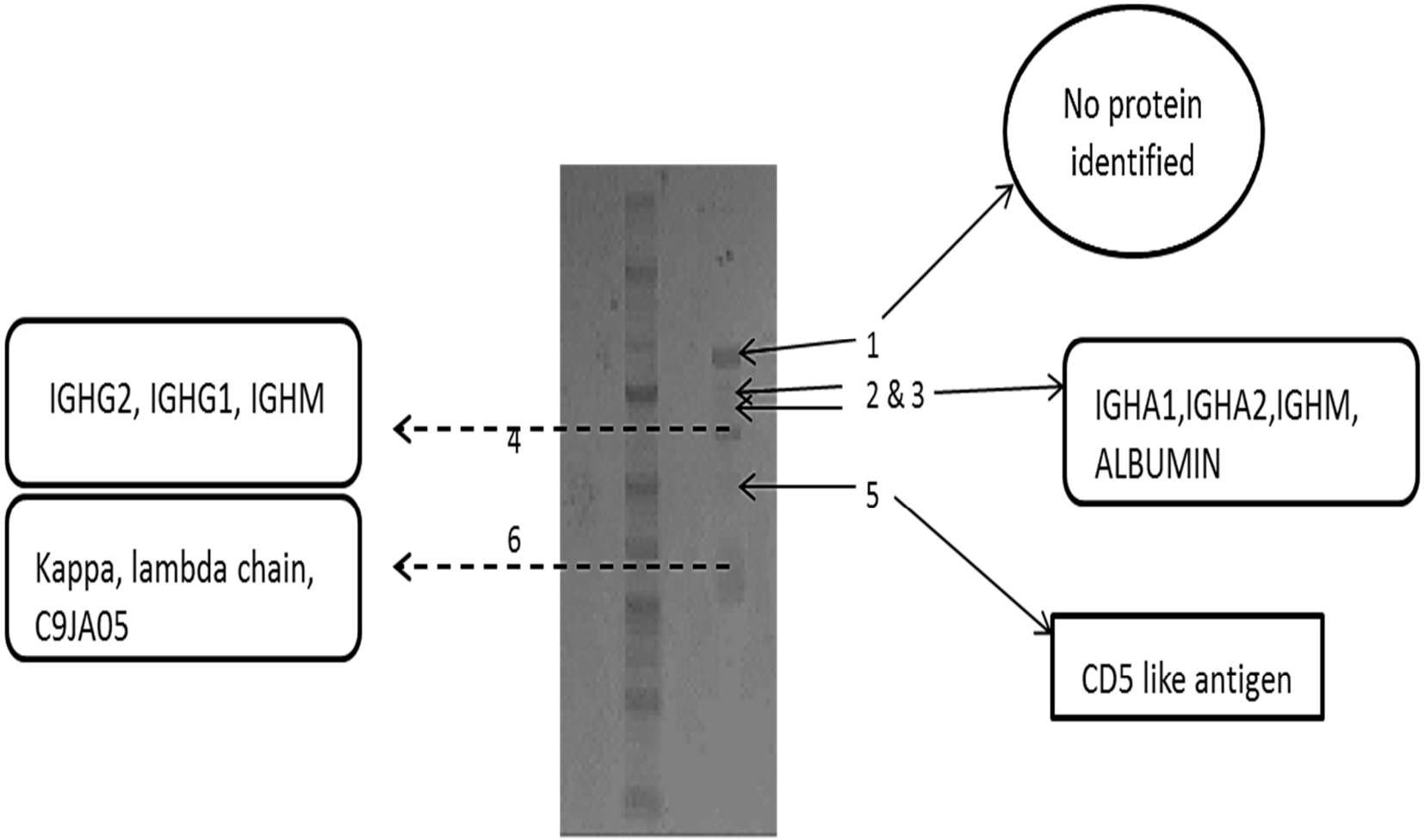


Figure 6-10 : Mass spectrometry analysis of processed sample from LT77 plasma effluent

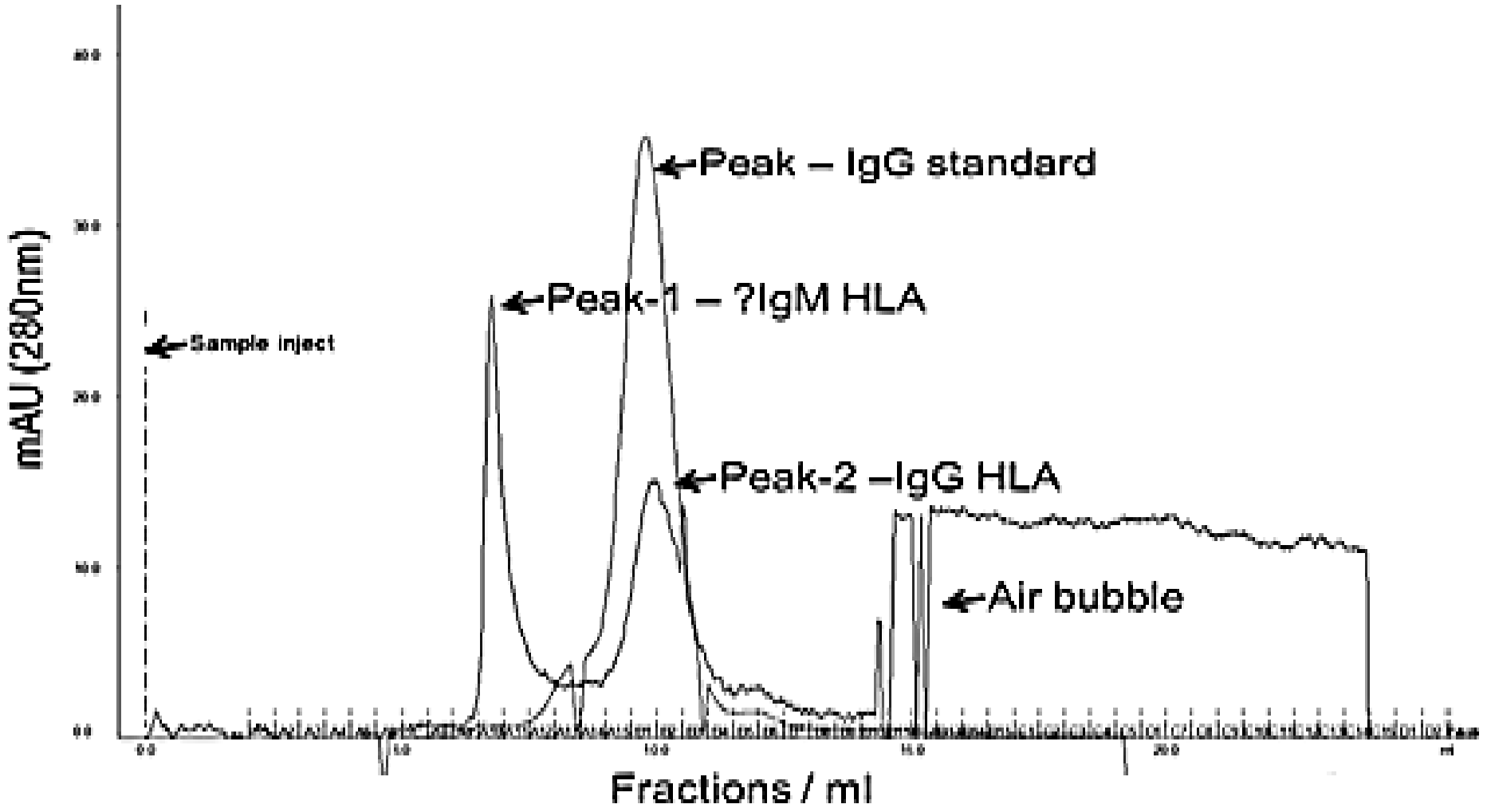


Figure 6-11 : Chromatogram following size exclusion chromatography of processed LT77 sample with an overlap of chromatogram from polyclonal IgG standard experiment.

Due to the clear presence of other impurities, size exclusion chromatography (SEC) was incorporated as a further purification step. This further separated proteins into two distinct peaks as seen in Figure 6-11. A principle of SEC is that larger molecules emerge early in the elution profile such that Peak 1 would contain high molecular weight proteins - very likely IgM. Peak 2 overlaps very well with the pre-run standard IgG peak, strongly suggesting that IgG-class antibodies from the clinical case are eluting at this point. SDS-PAGE analysis of SEC fractions supported separation of high molecular weight bands (Figure 6-12, Lane 2) from IgG heavy chain (Figure 6-12, Lane 3) compared to pre-SEC sample (Figure 6-12, Lane 1).

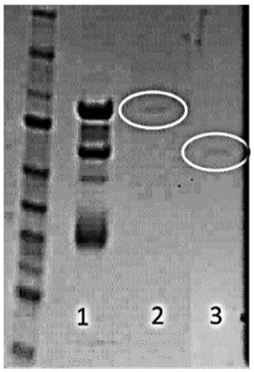


Figure 6-12 : SDS PAGE showing bands on pre (Lane 1) and peak fractions (Lane 2 – Peak 1; Lane 3 – peak 2)

The fraction corresponding to the IgG peak was quantified using Lowry's assay and there was substantial reduction in amount (from 0.38 mg/ml to 0.08mg/ml). However, Luminex MFI values were over 10,000 for HLA-A2 and B57 (see Table 6-1). Using neat fractions corresponding to the IgG SEC peak (estimated to contain 500 nM antibody from Lowry's assay), a discernible binding signal to HLA-A2 was observed and compared with the SN607D8 monoclonal antibody control (6.25 nM), as shown in Figure 6-13. Given the high MFI value of the this material, the apparently poor comparative binding response suggested that in spite

of using the HLA-protein affinity steps, only a small proportion of the purified IgG recovered was truly HLA-specific. However, the overall quality of the binding curve and its mirroring of the monoclonal antibody signal showed more reassuring evidence that SEC was capable of separating out the offending impurities responsible for a lot of the previous signal noise and high non-specific binding responses.

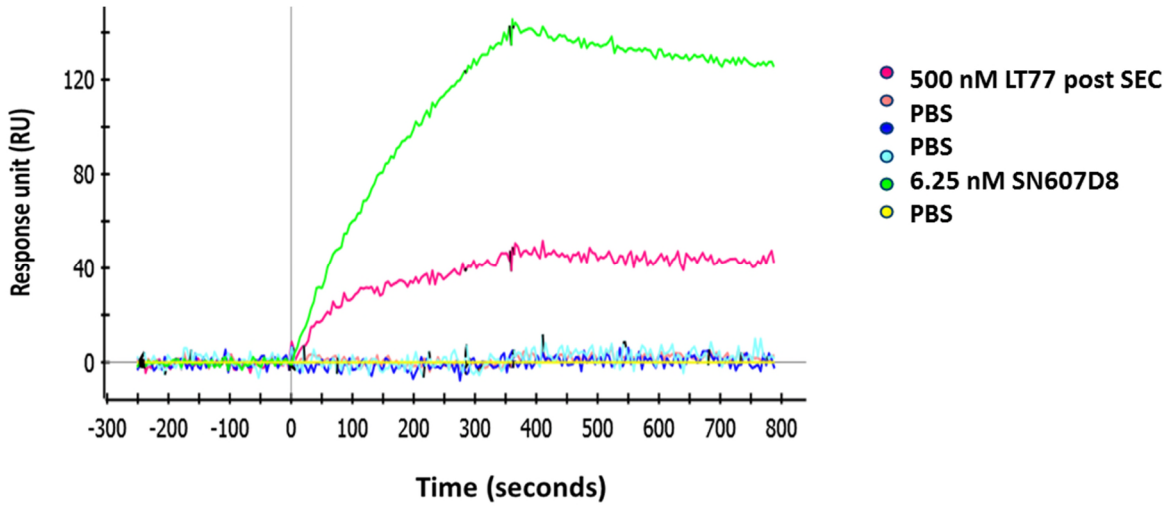


Figure 6-13 : Binding curves from interactions of processed LT77 and HLA-A2 protein

6.3.2.4 *Serial affinity and size exclusion chromatography*

Alternative affinity chromatography strategies using HLA-A2-Sepharose or Protein G-Sepharose with downstream SEC, were tested for a selection of patient cases.

1.1.1.1.1 Serial HLA-affinity and size exclusion chromatography:

Plasma effluents (400 ml volumes) from three cases (LT33, LT68 & LT79) were used. Following 1:1 dilution with PBS to reduce viscosity, samples were applied to the 60 ml of HLA-A2, eluted as described in Chapter 2.

Following sample concentration and exchange into PBS using viva spin concentrators, second runs of HLA-A2 affinity chromatography were performed using the 4 ml column. The protein eluted was again concentrated down to a 1 ml volume and further purified via SEC at a flow rate of 0.5 ml/ml. Fraction 14F, eluting at 13 ml/26 mins (Figure 6-14), consistently corresponded to IgG (as determined via commercial standard) and was retained for analysis for each patient case.

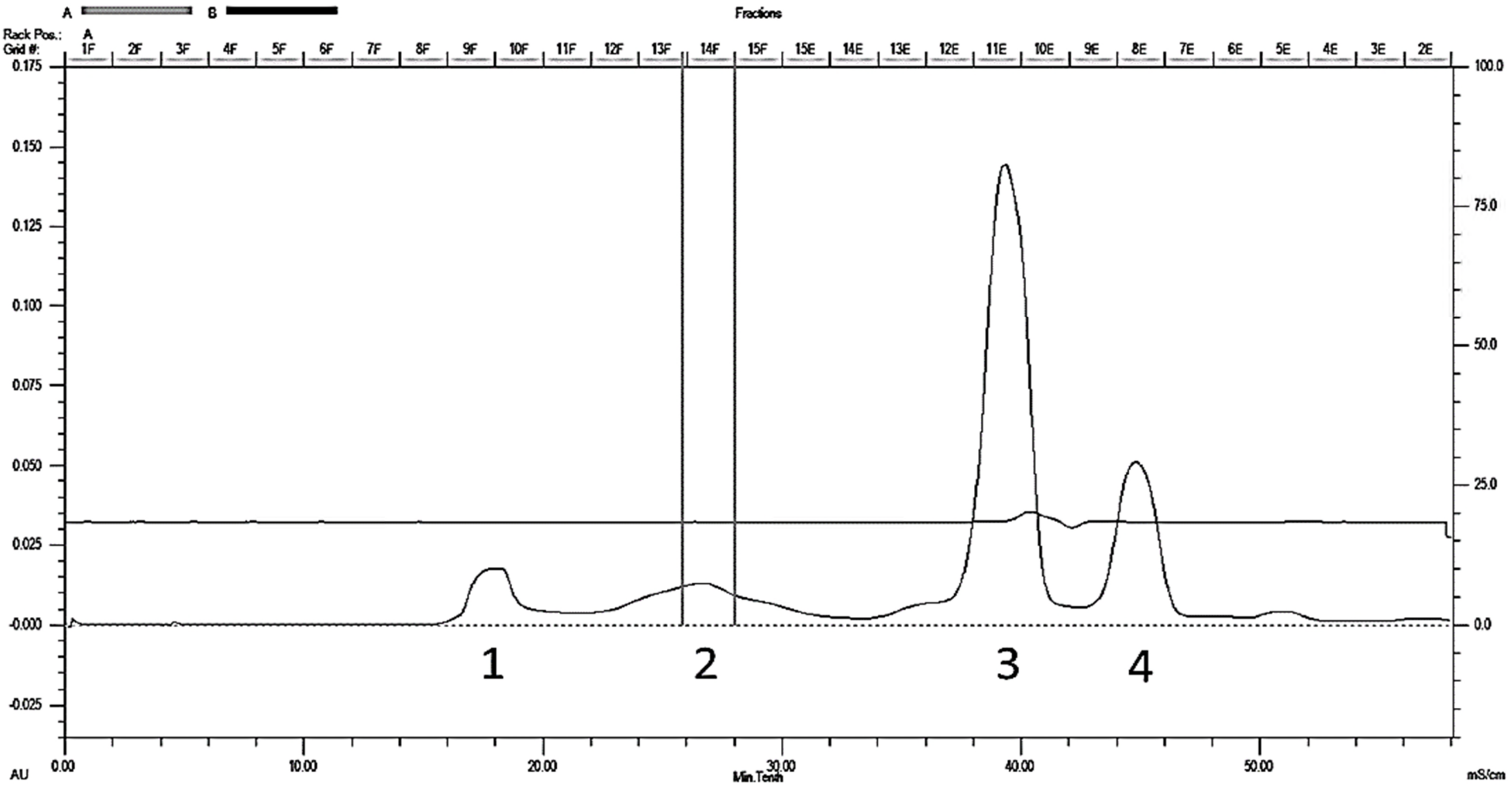


Figure 6-14 : Size exclusion chromatography of processed polyclonal samples showing peaks corresponding to large molecular weight proteins (1), IgG (2) and low molecular weight proteins (3-4).

Case LT33:

Case LT33 and concentrated IgG HLA-specific antibodies as seen in MFI values indicated in Table 6-2. The concentration of protein from Fraction 14F, measured by Lowry's assay, was 0.1 mg/ml (compared to pre-SEC concentration of 0.54 mg/ml). This processed LT33 material was studied via SPR (concentration range 0.03 to 1.2 nM measured by quantitative ELISA) and gave dramatically improved signal for interactions with HLA-A2 and HLA-B57 (as seen Figure 6-15) from which meaningful, apparent kinetic measurements could be derived.

LT33	HLA-A2	HLA-B57	HLA-A69
PI effluent	6626	3800	6507
Post 60 ml AC	4782	2488	3904
POST SEC	23215	21868	2700

Table 6-2 : MFI values measured by SAB assay on samples during enrichment of IgG in case LT33

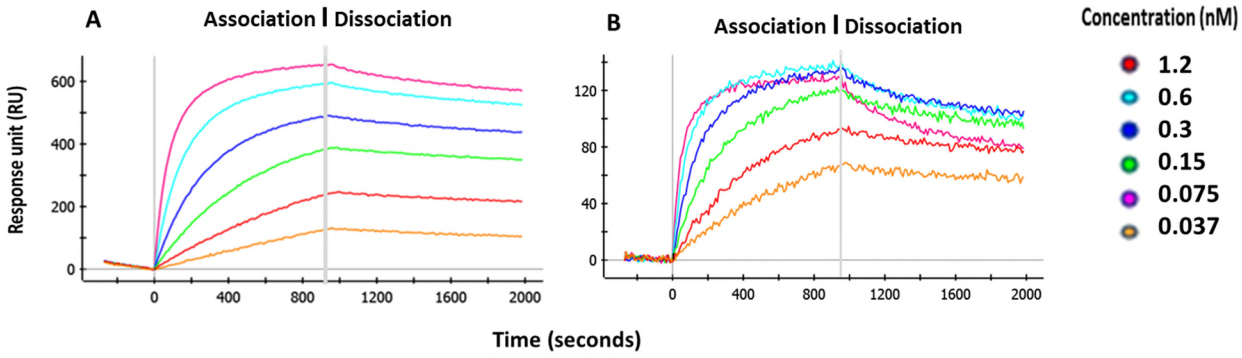


Figure 6-15: Binding sensorgrams of LT33 polyclonal antibodies interacting with HLA-A2 (A) and HLA-B57 (B)

The binding data were calculated for interactions with HLA-A2 using equilibrium analysis (Figure 6-16), yielding an apparent dissociation constant K_D value of 0.267 nM. The quality of HLA-B57 data was questionable and not considered accurate.

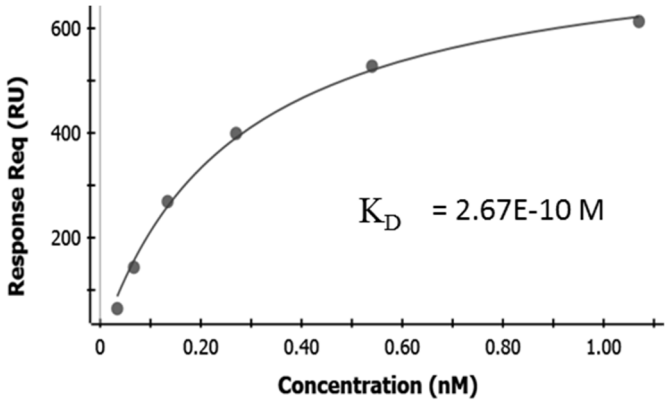


Figure 6-16: Equilibrium analysis of binding data for LT33 polyclonal antibody interactions with HLA-A2)

Case LT79:

Following polyclonal antibody purification, MFI values were measured, as seen in Table 6-3. Lowry's assay indicated a protein concentration of 1 mg/ml. The LT79 antibodies (concentration range 1.125 to 36 nM measured by quantitative ELISA) gave a clean and impressive SPR signal for interactions with both HLA-A2 and HLA-B57 (Figure 6-17).

LT79	HLA-A2	HLA-B57	HLA-A69
Pl effluent	15824	5627	11435
Post 60 ml AC	14914	4311	9055
PRE SEC	21516	18964	17621
POST SEC	23289	21277	20906

Table 6-3 : MFI values from Luminex SAB during purification process of case LT79 plasma effluent

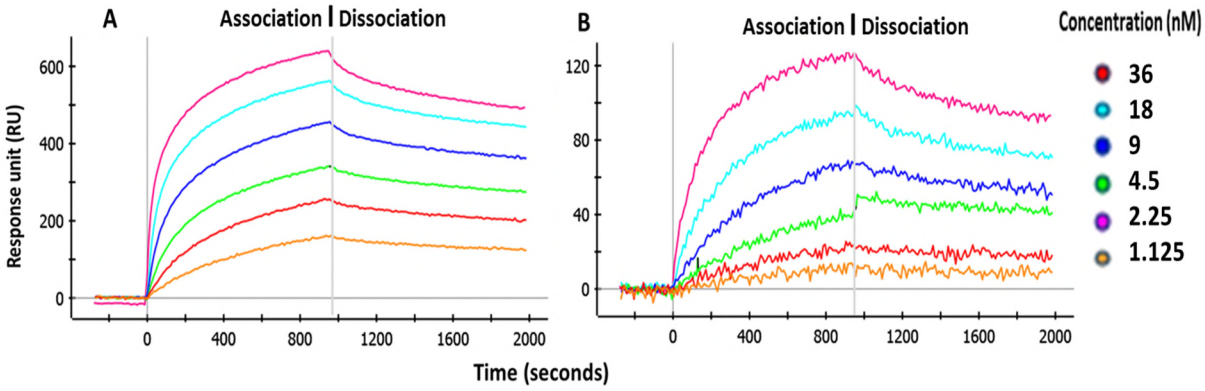


Figure 6-17 : Binding interactions between polyclonal antibodies from case LT79 and HLA-A2 (A) and HLA-B57 (B)

Equilibrium analysis is indicated in Figure 6-18 and early indications suggest varying affinity values between interactions measured simultaneously for the same polyclonal antibody preparation for different HLA proteins.

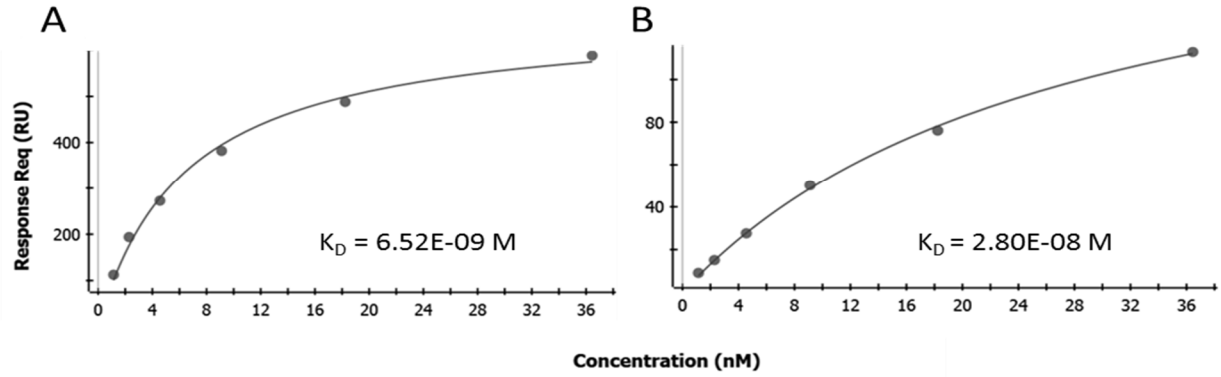


Figure 6-18 : Equilibrium analysis of binding data from interaction between polyclonal antibodies from case LT79 and HLA-A2 (A) and HLA-B57 (B).

Case LT68

Polyclonal antibodies from case LT68 gave distinct binding signal, however compared to LT33 and LT79 the maximal response was lower and was keeping with lower concentration measured by quantitative ELISA (range 0.26 to 0.016 nM) (Figure 6-19). The MFI values pre and post SEC are given in Table 6-4. As only few points were in the equilibrium levels, the dissociation constant was not estimated.

LT68	HLA-A2	HLA-B57	HLA-A69
PRE SEC	20431	20105	2673
POST SEC (1:2)	9396	8848	200

Table 6-4 : MFI values measured using Luminex SAB pre and post SEC on enriched plasma effluent from case LT68

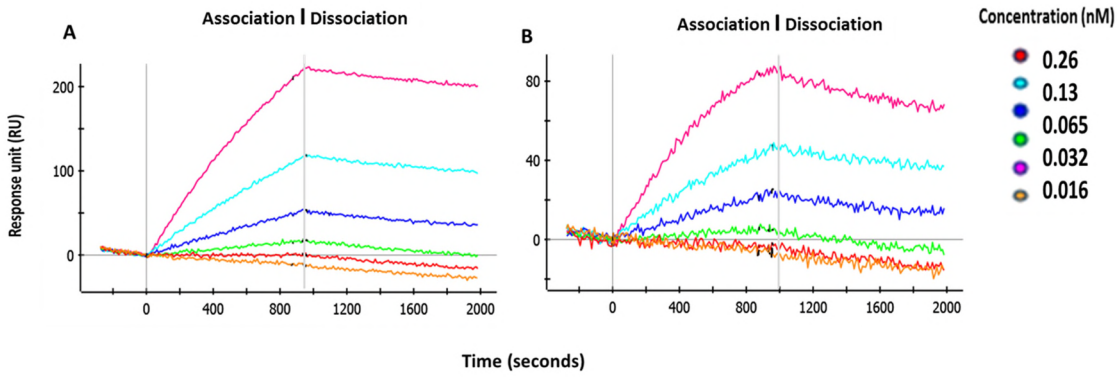


Figure 6-19 : Binding interactions between polyclonal antibodies from case LT68 and HLA-A2 (A) and HLA-B57 (B)

Thus a protocol based on serial HLA-protein affinity chromatography followed by size exclusion chromatography allowed for the purification of polyclonal IgG HLA-specific antibodies and removal of materials giving non-specific binding signals on SPR.

1.1.1.1.2 HLA-protein followed by protein-G affinity chromatography prior to size exclusion chromatography

Substantial loss of IgG between the two runs of HLA-affinity chromatography runs was noted. Therefore, use of Protein G-Sepharose was considered as an intermediate purification step. Plasma effluents from three cases (LT45, LT66 and LT68) were processed through HLA-A2 affinity chromatography (see section 6.3.1.1) and the eluted proteins were concentrated prior to running through a Protein G-Sepharose (4 ml) column as described in Chapter 2. This was followed by SEC as described above.

The Protein G-Sepharose column used for these experiments had previously been used for the enrichment of human monoclonal antibody WK1D12 (Chapter 4) and given the considerable expense of Protein G-Sepharose resin, it is customary in many laboratories to re-use resin from which antibodies have been ostensibly eluted. Despite prolonged re-generation after the particular previous use, WK1D12 remained attached which resulted in contamination of polyclonal antibody preparations from this set of experiments (as seen in Figure 6-20, B). However, it also gave specific binding signals to HLA-A2 which would not be derived from the contaminating WK1D12 monoclonal antibody (Figure 6-20, A). This interesting set of findings highlights the sensitivity of the SPR system and its ability to detect antibodies even as contaminants and also the vulnerability of established antibody purification techniques such as Protein G-Sepharose affinity chromatography.

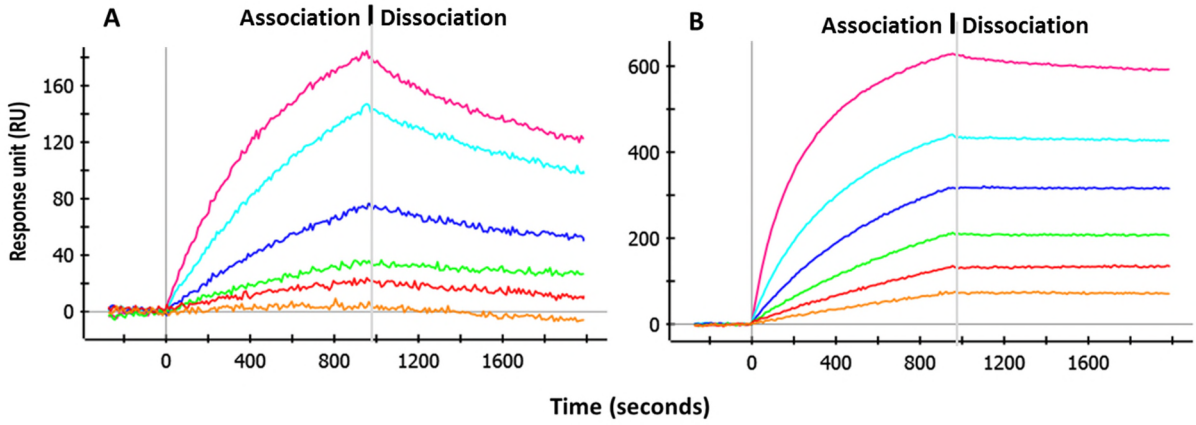


Figure 6-20 : Binding interactions between LT45 polyclonal antibodies to HLA-A2 (A) and contamination from WK1D12 to HLA-B27 proteins (B), measured simultaneously. Curves represent diluting concentration of purified antibodies.

Thus although the results obtained here are in some ways similar to serial HLA-protein affinity chromatography, compromised regeneration of Protein G-Sepharose columns meant that these could not be used repeatedly. Due to significant cost issues associated with this approach, it was not used for subsequent experiments. HLA-protein chromatography is more amenable to regeneration but the efficiency differs between effluent samples. Plasma effluent from LT45 gave two elution profiles when tested on Luminex (Figure 6-21). Thus this is dependent on epitope and as well as affinity of the antibodies. Additionally there was concern that low affinity antibodies in a polyclonal clinical sample may not bind as effectively to HLA-Sepharose and that the enrichment process would be biased for high affinity antibodies. Therefore, for subsequent experiments, affinity chromatography as an intermediate step was omitted and only sequential precipitation followed by size exclusion chromatography was considered.

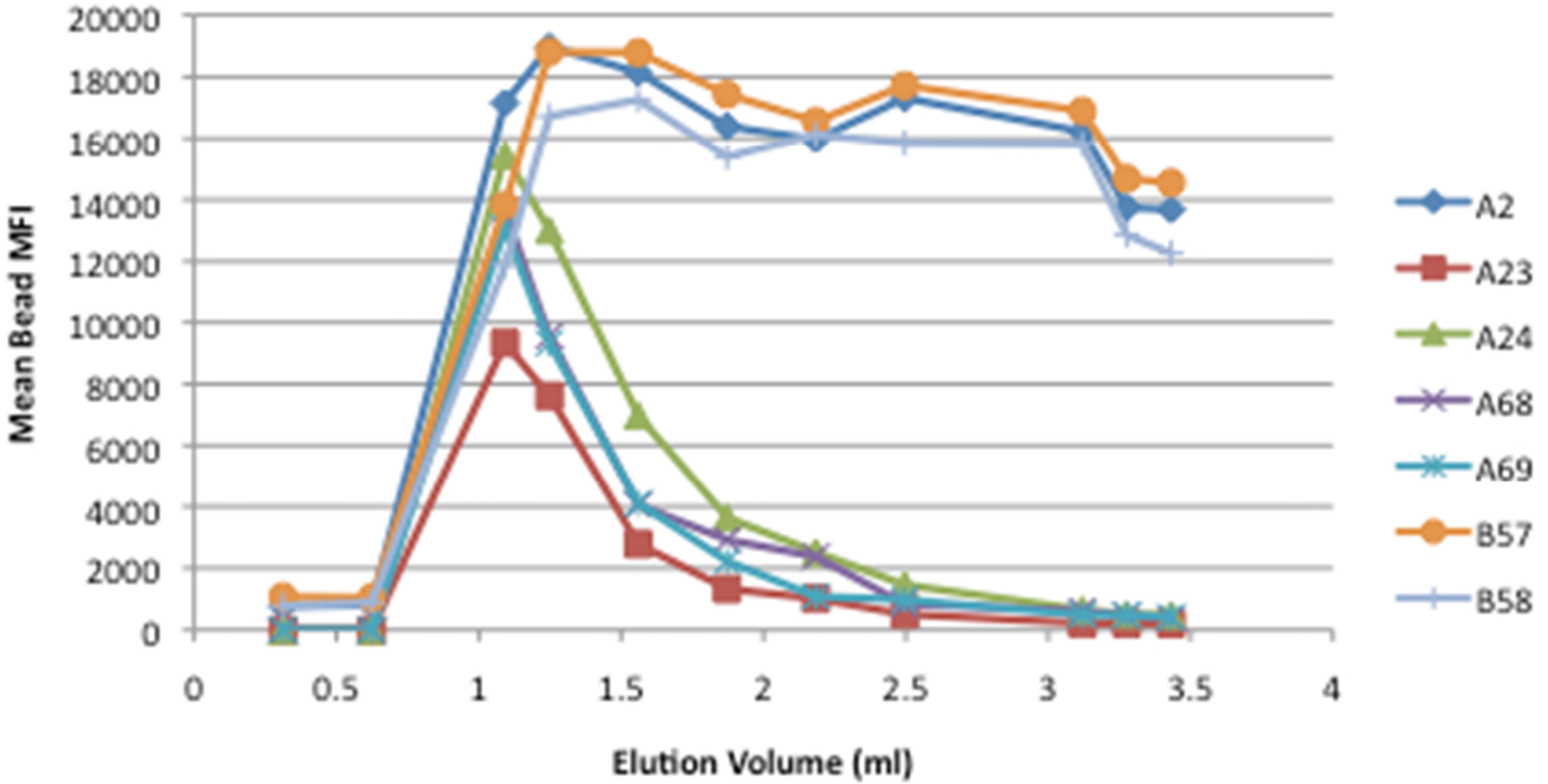


Figure 6-21 : Different elution profiles following HLA-A2 affinity chromatography of polyclonal antibodies from case LT45

6.3.3 Enrichment/purification protocols for serum samples

A major aim of this Project was to develop assays for the study of antibody binding kinetics using amenable clinical samples such as serum obtained via phlebotomy. Based on experiences from enrichment protocols of large volumes of plasma effluent described above, two protocols for polyclonal antibody recovery from clinical serum samples were attempted.

6.3.3.1 *Sequential precipitation and HLA-protein chromatography followed by size exclusion chromatography*

Six serum samples (20 - 30 ml each) from NHSBT, Birmingham were obtained. These were typing sera from multi-parous women and were known to have high CDC titre for HLA-A2 and HLA-B7 specificities.

Serum sample volume over 20 ml:

Serum sample B518

Serum from case B518 (20 ml), as distinct from plasma effluent, was processed using sequential precipitation and HLA-A2 affinity column (4 ml) followed by SEC as described above. The MFI values for HLA-A2, HLA-A69 and HLA-B57 specificities were 23207, 21967 and 13687 respectively. Concentration measured using quantitative ELISA was 3.2 nM. The binding interactions observed are shown in Figure 6-22. The Rmax values were highest for interactions with HLA-A2 protein followed by HLA-A69 and HLA-B57 and follow the trend with MFI values. HLA-specific antibody quantification was carried out using ELISA as described below in section 6.2.3. Equilibrium analysis is indicated in Figure 6-23.

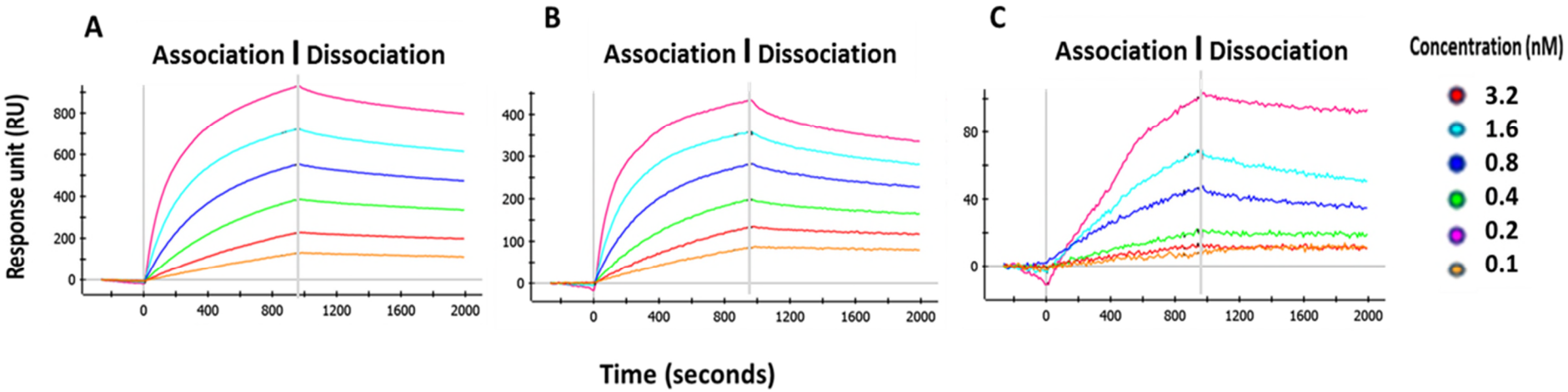


Figure 6-22 : Binding curves of interactions between polyclonal antibodies from case B518 and HLA-A2 (A), HLA-A69 (B) and HLA-B57 (C)

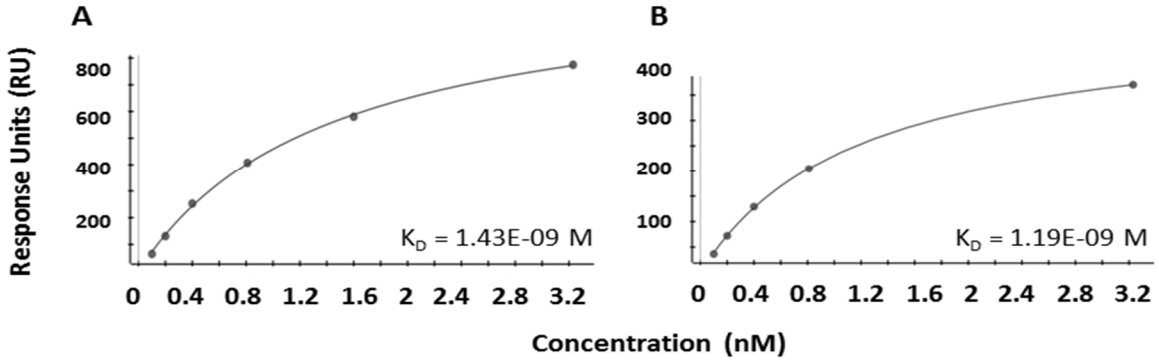


Figure 6-23 : Equilibrium analysis of binding data from interaction between polyclonal antibodies from case B518 and HLA-A2 (A) and HLA-A69 (B)

Serum sample B2007

Serum from case B2007 was processed as above, using sequential precipitation and HLA-B7 affinity column (4 ml) to enrich the HLA-B7-specific polyclonal antibodies (as opposed to HLA-A2 used previously), followed by SEC. The MFI value was 22778 and binding interactions with immobilised HLA-B7 via SPR are shown in Figure 6-24. The concentration measured by quantitative ELISA was 7 nM. The dissociation constant measured using equilibrium analysis is shown in Figure 6-25.

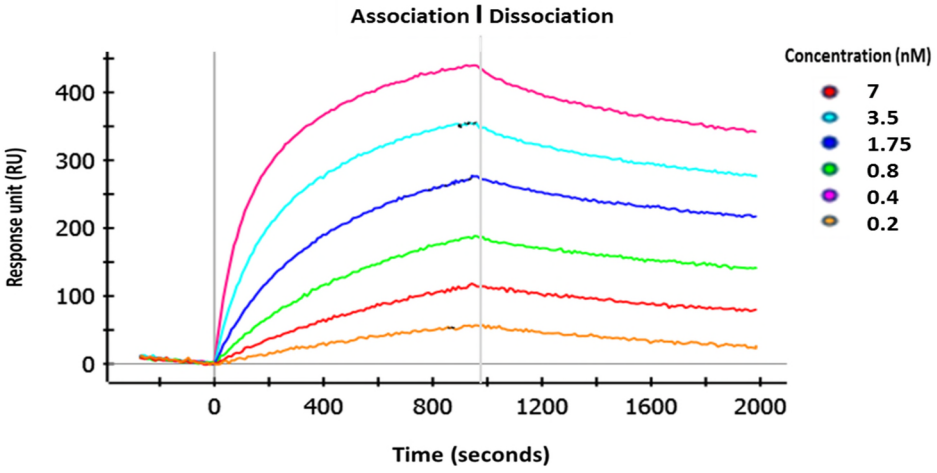


Figure 6-24 : Binding interactions between polyclonal antibodies from case B2007 and HLA-B7 protein

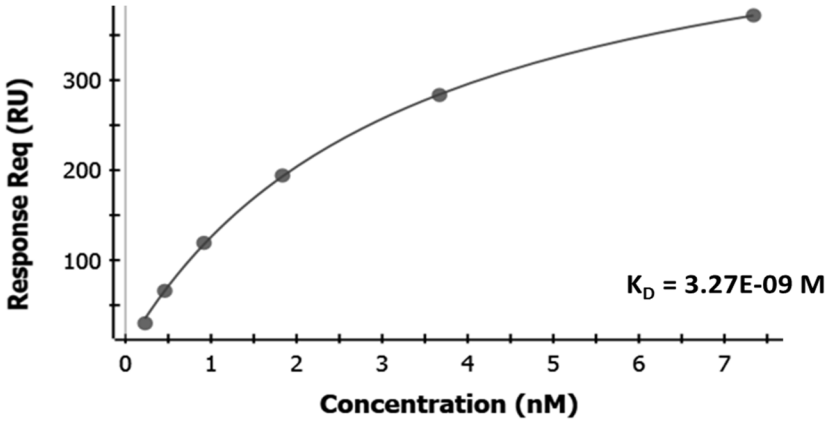


Figure 6-25 : Equilibrium analysis of binding data from interaction between polyclonal antibodies from case B2007 and HLA-B7

Serum sample volume of 5 ml

Serum sample B572

Serum from case B572 was processed as a 5 ml sample using HLA-A2-Sepharose. MFI values measured by Luminex SAB and were for HLA-A2, HLA-A24, HLA-A69 and HLA-B57 were 20639, 4956, 8139 and 16814 respectively. In spite of a lower volume of starting material, good-quality binding interactions were still obtained with a discernable range of specificities for a HLA-protein panel as shown in Figure 6-26. Non-specific binding artefacts were also low. The dissociation constant calculated for interaction with HLA-A2 was 1.06E-07 M. For other interactions the binding response gave only few data point to calculate dissociation constant using equilibrium analysis.

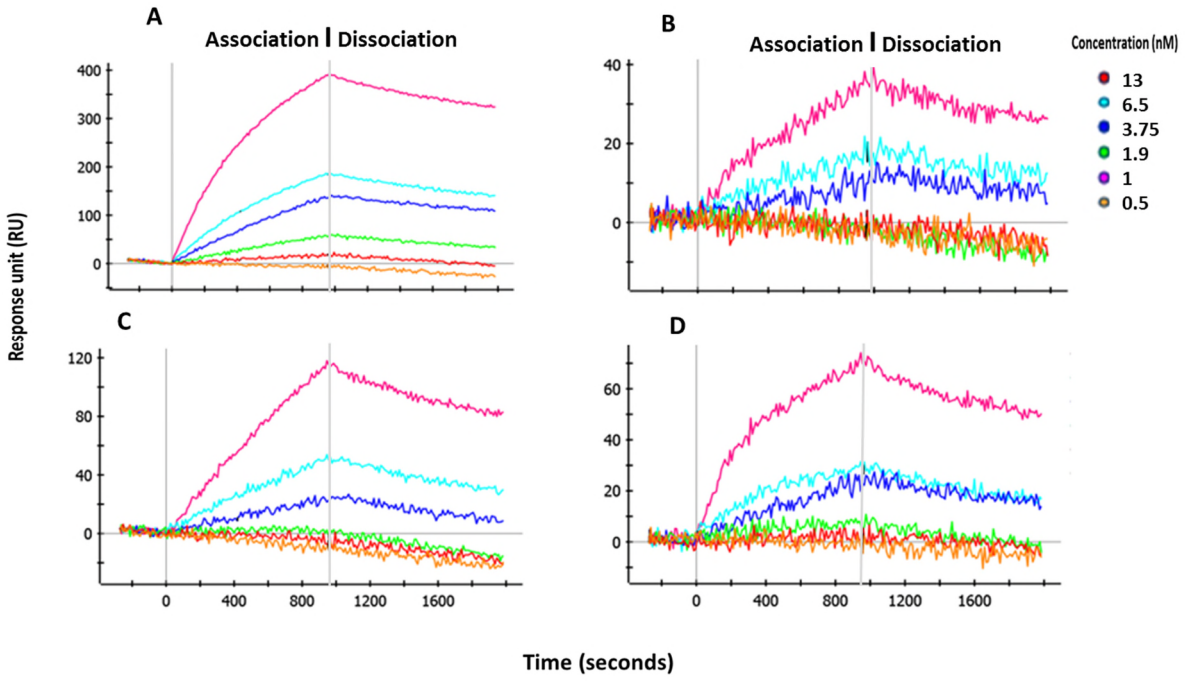


Figure 6-26 : Binding curves from interactions of B572 and HLA-A2 (A), HLA-A24 (B), HLA-A69 (C) and HLA-B57 (D)

6.3.3.2 *Sequential precipitation and size exclusion chromatography*

Following the promising results from using 5 ml serum, the next step was to consider further refinement to allow testing of antibodies recovered from 2 ml of serum. To achieve maximum polyclonal antibody recovery and quantification, the protocol was simplified to a two-step process that consisting of IgG precipitation with octanoic acid then ammonium sulphate, followed by SEC. Affinity chromatography steps were removed so as to reduce the considerable losses of IgG seen with the multi-step protocols, and also provide a more cost-effective protocol for the preparation of meaningful polyclonal antibody samples suitable for SPR analysis.

Two sera specific for HLA-B7 (cases B1170 & B1175) were used and the MFI values for B1170 were 23169, 18128 and 19200 against HLA specifities-B7, B40:01 and B40:02 respectively; and B1175, the values were 21958, 16234 and 16566 against HLA-specifities-B7, B40:01 and B40:02 respectively. The binding kinetics are shown in the Figure 6-27 and Figure 6-28 respectively.

These experiments demonstrated that clean and specific binding signals can be obtained using this simplified protocol. The challenge ahead would consist of reliably quantifying the HLA-specific antibodies within the enriched IgG pool eluting from the SEC column. Interestingly both the above cases used were known to be highly CDC positive (personal communication from NHSBT, Birmingham).

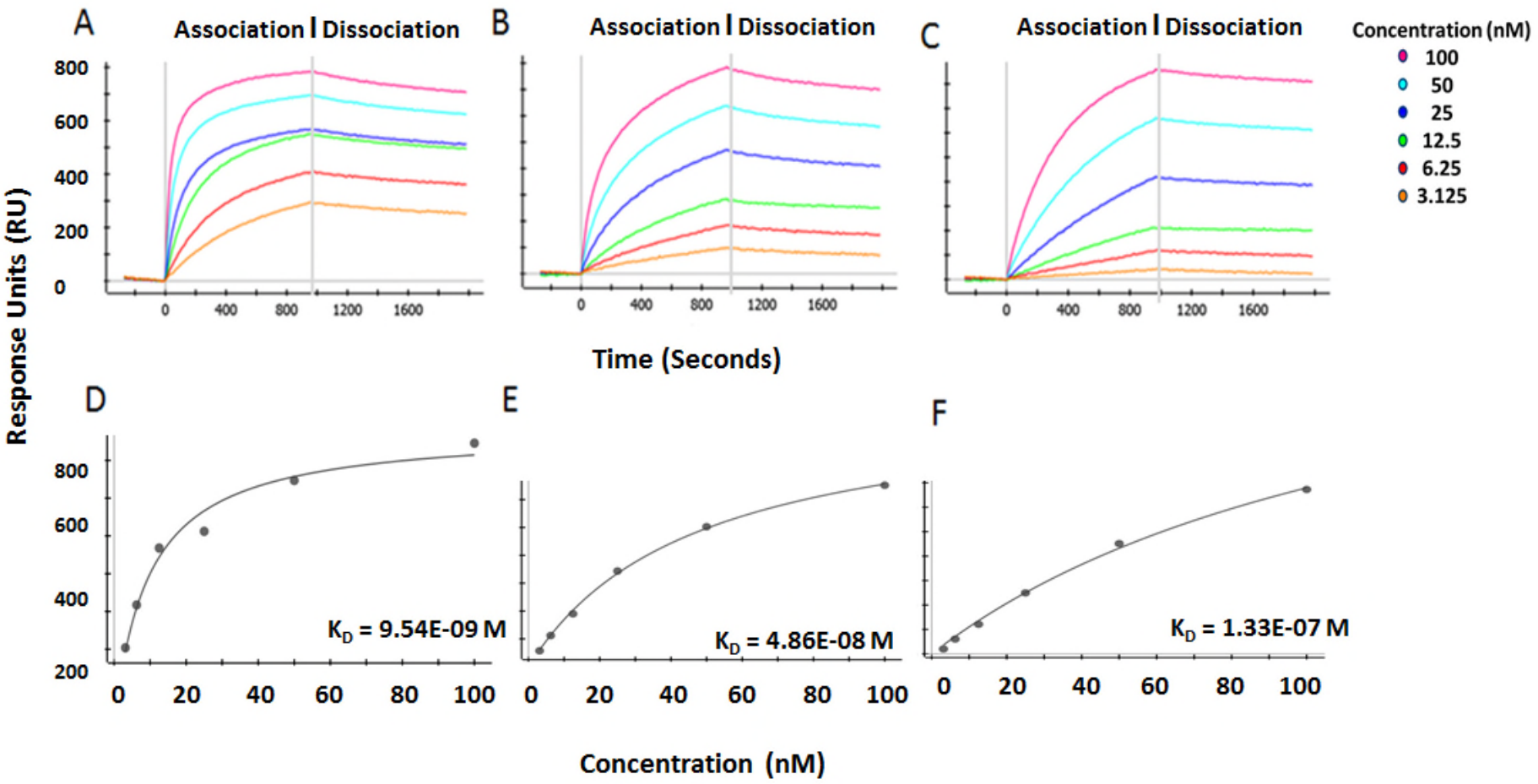


Figure 6-27 : Binding kinetics of interactions of polyclonal antibodies B1170 with HLA-B7 (A), HLA-B40:01 (B), HLA-B40:02 (C) and corresponding dissociation constant in D, E & F respectively.

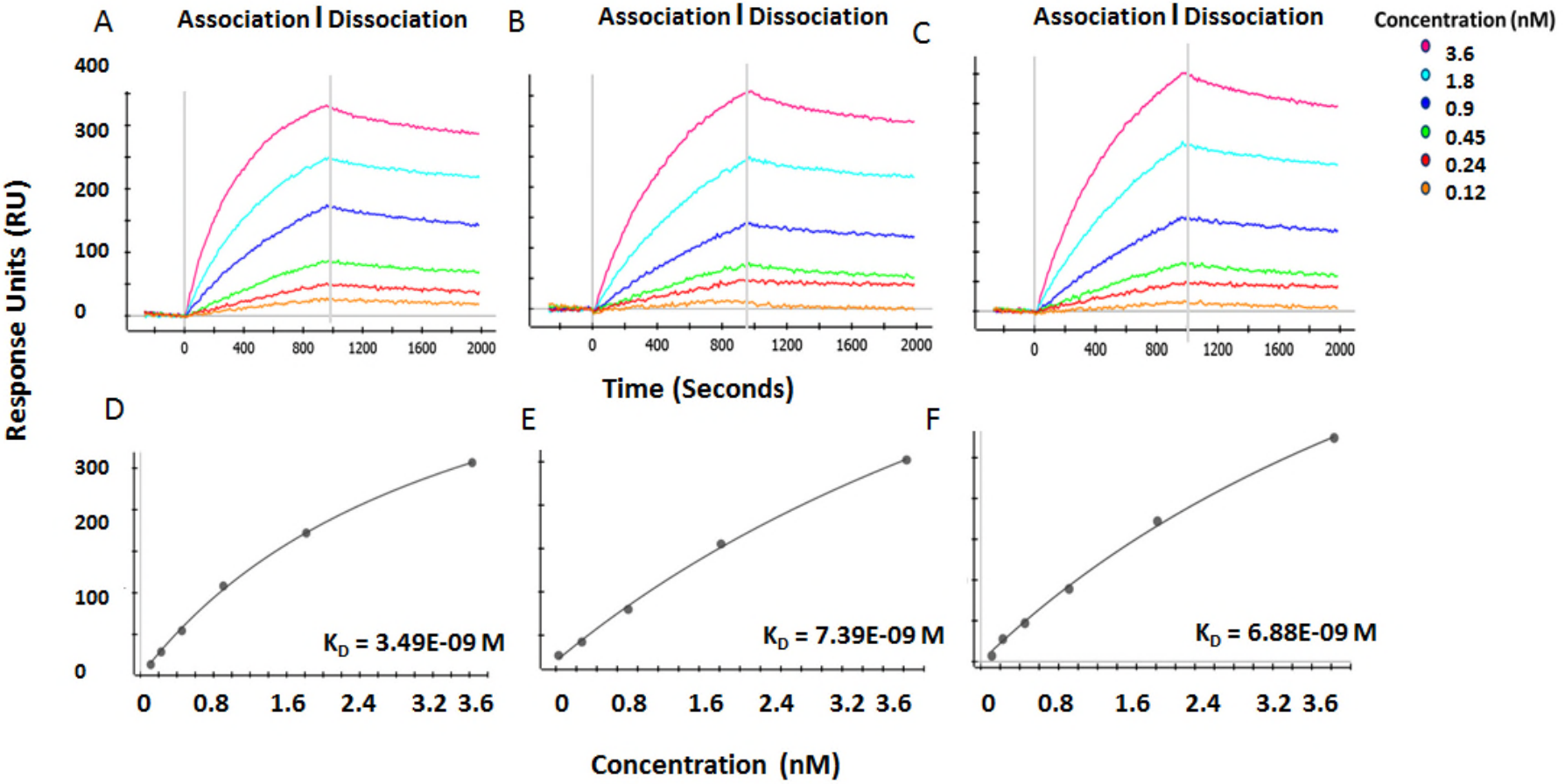


Figure 6-28 : Binding kinetics of interactions of polyclonal antibodies B1175 with HLA-B7 (A), HLA-B40:01 (B), HLA-B40:02 (C) and corresponding dissociation constant in D, E & F respectively.

6.4 Quantification of HLA-specific antibodies

Current assays do not quantify HLA-specific antibodies as a function of mass, from which specific molar concentrations can be derived. This presents an impediment to binding analysis via SPR, which requires knowledge of analyte molarity in order to generate rationalised, quantitative data sets. Popular current assays such as Luminex SAB are semi-quantitative and assays such as complement dependent cytotoxicity (CDC) yield important functional information but do not express quantities that can be directly translated to SPR experiments. Details of these assays are described in Chapter 2.

The development of quantitative assays for circulating HLA-specific antibodies is limited by the diversity and heterogeneity of the HLA system. In clinical practice, quantitative ELISAs are available for measuring serum antibody levels in conditions such as anti-GBM disease and ANCA-associated vasculitis. For both of these conditions, international calibrating standards have been agreed upon and are used in commercial diagnostic ELISAs worldwide. For example, in the ANCA ELISA kit, the standard consists of a sample from a clinical case containing a very high titre of polyclonal antibodies against ANCA autoantigens. In HLA-sensitised patients with polyclonal antibodies directed against multiple epitopes that could be on the same antigen or shared between many antigens, an attempt to develop an ELISA/Luminex standard has not been explored in depth. However, with the availability of several human monoclonal HLA-specific antibodies, early attempts to develop standards for quantitative polyclonal antibody assays have been made herein.

With respect to interaction techniques such as SPR, reliable quantification of HLA-specific antibodies as a function of molar concentration is one of the prerequisites for the calculation of binding kinetics. A highly accurate antibody concentration can only truly be obtained if the sample is pure IgG with no interfering proteins and in sufficient quantity to yield a high quality protein assay result. Various strategies were used to determine HLA-specific antibody concentration as described in Chapter 2. Two broad strategies were approached – 1) direct measurement of purified protein content from large clinical samples, purified using specific HLA-protein affinity chromatography; and 2) measurement of the HLA-specific component of isolated IgG fractions via ELISA using purified HLA proteins and an anti-HLA calibration standard comprised of monoclonal antibodies.

6.4.1 Determination of protein concentration

1.1.1.2 *Nanodrop spectrophotometry*

This assay was used as a rough guide for estimating of protein content during purification protocols. This was typically used for monitoring elution profiles during IgG purification from plasma effluent via affinity chromatography. Figure 6-29 demonstrates gross changes in protein concentration, as determined via A280nm, during various phases of affinity chromatography. There was larger protein content in plasma effluent from the flow through phase, as expected, followed by diminishing protein content during washing fractions. In the elution phase, a relatively small peak of protein appears (typically at fraction 4 in the elution protocol used - corresponding to approximately one column bed volume).

When these elution profiles were analysed via Luminex, a prolonged "tail" of anti-HLA reactivity was seen (Figure 6-30). Surprisingly, the standard 4 x column volume elution length in high pH buffer was not sufficient for complete antibody recovery and regeneration of the HLA affinity column. This "tail" could be due to high affinity and high pH-resistant antibodies that either form very stable complexes with the immobilised HLA under the extreme elution conditions, or require a much longer elution time in order to dissociate from the column. Henceforth, larger elution volumes (20 x column volume) were used.

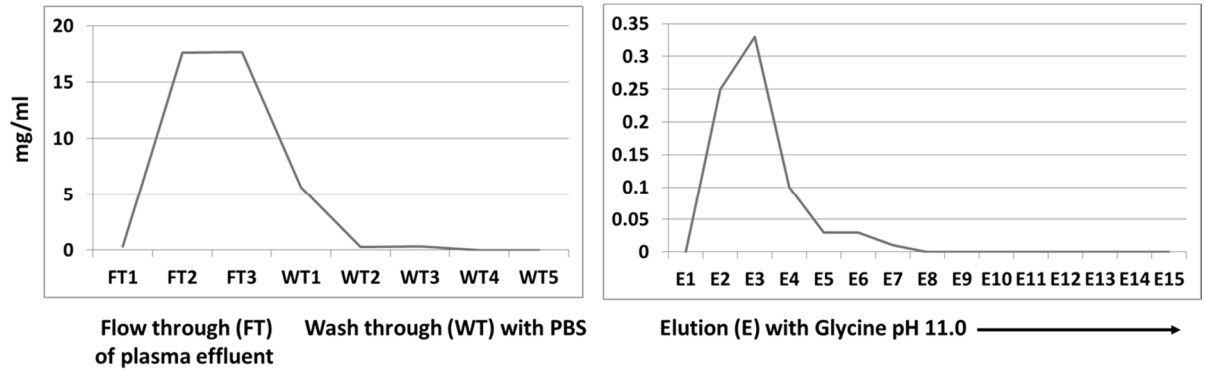


Figure 6-29 : Profile of protein content during different phases of HLA-A2 affinity chromatography using plasma effluent from case LT79.

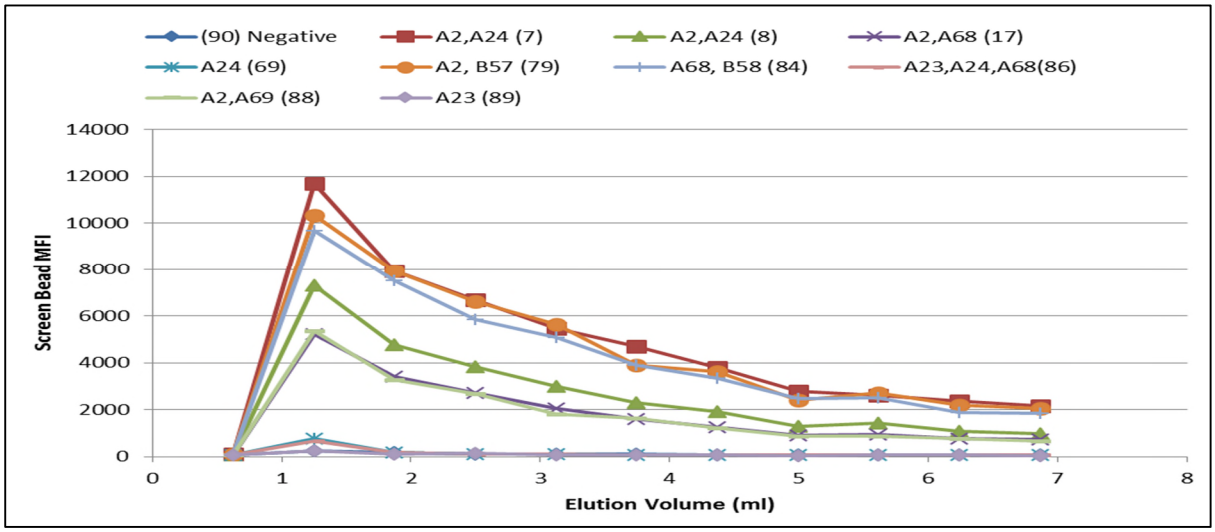


Figure 6-30 : Elution profile of case LT45 using the HLA-A2 affinity column

Hence concentration measurement with Nanodrop is limited by the sensitivity and if used alone can miss the tail end of elution fraction.

6.4.2 Determination of polyclonal HLA-specific antibody concentration

1.1.1.3 *Luminex quantification*

Two different purified monoclonal HLA-A2 specific antibodies of known concentration (determined via Lowry's and BCA protein assay) were used to study the effect of titration on MFI levels using single antigen beads. Despite being used at identical concentrations, there was a 10-fold difference in EC_{50} (Effective concentration that gives 50% of maximal response) between these two antibodies with respect to HLA-A2 (10.4 and 1.3 ug/ml) (Figure 6-31). The EC_{50} concentration value was lower for SN230G6, confirming earlier observations (Chapter 4) that it is a higher affinity antibody compared to SN607D8.

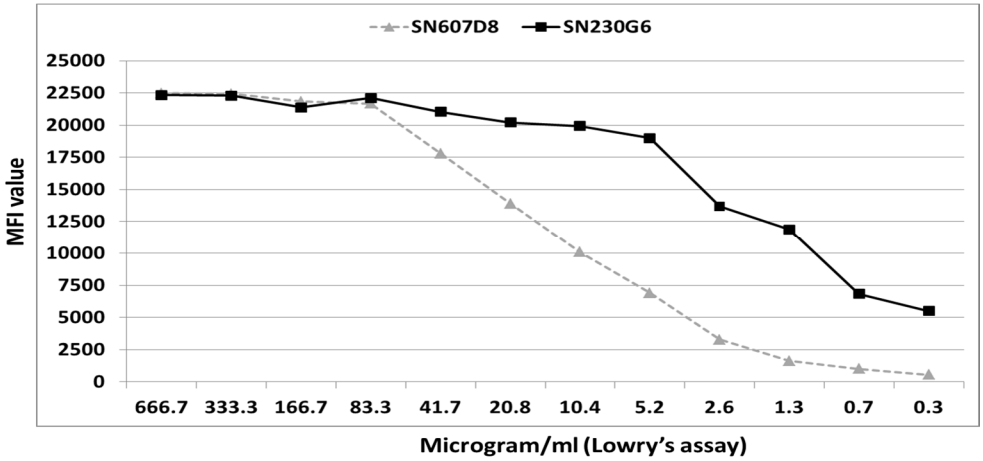


Figure 6-31 : Titration curves showing relationship between sample dilution and MFI values obtained via Luminex.

These data indicated discrepancies in antibody concentration in relation to MFI and given the expense of Luminex assays, optimisation and future use of this platform for explicit determination HLA-specific antibody concentration would not be cost effective. Therefore the ELISA platform was considered as a more practical alternative.

6.4.2.1 *Sandwich ELISA quantification*

Although ELISA and Luminex are similarly end-point immunoassays, the cost benefit and adaptability of ELISA makes it easier to set up and optimise rapidly in the laboratory. The HLA-A2-specific monoclonal antibodies SN607D8 and SN203G6 were tested in triplicate via ELISA using HLA-A2-coated microwell plates to derive the titration curve (Figure 6-32). EC_{50} values were calculated using dose-response curve fitting using PRISM software.

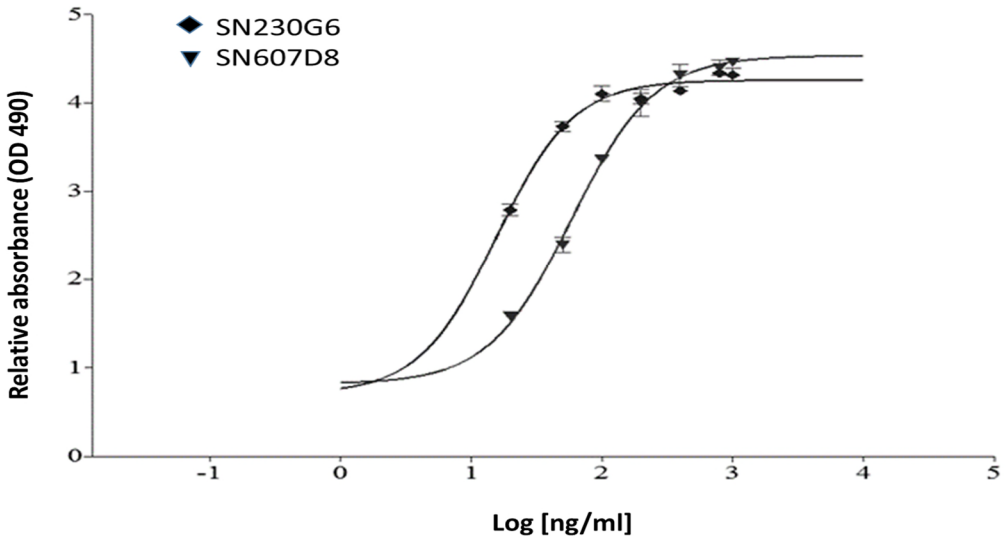


Figure 6-32 : Titration curve showing relationship between concentration and relative absorbance measured via ELISA for HLA-A2 specific monoclonal antibodies. (X-axis values are log conversion of concentration measured in ng/ml).

The ELISA assay also confirmed that the SN230G6 stronger than SN607D8 as EC_{50} for SN230G6 was 3.7-fold lower than SN607D8. EC_{50} values were 15.68 and 58 for SN230G6 and SN607D8 respectively. The stability of the SN607D8 antibody, its good performance in SPR, and its abundance in the laboratory made it the preferred calibration curve standard. For HLA-B7 quantification, the monoclonal antibody WK1D12 was used.

Five polyclonal antibody samples (3 derived from plasma effluent (LT33, LT68 & LT79 cases) and 2 from serum (B518 & B572 cases)) that gave distinct binding to HLA-A2 via SPR were quantified using the HLA-A2-specific sandwich ELISA. These samples were tested as neat fraction, and also as 1:10, 1:100 and 1:1000 dilutions to obtain a titration range. The points in the linear range of the standard curves obtained using SN607D8 were used to derive polyclonal concentrations (see Figure 6-33). The concentrations of the neat polyclonal anti-HLA-A2 preparations were determined to be as follows 8.55 nM for LT33, 36.45 nM for LT79, 3.23 nM for B518, and 1.07nM for samples B572 cases.

Similarly, a HLA-B7-specific sandwich ELISA assay was used to quantify three polyclonal antibody samples as neat post-SEC fractions, and also dilutions of 1:10, 1:100 and 1:1000. The concentrations derived were 7.13 nM for B2007, 71 nM for B1170 and 3.53 nM for B1175 (see Figure 6-34). This sandwich ELISA protocol was therefore able to measure polyclonal antibody concentration in nanomolar range.

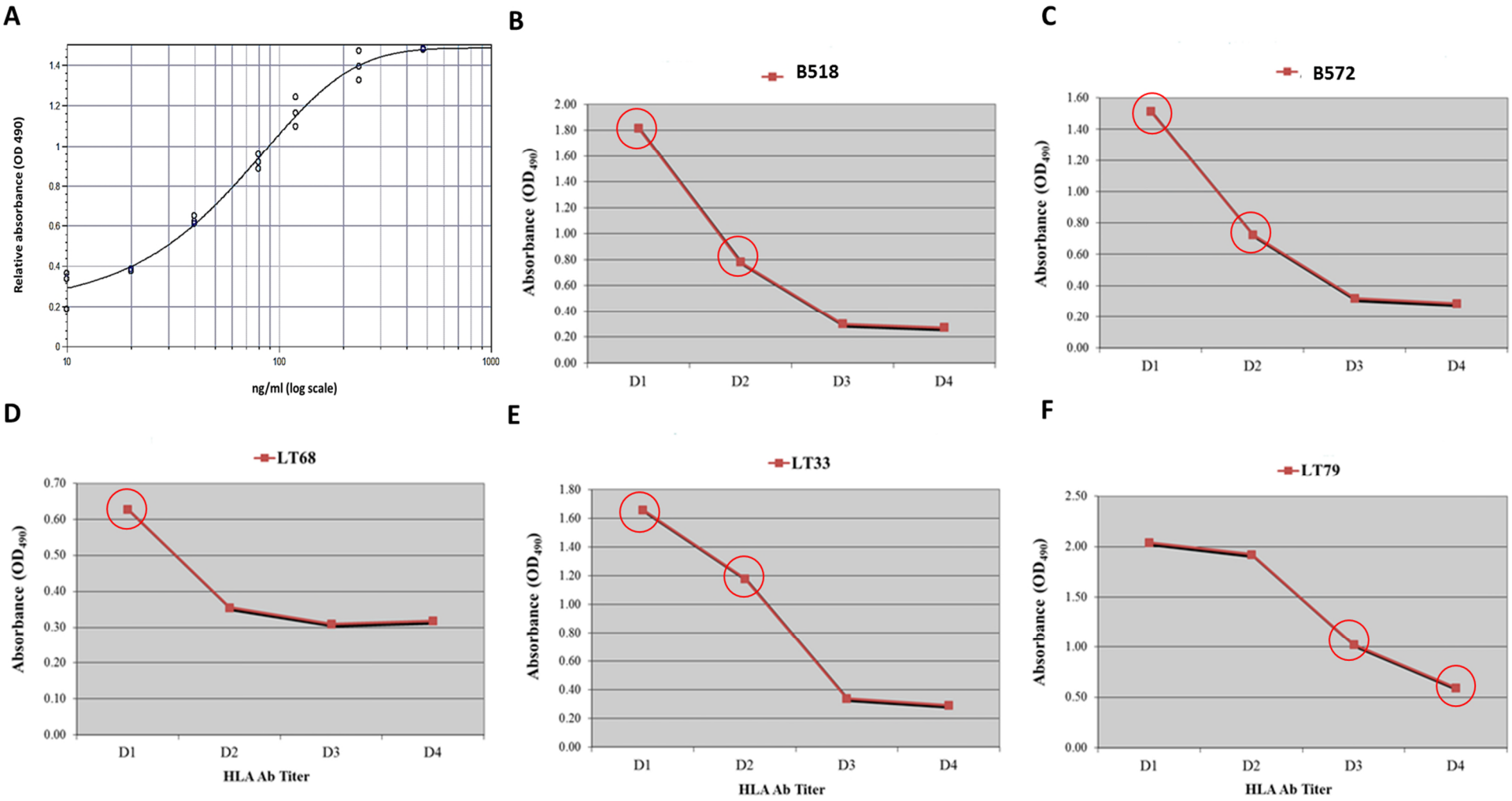


Figure 6-33 : Quantitative HLA-A2 specific ELISA. A – Standard curve using monoclonal antibody SN607D8, B-E – polyclonal HLA-A2 specific antibodies in clinical samples (Red circle indicates values in the linear range of the standard curve)

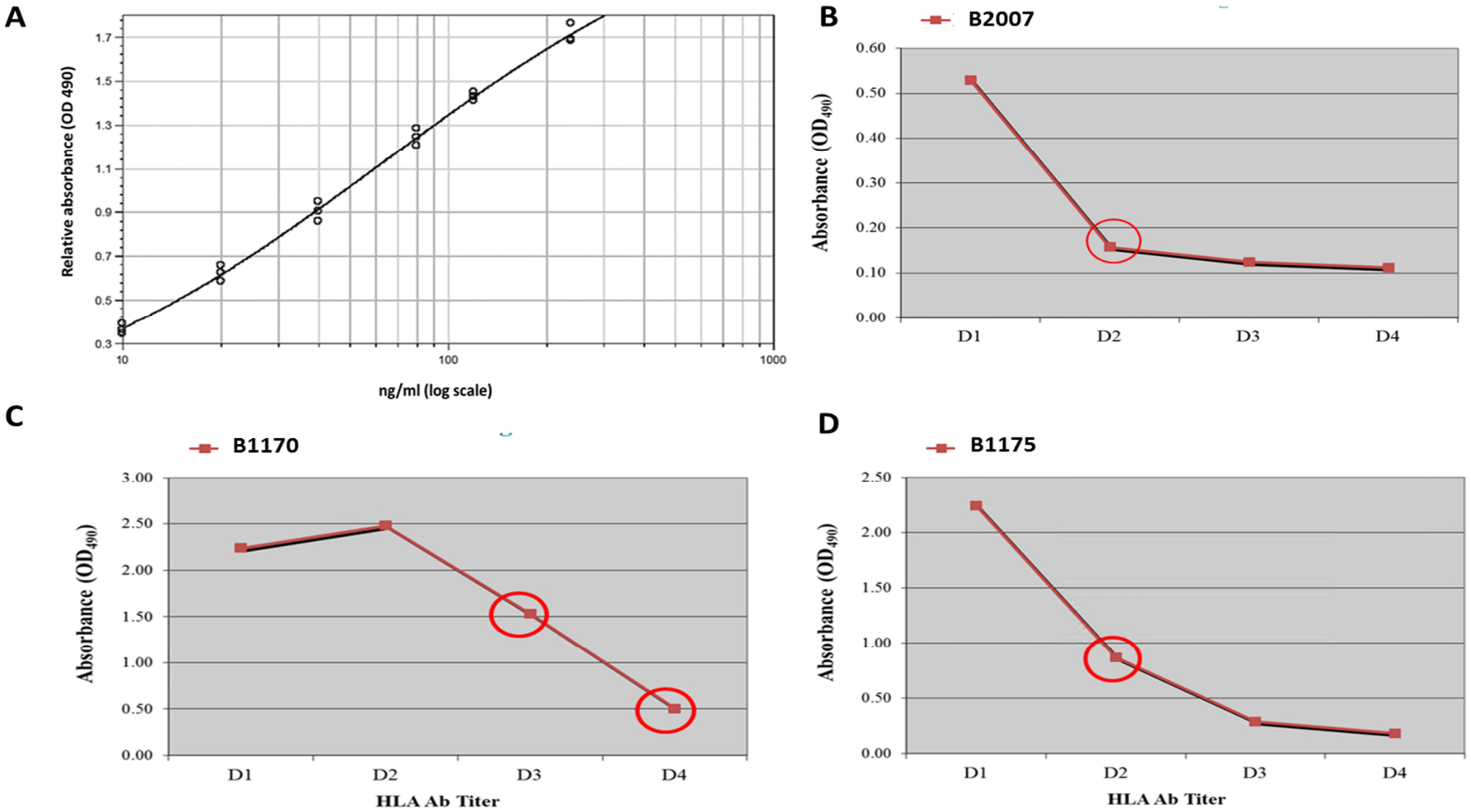


Figure 6-34 : HLA-B7 specific sandwich ELISA. A – Standard curve obtained from monoclonal antibody WK1D12, B-D – clinical, polyclonal samples (Red circle indicates values in the linear range of the standard curve)

The relative absorbance (OD494) obtained from ELISA were also correlated with associated Luminex MFI values for samples quantified above (five samples against HLA-A2 and three samples against HLA-B7) and Spearman correlation coefficient was determined as 0.952 ($P < 0.001$). See Figure 6-35.

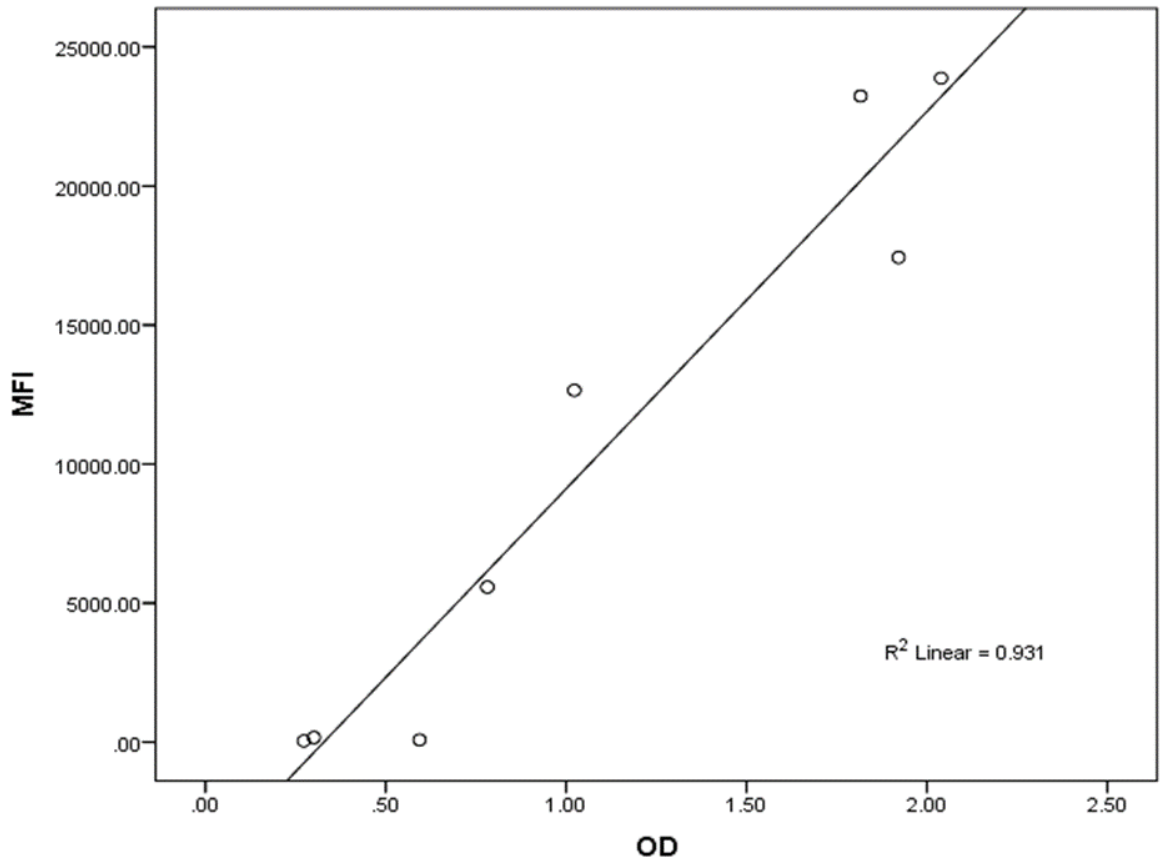


Figure 6-35 : Correlation of Luminex MFI values and ELISA absorbance units at 494 nm

6.4.2.2 *Protein concentration determination via surface plasmon resonance*

In addition to measurement via ELISA, there are various ways by which relevant analyte concentrations can be calculated using surface plasmon resonance.

i. Using standard calibration curve

Purified human monoclonal HLA-specific antibodies of known concentration can be used to obtain a standard curve of response units (RU) versus concentration. Polyclonal HLA-specific antibody concentration can be derived from the standard curve. (Yurugi et al., 2007).

ii. By using prior knowledge of kinetic constants

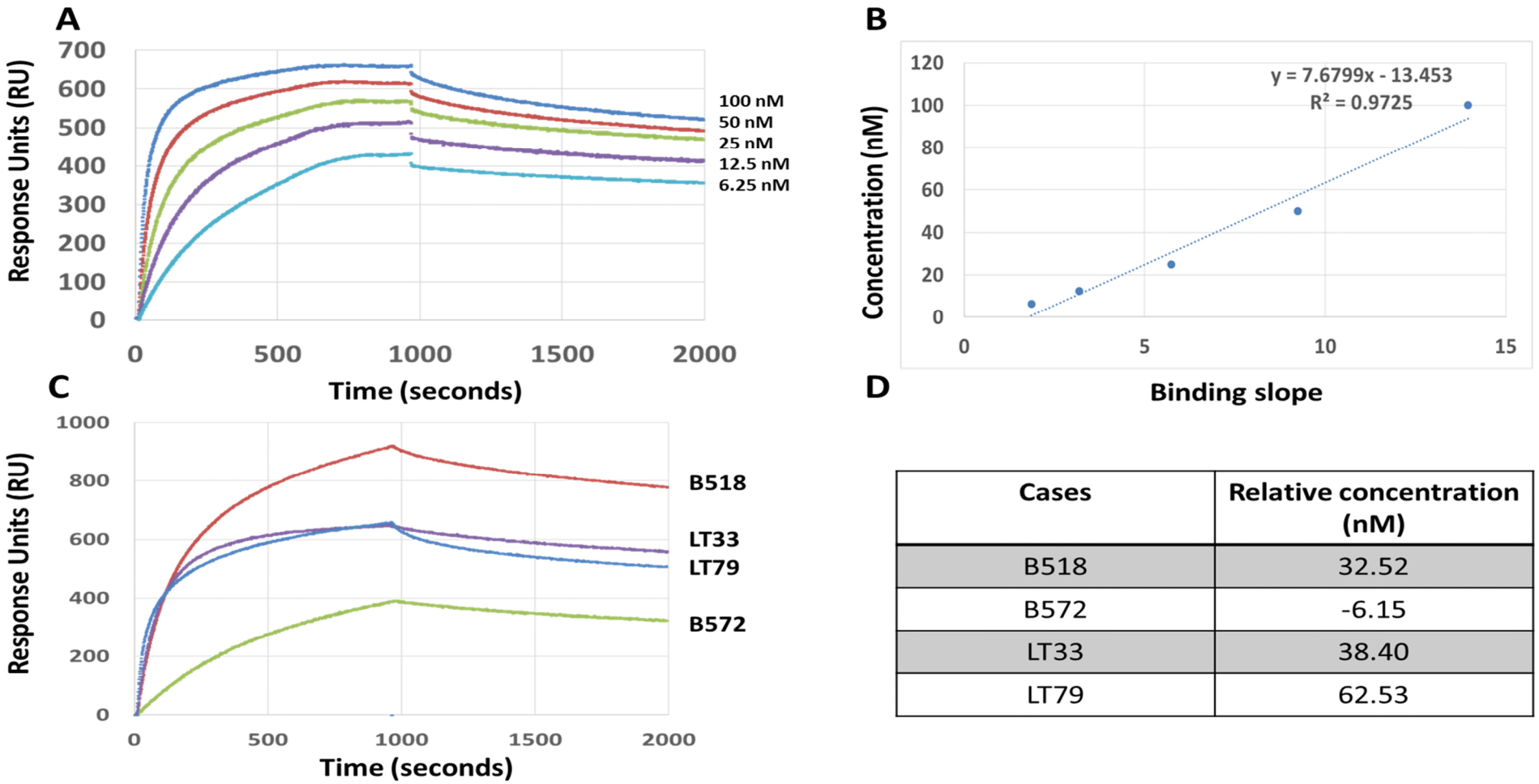
In this approach the kinetic constant is already known and concentration parameters are identified by fitting to binding data. An advantage of this approach, if applicable, is that it does not require a calibration curve (Mehand et al., 2011).

iii. From initial reaction rate correlation

Under mass transport limitation, the initial (first 30 seconds) reaction rate is proportional to antibody concentration and is independent of binding kinetics (Karlsson et al., 1991, Lynch et al., 2014, Chavane et al., 2008).

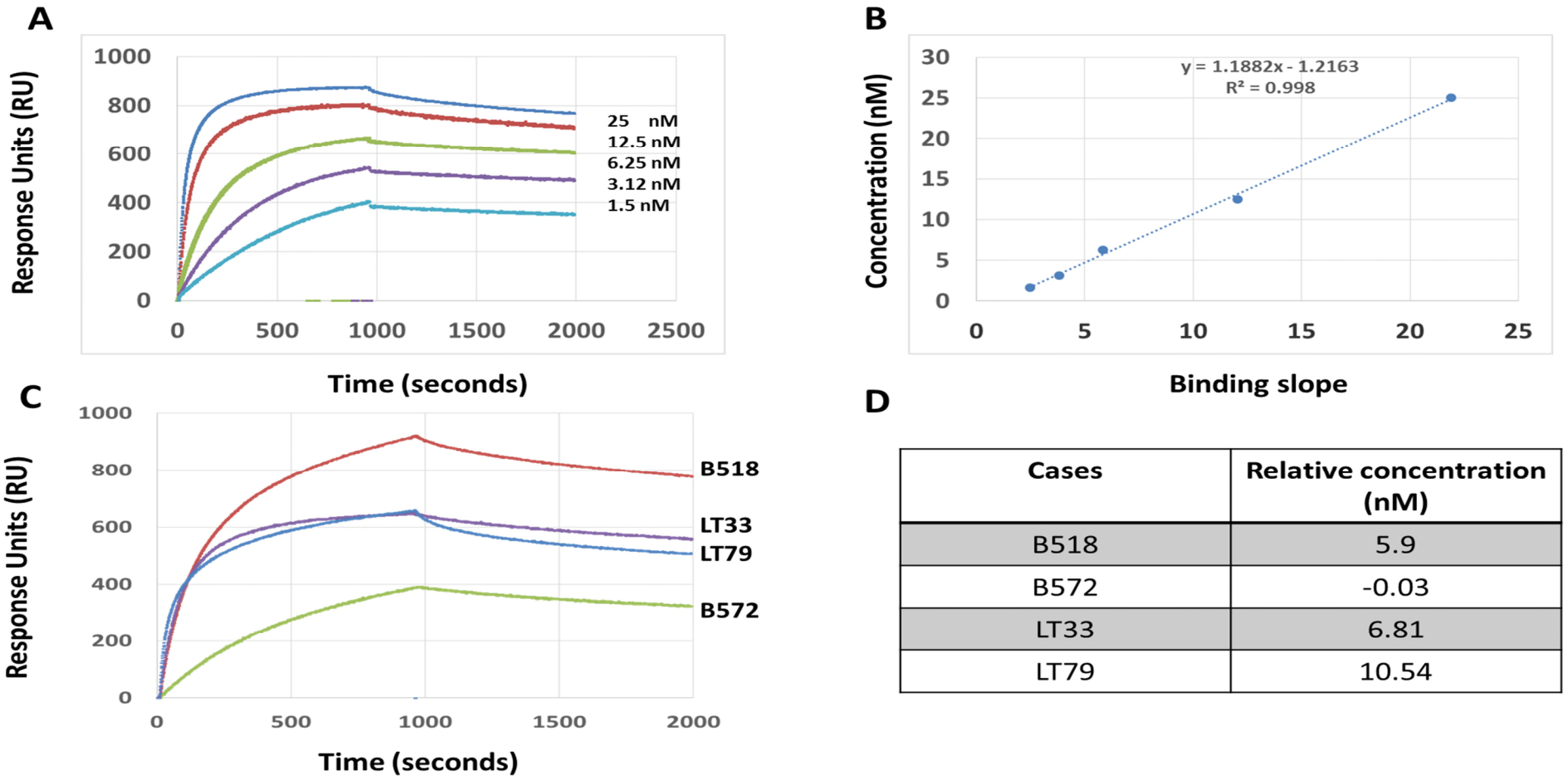
The first approach, using a standard curve of RU values had the inherent limitation of affinity dependence, as it uses maximal response (which can be affinity dependent) and since it is known that different interactions give rise to variations in Rmax, this method for quantification in polyclonal antibodies is not reliable. The second approach assumes that affinity is same for all HLA-specific antibodies and can only conceivably be used for quantifying antibodies with the same or very similar physiochemical properties. It is known from monoclonal antibody work (Chapter 4) that variation in affinity is wide and substantial.

The third approach can be used in conjunction with binding experiments and can be used off-line to calculate the concentrations. The concentrations of enriched polyclonal HLA-A2 specific antibodies (LT33, LT79, B518 and B572) were calculated and compared results from two different human monoclonal HLA-A2 specific antibodies as shown in Figure 6-35 and Figure 6-37. Despite previous reports, it was noted that the concentration derived for each monoclonal HLA-A2 specific antibody was different - the higher affinity antibody SN230G6 gave a 6-fold lower concentration reading than SN607D8. When calculating binding slope versus concentration using the first 30 seconds of analyte contact, the SN230G6 sensorgram already started to plateau. Hence only first 10 seconds were used for the standard curve for this antibody. Figure 3-38 shows quantification of polyclonal HLA-B7-specific antibodies, studied alongside the B7-specific WK1D12 monoclonal. The Rmax values correlated well with the relative concentrations of the HLA-B7 specific antibodies but less so for the HLA-A2 specific antibodies. The anomalous negative value obtained for B572 case suggested that the affinities some of the polyclonal antibodies were lower than both of the monoclonal standards used, and lower limits of meaningful detection were being reached.



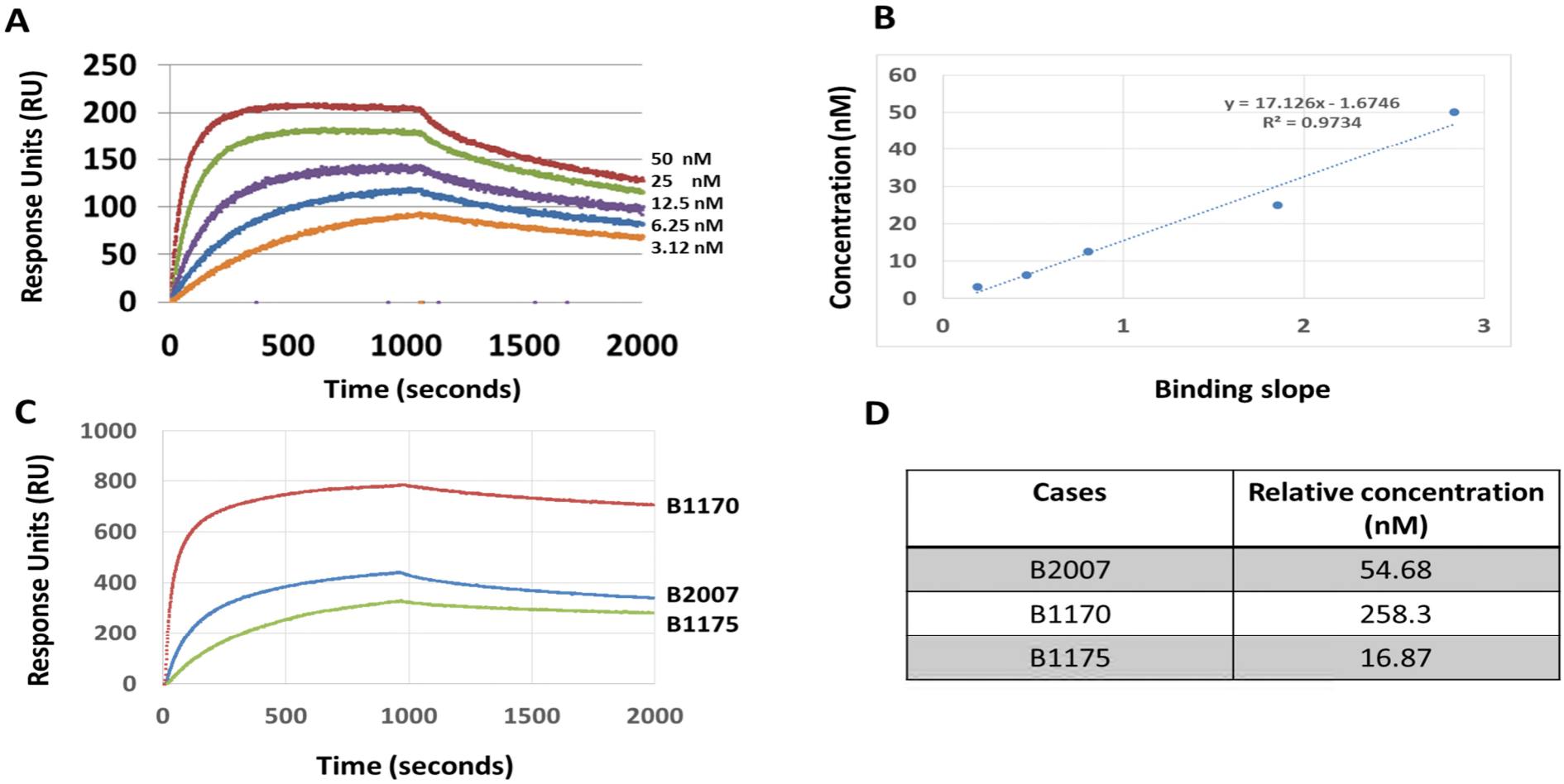
Cases	Relative concentration (nM)
B518	32.52
B572	-6.15
LT33	38.40
LT79	62.53

Figure 6-36 : Determination of anti-HLA-A2 polyclonal antibody concentration. A – Calibration curve of monoclonal HLA-A2-specific antibody SN607D8; B – relationship of concentration and binding slope giving standard curve; C – Binding curves of polyclonal HLA-B7 specific antibody samples, D – concentrations of samples



Cases	Relative concentration (nM)
B518	5.9
B572	-0.03
LT33	6.81
LT79	10.54

Figure 6-37 Determination of anti-HLA-A2 polyclonal antibody concentration. A – Calibration curve of monoclonal HLA-A2-specific antibody SN203G6; B – relationship of concentration and binding slope giving standard curve; C – Binding curves of polyclonal HLA-B7 specific antibody samples, D – concentrations of samples



D

Cases	Relative concentration (nM)
B2007	54.68
B1170	258.3
B1175	16.87

Figure 6-38 : Determination of anti-HLA-B7 polyclonal antibody concentration. A – Calibration curve of monoclonal HLA-B7-specific antibody WK1D12; B – relationship of concentration and binding slope giving standard curve; C – Binding curves of polyclonal HLA-B7 specific antibody samples, D – concentrations of samples

1.1.1.4 *Comparisons of antibody quantification methods*

The concentrations of polyclonal HLA-specific antibodies obtained by sandwich ELISA and SPR methods were compared and correlated relatively well (Linear R^2 = 0.88; Pearson correlation coefficient = 0.98, P = 0.002) (Figure 6-39) and were in agreement within one order of magnitude. As seen in Table 6-5, the SPR calculations were generally higher than those determined by ELISA, although low concentration/low affinity antibody samples highlighted the lower limit of sensitivity within the SPR method.

Cases	Relative concentration (nM) SPR	Relative concentration (nM) ELISA
LT33	38.40	**8.55**
LT79	62.53	**36.45**
B518	32.52	**3.23**
B572	-6.15	**1.07**
B2007	54.68	**7.13**
B1170	258.3	**71**
B1175	16.87	**3.53**

Table 6-5 : Relative concentration of enriched clinical samples – comparison between SPR and ELISA

Despite of these limitations, the SPR method would in principle allow for measurement of concentrations from interactions across a range of different HLA proteins simultaneously on the

XPR36 system. It is also represents a self-contained system for measuring both concentration and subsequently affinity. Hence for the clinical studies involving polyclonal antibodies, the SPR method was used to measure antibody concentration.

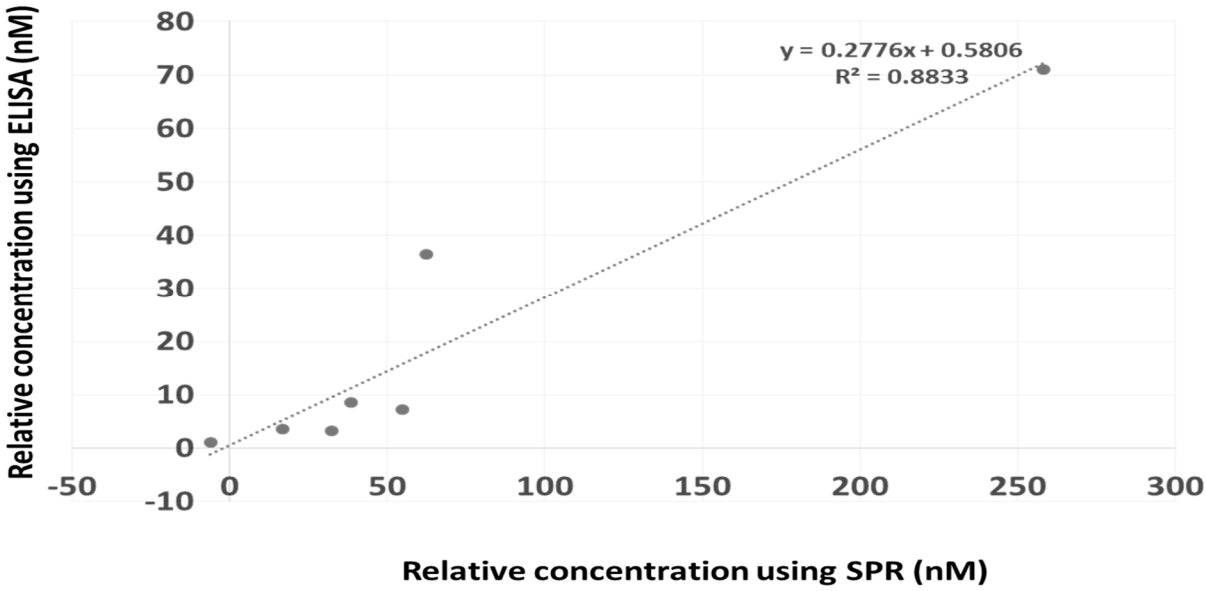


Figure 6-39 : Correlation between concentration calculated using SPR (initial 30 seconds binding approach) and sandwich ELISA

6.5 Summary and Discussion

This Chapter covers two important aspects in developing SPR techniques for measuring binding kinetics of polyclonal HLA-specific antibodies derived directly from clinical samples with realistic starting volumes of serum. Firstly, although labour-intensive at the outset, the protocols described here clearly demonstrate that polyclonal HLA-specific IgG can be enriched and yield meaningful SPR sensorgrams via a refined purification and sample polishing process that, very importantly, was able to remove persistent, serious and impeding non-specific binding factors in polyclonal antibody preparations otherwise considered acceptable in other assays. This work demonstrates the effective combination of precipitation and chromatography techniques and highlights the importance of diligent protein chemistry when approaching extremely sensitive biophysical techniques such as SPR.

The second important aspect was quantifying HLA-specific antibodies. Mathematical models used for calculating binding kinetics require a concentration value input. Currently there are no assays that routinely quantify polyclonal HLA-specific antibody levels with accuracy. Luminex assays measure HLA-specific antibody levels semi-quantitatively and the experiments have shown that MFI vales can be influenced by the intrinsic affinity of antibodies. Protein assays such as Lowry and BCA can quantify high purified HLA-specific antibodies following serial affinity and size exclusion chromatography steps, but they are limited by sensitivity and consume valuable amounts of protein, being only useful following large scale purification using plasma effluent. With the availability of human monoclonal IgG HLA-specific antibodies, the developing of quantitative assays using a standard curve approach was sought. ELISA work showed that calibration curve characteristics were influenced by varying antibody affinities. Concentration

determination via SPR that derives data from initial binding events in first 30 seconds was more appealing, due to being applicable to multiple HLA-specificities simultaneously. Whilst possessing a wider range, this unique SPR technique still presents imperfections and, ultimately, further optimisation will be required.

7 Binding kinetics and affinity measurement on polyclonal clinical sample of highly sensitised patients

7.1 Introduction

One in four patients on transplant waiting list are highly sensitised and have HLA-specific antibodies of various strength and specificities. The assessment of these HLA-specific antibodies possesses a particular challenge pre-transplantation. With current assays it has been observed that not all HLA-specific antibodies pose similar risk and various strategies described in chapter 1 are utilised in clinical practise to allow direct transplantation in presence of donor HLA-specific antibodies (DSA). With the limitations of available clinical assays, the current PhD project aimed at developing and validating an assay to measure binding characteristics of HLA-specific antibodies to the HLA proteins in an in vitro assay based on biosensor platform.

7.2 Methods and materials

In previous chapter-6, the protocols were established that allowed purification of IgG HLA-specific antibodies from larger plasma effluent samples and enrichment of IgG HLA-specific antibodies from smaller volume of serum samples (2-5 ml). Enrichment of IgG polyclonal HLA-specific antibodies together with ability to measure relative concentration allowed us to study clinical samples.

Samples from 32 highly sensitised patients who underwent HLA-incompatible kidney transplantation at University Hospital, Coventry were used. Cases were selected based on presence of HLA-A2 and / or HLA-B7 specific antibodies detected by Luminex single antigen beads. Ethical permission (CREC-055/01/03 and 13/WM/0090) and written informed consent were taken from these patients. Table 7-1 shows baseline characteristics of these 32 highly

sensitised patients. 1.5 to 3 ml of stored serum samples were used to characterise binding kinetics pre-transplantation.

Characteristics	results
Age median (IQR)	44 (38 to 49)
Gender (M:F)	15:17
Previous Transplant	0.87
ESRF median (IQR) years	13 (6 to 19.5)
Time on waiting list in years median (IQR)	3 (1.25 to 7.5)
Mismatch (mean)	3.13
Pre-transplant cross match status CDC FXM (Flow cross match) Bead only	 11 19 2

Table 7-1: Baseline characteristics of patients studied for binding kinetics

7.2.1 Purification and quantification

Serum samples with starting volume of 1.8 to 3 ml were processed using protocol consisting of sequential precipitation and size exclusion chromatography. The peak fraction from size exclusion chromatography was tested for enrichment and HLA-specificities using Luminex single antigen bead (SAB) assay by One Lambda and Life code kits. A positive cut off for One Lambda SAB assay was set as over 500 MFI. Whilst Life code SAB assay, the positive assignment is based on company's recommendation of two of more positive adjusted ratio relative to control beads.

The enriched samples corresponding to the IgG peak on Size exclusion chromatography were quantified using sandwich ELISA and SPR using initial binding reaction. Monoclonal HLA-specific antibodies; SN607D8 (epitope – 142T) and WK1D12 (epitope – 163E) were used as standards for calculating concentration of HLA-A2 and HLA-B7 specific antibodies respectively.

7.2.2 SPR experiments on enriched samples

The experimental condition and protocols were same as described in chapter 3. The only difference was the association phase was reduced to 720 seconds due to limited volume of samples. Initially the experiments were performed using ligand immobilisation of RU 400 (0.25 microgram/ml) but latter this was increased to RU of 1200 (2.5 microgram/ml). The data obtained were modelled with heterogeneous ligand model.

7.3 Results

7.3.1 Quantification of HLA-specific antibodies

The relative concentration obtained from sandwich ELISA and SPR (Initial binding response) were correlated using linear regression. As seen in Figure 7-1, there is moderate linear relationship between the measurements.

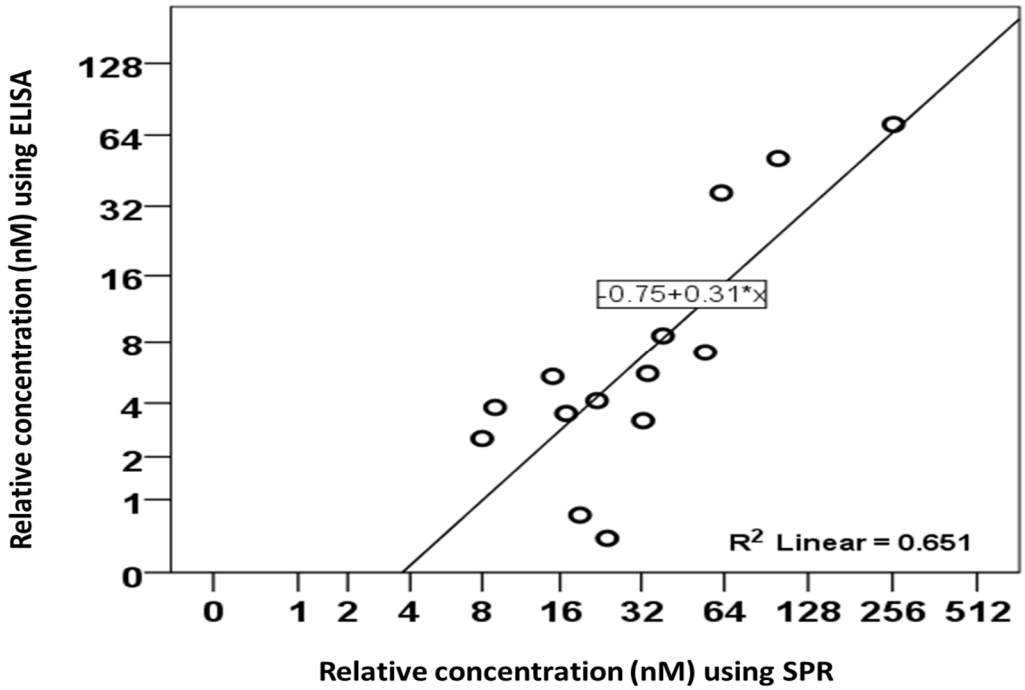


Figure 7-1 : Correlation of relative concentration measured by ELISA and SPR

There was poor relationship between concentration measurements and MFI values determined by Luminex SAB assay (See Figures 7-2 and 7-3). Moreover the relative concentration measurements using sandwich ELISA could only be used for HLA-specific antibodies against HLA-A2 or B7 and cannot be extrapolated to antibodies against other HLA proteins.

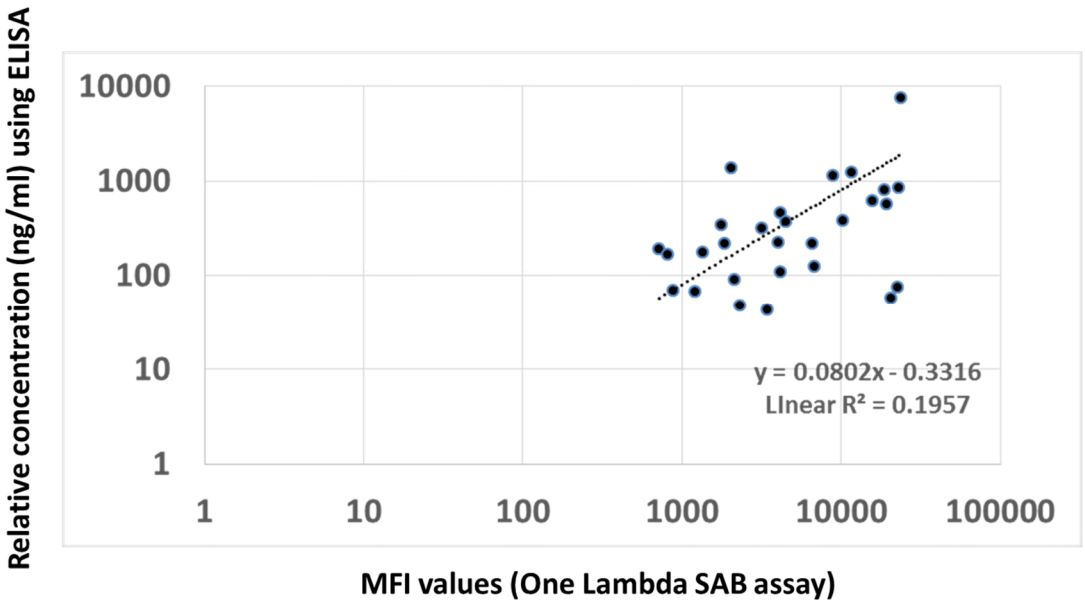


Figure 7-2 : Correlation between raw MFI (One Lambda SAB assay) and relative concentration (ELISA)

The relative concentration obtained using SPR (initial binding slope) were not affected by binding kinetics and affinity of interactions with HLA proteins. Additionally SPR method could be used for quantification of antibodies interacting against various HLA proteins. Hence relative concentration of antibodies obtained from SPR is more reliable and favoured for calculating the binding parameters from the overall experiments.

7.3.2 Binding response (qualitative assessment)

Out of 32 patients, five patients were excluded as the MFI was less than 500. There was no specific binding response observed for nine patients. The MFI of the corresponding HLA specificities were below 2700 for all except one case (MFI 7578). One case was further excluded due to error in labelling. Rest enriched samples from 17 patients gave distinct signal against 26 out of 50 HLA-specificities tested. When the binding response was broken as per MFI values obtained on the enriched samples, a MFI greater than 10,000 predicted successful binding (p = 0.0047, Fisher Exact 2-tail test) (Table 7-2).

MFI	Binding on SPR	
	Yes	No
<10,000	10	19
>10,000	16	5

Table 7-2: Correlation of MFI and binding response on SPR (Fisher exact two tail test used to calculate significance).

When the binding response on SPR was correlated with positive response on Life code single antigen beads, the relationship between positive response on both assay was statistically significant (p=0.0001). Table 7-3 shows the relationship of responses on both the assays.

Life code	Binding on SPR	
	Yes	No
Positive	21	4
Negative	6	19

Table 7-3 : Correlation of positive response on Life code single antigen bead and binding on SPR (Fisher exact two tail test used to calculate significance)

A higher concentration is required to give binding response on surface plasmon resonance with HLA-protein density of 2.5 mcg/ml. A mean relative concentration for binding responses for HLA-A2 and HLA-B7 was 1381 ng/ml compared to 281.52 ng/ml for non-binding responses on SPR. The median value was similarly higher for a binding response (617.74 ng/ml) compared to non-binding response (171.35 ng/ml).

7.3.3 Binding kinetics and affinity (quantitative)

The binding affinities were calculated using equilibrium and kinetic analysis. As shown in the Table 7-4, the dissociation constants derived from kinetic analysis can be divided into four groups based on binding affinity. The green colour corresponds to low affinity antibodies, orange for intermediate affinities, red for high affinities and dark red for very high affinity antibodies. The kinetic values were measured using heterogeneous ligand mathematical model (See Figure 7-3). Different kinetic values were observed for interaction against same HLA-A2 protein, suggesting diverse binding characteristics of polyclonal HLA-specific antibodies in patients.

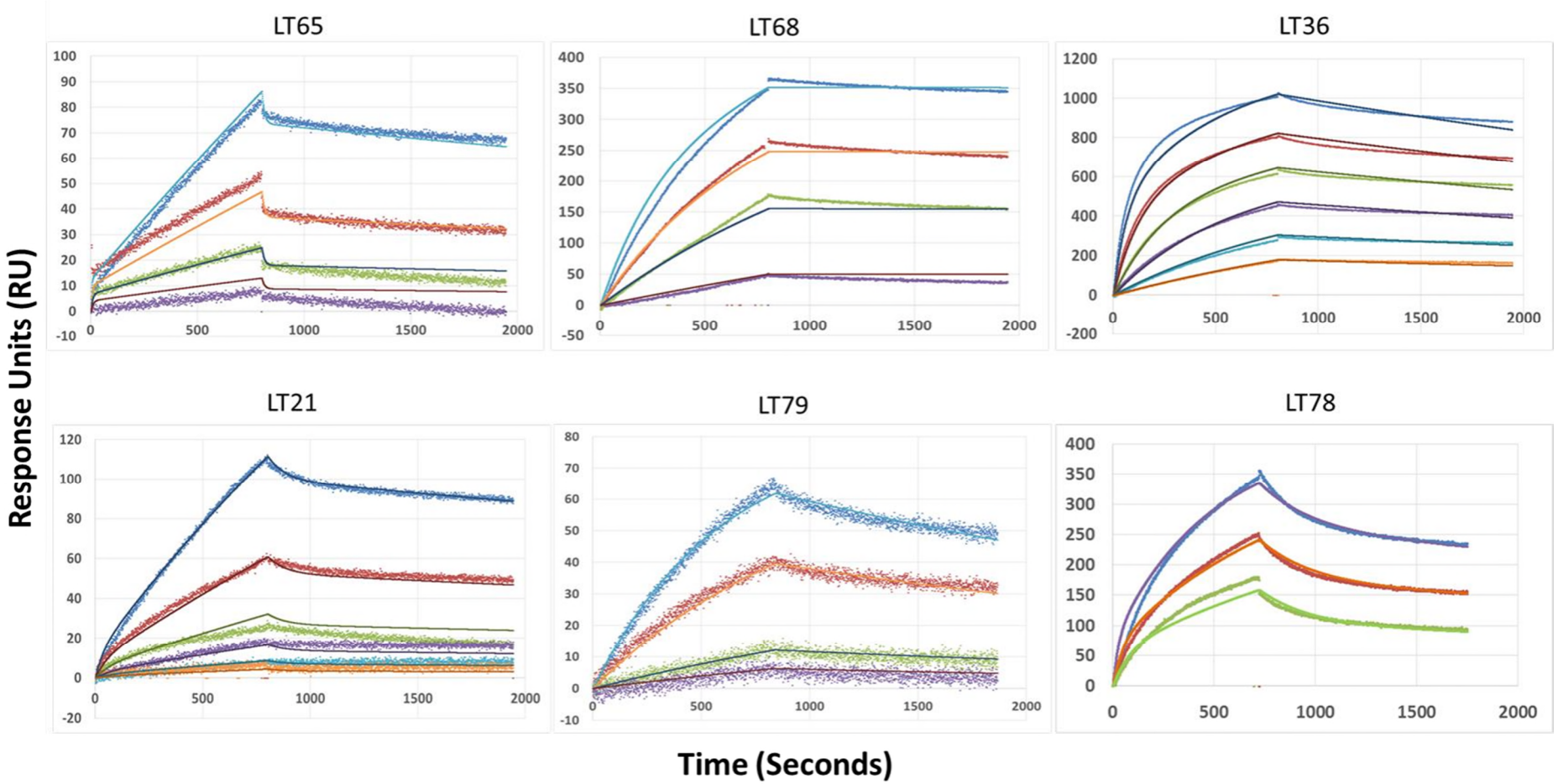


Figure 7-3 : Binding sensorgram of polyclonal HLA-A2 specific antibodies and HLA-A2 proteins (solid lines are data fitting from heterogeneous ligand model. Binding curves from top to bottom corresponds to diluting concentration of purified IgG HLA-specific antibodies

		Equilibrium	Kinetic (Heterogeneous Ligand model)		
		KD	ka	kd	KD
LT42	B27	6.09E-08	3.27E+01	7.57E-02	2.31E-03
LT50	B7	4.83E-04	8.45E+00	2.77E-03	3.28E-04
LT28	A2		2.78E+00	2.56E-04	9.20E-05
LT43	B27		1.29E+01	5.43E-04	4.20E-05
LT43	B40:01	3.95E-04	3.84E+00	1.04E-04	2.99E-05
LT39	B37	7.87E-05	1.55E+01	2.63E-04	1.69E-05
LT35	A24	3.47E-05	4.98E+01	5.75E-04	1.15E-05
LT33	B40:01		8.93E+00	5.26E-05	5.90E-06
LT66	A2	1.30E-05	9.21E+01	5.10E-04	5.53E-06
LT2	B27	2.77E-08	1.12E+04	3.42E-04	3.04E-08
LT43	B40:02		2.01E+00	4.88E-08	2.42E-08
LT36	A2		1.83E+04	2.00E-04	1.09E-08
LT65	A2		1.37E+03	1.28E-04	9.37E-08
LT42	B7	3.25E-08	4.56E+05	3.72E-03	8.16E-09
LT40	B27	1.26E-08	1.51E+06	9.66E-03	6.37E-09
LT40	B40:02	7.10E-09	1.20E+06	6.74E-03	5.61E-09
LT21	B57		2.71E+04	1.14E-04	4.20E-09
LT2	B7	8.12E-09	2.01E+06	6.98E-03	3.47E-09
LT36(LD)	A69		5.09E+04	1.52E-04	2.99E-09
LT79	A2 (LD)		1.21E+05	2.66E-04	2.20E-09
LT42	B40:02	7.11E-08	6.62E+04	8.77E-05	1.33E-09
LT78	A2		1.35E+07	7.39E-03	5.46E-10
LT68	B57		1.97E+05	7.43E-05	3.78E-10
LT68	A2		2.44E+05	2.12E-06	8.70E-12
LT16	A1		1.17E+04	1.19E-09	1.02E-13
LT40	B8		1.21E+06	1.01E-11	8.38E-18
LT35	A1	4.94E-09	6.55E+06	2.36E-11	3.61E-18
LT43	B7	1.96E-08	1.06E+06	3.48E-16	3.28E-22
LT40	B40:01	7.30E-09	5.04E+06	1.91E-17	3.78E-24

Table 7-4 Binding kinetics and strength highly sensitised patient

Although patients can be grouped in to four groups based on dissociation constants derived from kinetic analysis, other parameters vary for patients with same overall dissociation constants. For example in Table 7-4, the apparent dissociation constant is same for patients LT42 and LT21 for HLA-B7 and HLA-B57 respectively, the association and dissociation rates are faster or patient LT21.

Similarly, the same patient can have range of affinities against different HLA-proteins, which may be due to different stages of affinity maturation for the clones producing the respective antibodies. Figure 7-4 shows different binding characteristics against different HLA-proteins from same patients (LT40 & LT42). As seen in Table 7-5, patient LT43 has low affinities for HLA-B27 and B40:01 but very high affinity for HLA-B7. Similarly, the apparent affinity values are different for different antibody specificities in the case of LT42 and LT35. Whilst cases such as LT40, LT66 and LT21 have consistently higher affinities for all of the specificities studied.

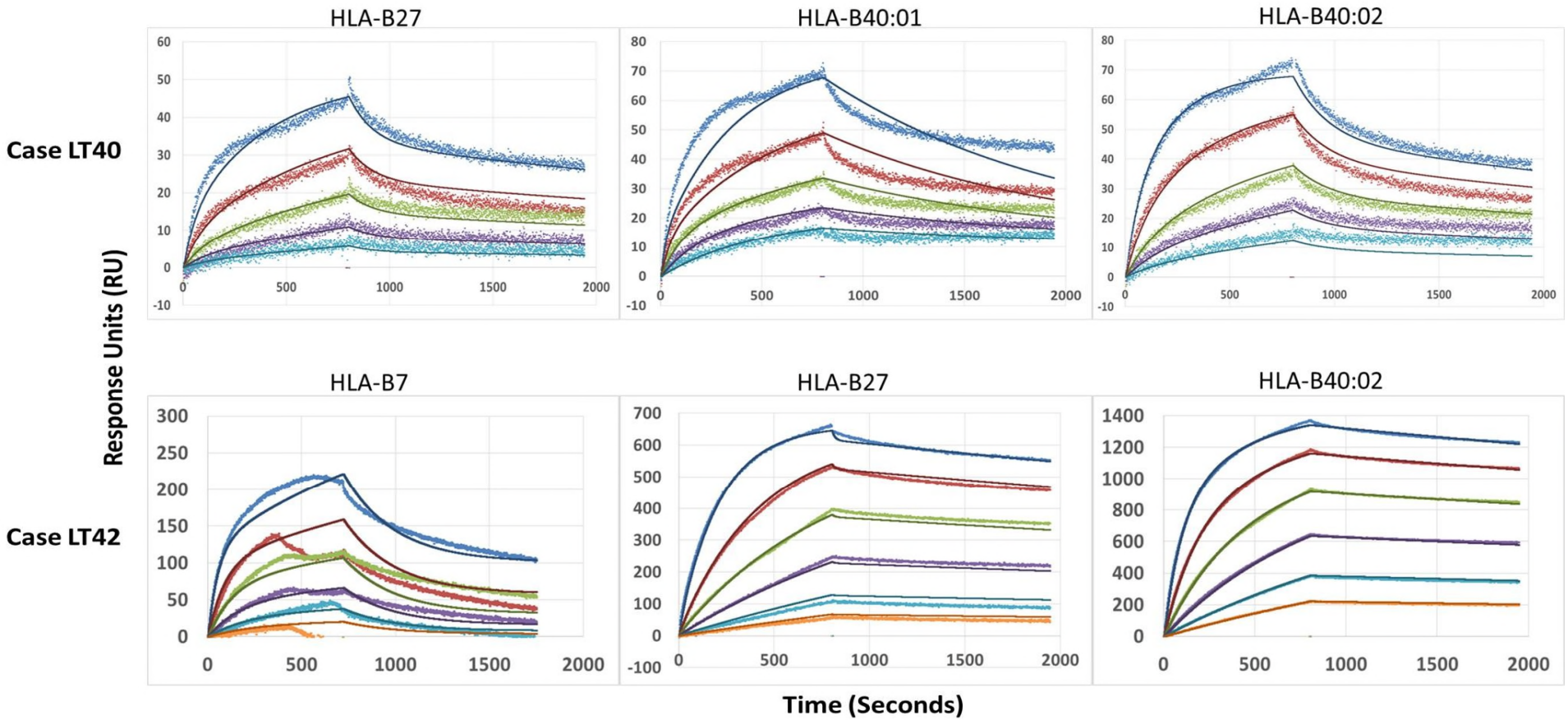


Figure 7-4 : Binding sensorgram of polyclonal HLA-specific antibodies and different HLA-proteins. Binding curves from top to bottom corresponds to diluting concentration of purified IgG HLA-specific antibodies

		Equilibrium	Kinetic (Heterogeneous Ligand model)		
		KD	ka	kd	KD
LT2	B27	2.77E-08	1.12E+04	3.42E-04	3.04E-08
	B7	8.12E-09	2.01E+06	6.98E-03	3.47E-09
LT35	A24	3.47E-05	4.98E+01	5.75E-04	1.15E-05
	A1	4.94E-09	6.55E+06	2.36E-11	3.61E-18
LT36	A2		1.83E+04	2.00E-04	1.09E-08
	A69		5.09E+04	1.52E-04	2.99E-09
LT40	B27	1.26E-08	1.51E+06	9.66E-03	6.37E-09
	B40:02	7.10E-09	1.20E+06	6.74E-03	5.61E-09
	B8		1.21E+06	1.01E-11	8.38E-18
	B40:01	7.30E-09	5.04E+06	1.91E-17	3.78E-24
LT42	B27	6.09E-08	3.27E+01	7.57E-02	2.31E-03
	B7	3.25E-08	4.56E+05	3.72E-03	8.16E-09
	B40:02	7.11E-08	6.62E+04	8.77E-05	1.33E-09
LT43	B27		1.29E+01	5.43E-04	4.20E-05
	B40:01	3.95E-04	3.84E+00	1.04E-04	2.99E-05
	B40:02		2.01E+00	4.88E-08	2.42E-08
	B7	1.96E-08	1.06E+06	3.48E-16	3.28E-22
LT68	B57		1.97E+05	7.43E-05	3.78E-10
	A2		2.44E+05	2.12E-06	8.70E-12

Table 7-5 : Comparisons of affinities and kinetic values from the same patient

7.3.4 Relationship between Luminex MFI values, antibody concentration and binding affinity of HLA-specific antibodies determined by SPR

The relationship between the affinity, concentration and MFI value (measured by One Lambda SAB assay) is not linear. Higher MFI values do not correlate with high antibody concentration nor higher affinity (Figure 7-5, A). As seen in Figure 7-5 (B), MFI values differed 4-6 fold for the same concentration and equally for a range of concentrations the MFI values were similar (right side of figure). There was no linear relationship between MFI values and dissociation constant (Figure 7-5, D) and similarly no linear relationship between relative concentration and dissociation constant (Figure 7-5, C).

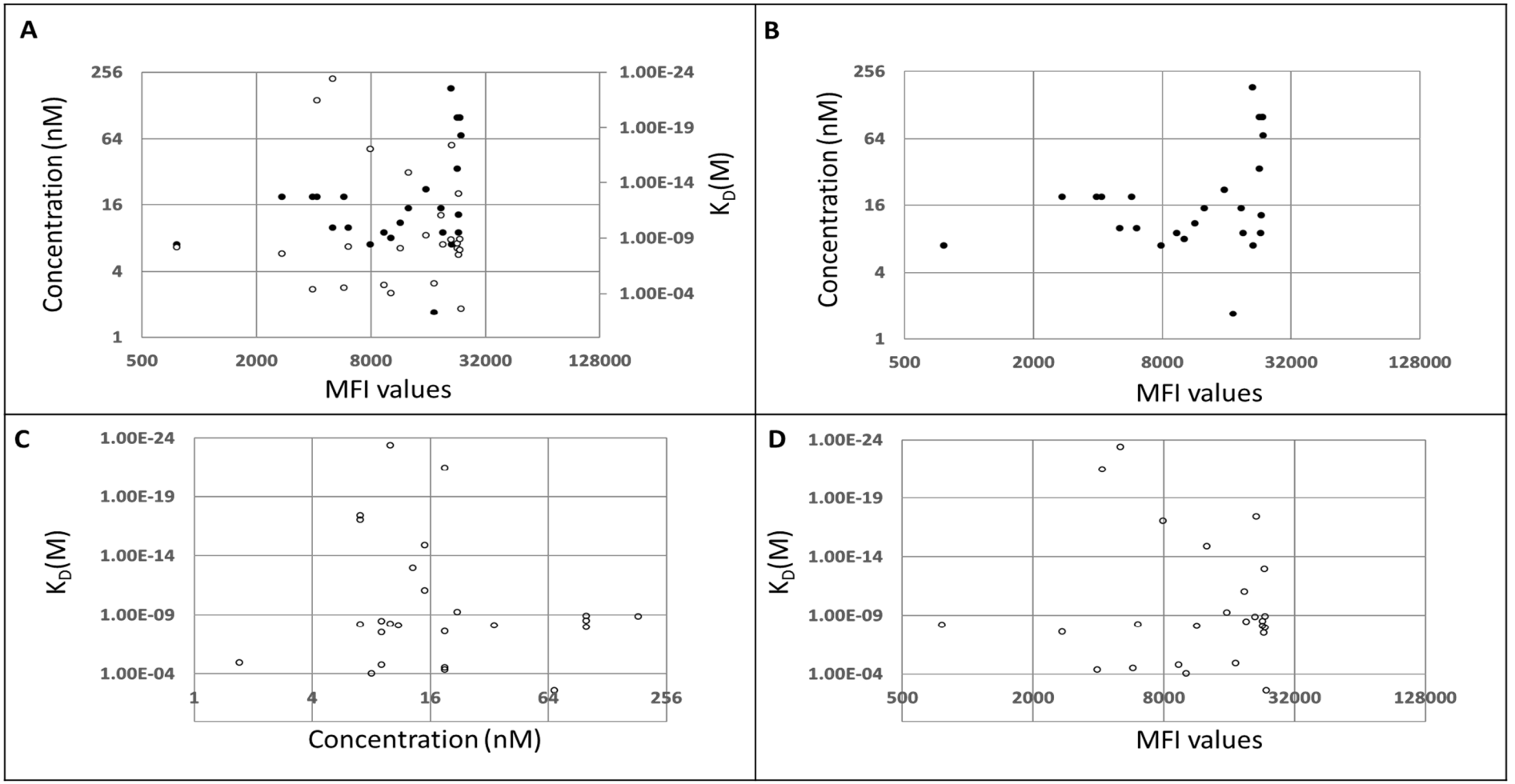


Figure 7-5 : Relationship between MFI values, concentration (nM) and dissociation constant (KD)

7.4 Relationship of affinity of polyclonal HLA-specific antibodies with transplant outcomes (rejection and graft loss)

Out of the 32 cases selected, 18 cases had donor-specific responses and 14 cases had non-donor specific responses against HLA-A2 or B7 on Luminex SAB assay using the One Lamba kit. HLA-A2 and B7 reactivity were chosen, since the quantitative ELISA can be performed against these specificities. However, out of 32 cases, only 10 cases demonstrated a meaningful SPR binding response against HLA-A2 or HLA-B7 proteins. Only two cases (LT68 and LT43) had quantifiable binding responses against donor-specific HLA proteins (see Figure 7-6). From a graft outcome perspective, Case LT68 had acute AMR on day-0 and the graft failed despite treatment with ecluzimab, ATG and plasma exchange in the early post-transplant period.

In contrast, Case LT43 was stable with no clinical evidence of rejection. This difference in graft outcome might be explained by differences in the affinities of other cross-reactive polyclonal HLA-specific antibodies. As seen from Table 7-5, LT68 has higher affinity antibodies against HLA-A2 and HLA-B57 whilst LT43 only has higher affinity polyclonal HLA-specific antibodies against HLA-B7; other specificities were classified as having low to intermediate affinities. The MFI values were lower for LT43 compared to LT68 (4000 versus 11,000).

Obviously, from only these two cases it is impossible to establish a reliable relationship between antibody affinities and adverse outcome. Nevertheless, data from future, larger studies are eagerly awaited, and the opportunity to incorporate antibody affinity parameters is now available.

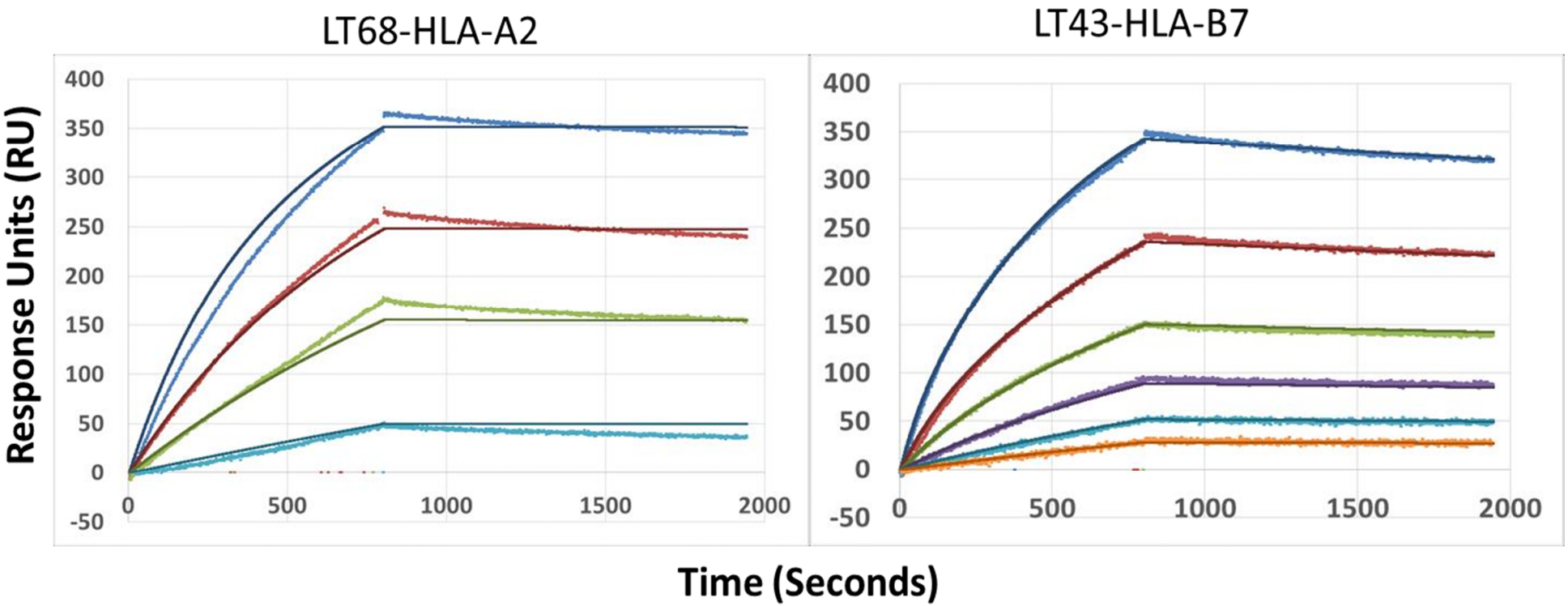


Figure 7-6 : Binding kinetics of donor HLA-specific polyclonal IgG in cases LT68 and LT43. Binding curves from top to bottom corresponds to diluting concentration of purified IgG HLA-specific antibodies and black line represents the fitting with mathematical model

7.4 Discussions

This preliminary study of polyclonal HLA-specific antibody interactions with HLA-proteins using antibodies recovered from manageable volumes of patient serum samples gave useful insights and is probably the first in the world to indicate the associated binding kinetics.

The apparent affinity of polyclonal HLA-specific antibodies can be grouped in to four distinct sets across a defined range of KD values. This has the potential to be used as additional tools to risk stratify cases considered for direct transplantation (both against low and high MFI levels). As seen in Figure 7-5, antibodies corresponding to high MFI could fall into the lower affinity range. The antibody concentration measurements using SPR-IBR had reasonable correlation to concentration measured by ELISA. The advantage of using SPR-IBR is that it allows the simultaneous measurement of concentration against a range of HLA proteins, which is important especially when studying clinical samples with very limited amounts of antibody.

In some cases the kinetics rates (ka, kd) differed between discrete antibody interactions that yielded similar overall dissociation constants (KD). Thus the real time nature of SPR analysis could provide additional useful parameters that further define antibody character which are unavailable to end-point equilibrium analyses such as ELISA. Within the same patients, the binding kinetics varied across different HLA proteins, and it is tempting to suggest that this might reflect different stages of antibody affinity maturation produced from different clones, something that may be due to differences in the duration following primary sensitization events

within the same patient. Hence, when deciding the relevance of HLA-specific antibodies in future, the binding kinetics of individual antibodies could be very valuable.

Dissociation constants calculated by equilibrium and kinetic analysis differed. This is because the assumption for equilibrium analysis is that all the binding interactions reach an equilibrium phase. However, due to limited amounts of patient sample and the sample loop volume limit of the SPR instrument, prolonged contact times were not feasible and calculations had to depend on data extrapolation by the analysis software. In principle, dissociation constants calculated via kinetic analysis are more accurate; however, these analyses can be limited by the fidelity current data fitting mathematical models that are designed around simpler interacting systems than comparatively complex IgG and HLA molecules.

Other limitations of these preliminary protocols include the requirement for higher starting concentration of HLA-specific antibodies for a distinct SPR binding signal compared with Luminex or ELISA, largely due to SPR being a direct rather than indirect form of measurement (i.e. no secondary conjugate step with a signal amplification capacity). The relative concentrations of HLA-specific antibodies derived from ELISA for non-binding responses on SPR were in the nanomolar range. Due to the scarcity of sample, these experiments examining a larger range of HLA proteins could not be done in duplicate and thus we were not able to establish reproducibility in full. However, the reproducibility and robustness of the analysis platform as demonstrated using monoclonal HLA-specific antibodies lent a considerable degree of confidence to the preliminary results.

8. Summary and future direction

8.1 Summary

HLA-specific antibodies have distinct and varied biochemical and biophysical characteristics (for example different profiles of class or sub-class) and these may explain varied immune responses associated with their presence in transplantation. Both class switching and affinity maturation are dependent on T cell help and thus measurement of class/sub-class profile and binding characteristics could offer further information regarding cellular and humoral immune system activity. In principle, cellular responses and function can be studied *in vitro* via assays such as ELISPOT. However, isolation of the relevant donor-specific B cells and T cells is extremely challenging. The biophysical properties of antibodies such as affinity could directly influence graft damage and could potentially serve as important indicators of both cellular and humoral immune responses. In this thesis, the class switching of *de novo* HLA- specific antibodies following standard transplantation and the binding kinetics of pre-formed HLA-specific antibodies are studied in detail. The objectives set out in Chapter-1 are tackled below:

8.1.1 Objectives and outcome:

1. To study the evolution of HLA-specific IgM and IgG antibodies in non-sensitised patients and study effects on graft outcome prospectively.

In order to study the evolution of *de novo* HLA-specific antibodies and their impact on transplant outcome, eighty patients receiving standard kidney transplantation were prospectively followed over three years. The serological sampling was rigorous and both IgM and IgG *de novo* responses were examined.

This study demonstrated that the prevalence of *de novo* IgM and IgG HLA-specific responses were low with 8.8 % for IgG and 3.8% for IgM. There were large numbers of non-donor specific antibodies present across the cohort. Baseline PRA of over 5% was associated with higher *de novo* antibody development. Most of the IgG occurred without apparent prior IgM indication, raising the question of whether genuine *de novo* responses were at play or whether this was a re-call from previously undetected sensitisation, or as a result of nonspecific immune up-regulation. IgG responses to class I HLA appeared earlier compared to class II HLA and IgM responses were not detected against class II HLA in this study.

When the patients were separated into four groups depending on the development of IgM or IgG HLA-specific antibodies, the group with dual IgM and IgG positivity had a higher number of adverse graft outcomes with poor eGFR (MDRD) at 36 months. There was no strong temporal relationship between prior antibody appearance and rejection episodes, with 3 out of 9 cases showing antibody prior to an antibody-mediated rejection episode. In fact, the majority of IgG-positive cases did not show episodes of rejection (13/21). IgM alone did not have any adverse effects. Thus either a class switch to IgG or the presence of both IgG and IgM together can lead to graft damage.

Preformed IgM DSA prior to kidney transplantation has been considered benign previously but recently small studies have shown them to be of importance. However, in this thesis we did not study binding kinetics of IgM HLA-specific antibodies. Studying IgM binding kinetics will require separate purification and experimental protocols due to its very different structure and pentameric form.

2. To develop and validate surface plasmon resonance as a technique for characterising HLA-specific antibody interactions with HLA proteins using available human monoclonal IgG HLA-specific antibodies.

Surface plasmon resonance using the BioRad ProteOn XPR system and NLC Sensor chips was successfully employed following extensive sample preparation to remove trace contaminants responsible for substantial signal noise. Purification of human monoclonal HLA-specific antibodies was achieved by a protocol consisting of affinity chromatography (HLA protein or protein G) followed by fast protein liquid chromatography (FPLC) techniques such as ion exchange and size exclusion chromatography. These purification protocols yielded pure IgG HLA-specific antibodies that could be accurately quantified and gave no non-specific binding noise in the SPR instrument. Optimal conditions were subsequently used for studying the binding interactions of HLA-specific antibodies, taking into account the stability of HLA proteins. Physiological conditions, including a running temperature of 37oC, pH 7.4 and isotonic buffer yielded high quality sensorgrams suitable for quantitative kinetic analysis.

3. To characterise the binding kinetics of epitope–paratope interactions using the same monoclonal HLA-specific antibodies with different HLA proteins that possess shared epitopes.

Human monoclonal HLA-specific antibody kinetics were studied against multiple HLA proteins that share various key epitopes. This showed that the binding kinetics of different human monoclonal HLA-specific antibodies varied between different HLA proteins expressing same epitope across a set of six monoclonal antibodies studied. This could be due to the differences in net binding between the antibody and structural epitope.

Structural epitopes consist of amino acid residues that result in specific interactions (functional epitope) and residues that interact with the antibody paratope. Thus both the best fit and surface area involved in the overall interaction may determine the stability of binding. Although the difference in dissociation constant is relatively small (within one order of magnitude), the kinetic parameters such as association and dissociation rates differed more widely. Thus for comparisons, dissociation constant alone may not give a full picture. These are useful observations as current solid phase assays do not distinguish subtle differences in binding characteristics which may be important in determining biological reactivity of the HLA-specific antibodies and downstream effects. Importantly, the data suggest that higher affinity and faster association rates exist for interactions involving HLA-specific antibodies and the selected HLA protein that was originally attributed as the immunising antigen for antibody productions.

The maximal response for certain HLA-proteins such as HLA-B7 and HLA-B57 were small despite high affinity interactions and this could be due to a different and less favourable immobilisation orientation for these particular HLA proteins. This could be due to the biotinylation technique used by Pure Protein LLC at Oklahoma, which is based on chemical and not enzymatic biotin conjugation. In future, an enzymatic biotinylation reaction via the BirA system could allow for the site-selective attachment of biotin to all HLA molecules and ensure homogenous orientation upon immobilisation.

In addition to kinetic analysis, preliminary thermodynamic analysis was also accomplished. Thermodynamic experiments suggested that the Gibbs free energy value was double for the interaction of SN607D8 (IHB-Hu-081) with HLA-A69 compared to HLA-A2 (Table 5-9). Both the enthalpy and entropy components were favourable for the interactions with HLA-A69. The capacity to study thermodynamics for HLA/anti-HLA interactions opens up new possibilities such as understanding bond-making and bond-breaking processes, and whilst SPR is not the most accurate technique, it can yield data with much less material than optimal techniques such as isothermal titration calorimetry.

The SPR system was also used to study dual and competitive binding of HLA-specific antibodies to the same HLA protein. Additive binding was observed when the target epitopes are located far from each other. The epitopes 142T and 62G on HLA-A2 are located more than 30 Angstroms apart and the antibodies against them - SN607D8 and SN230G6 respectively demonstrated this additive binding effect (Figure 5-12). However if the epitopes are located within 20 Angstroms, then, competitive binding occurs, as seen with the monoclonal HLA-B40:01-specific antibodies

with different epitope specificities (WK1D12 – epitope 163E and OUW4F11 – epitope 80I) (Figure 5-14). Similar observations were made when two different monoclonal anti-HLA-B27 antibodies (WK1D12 & VTM1F11) with the same epitope specificities (163E) were studied. This is useful as it confirmed similar epitope specificities of antibodies obtained from different patients.

4. To develop methods for the molar quantification of polyclonal HLA-specific antibodies recovered from clinical samples.

Absolute quantification of HLA-specific antibodies can be achieved using purified material from large volumes of sample such as plasma effluent via serial HLA-affinity chromatography followed by size exclusion chromatography followed by standard protein assays such as BCA assay. This purification and quantification approach is not feasible for low volume samples such as those from standard serum collection. Hence, concentration using a sandwich ELISA calibrated with purified monoclonal HLA-specific antibodies was achievable. Cutting edge concentration measurement via SPR-IBR was also achieved. The rigorous IgG purification protocols were central to the success of this Objective.

5. To develop protocols for measuring the affinity of polyclonal HLA-specific antibodies purified from the serum of highly sensitised patients.

Unprocessed serum from patients caused significant and persistent non-specific binding to the SPR sensor chips and severe signal distortion such that specific binding was unquantifiable. The purification protocols for polyclonal HLA-specific antibodies from serum required substantial investigation. Sequential use of HLA affinity chromatography followed by size exclusion chromatography provided pure HLA-specific IgG antibodies but this protocol could only be applied to large volumes such as plasma effluent. Use of protein G affinity chromatography was surprisingly limited with incomplete regeneration.

For clinical patient serum samples, a simplified protocol using sequential precipitation followed by size exclusion chromatography to enrich IgG was used and the polyclonal HLA- specific antibodies were quantifiable by both ELISA and SPR-IBR. The material gave distinct and specific binding responses from which defined kinetic data were obtained.

8.1.2 Primary objective

To study and determine the distribution of affinities of HLA-specific antibodies in highly sensitised patients and to stratify the HLA-specific antibodies into groups based on the level of affinity.

To study this, 32 highly sensitised patients receiving HLA-incompatible kidney transplants at UHCW, Coventry were used. The analysis of binding kinetics of donor and non-donor specific antibodies demonstrated a wide heterogeneity of affinity. This heterogeneity was observed between both patients and different antibody specificities within the same patient. The antibodies could be divided into four groups based on the quantitation of affinity. There was no linear relationship between antibody concentration or MFI value with the dissociation constants, suggesting that binding kinetics can serve as an additional, independent biomarker(s) for risk stratification.

Although this opening study set out to distinguish binding kinetics for 14 DSA-positive cases, only two yielded quantifiable binding signals on SPR. Interestingly, one of these two cases rejected and was shown to have multiple higher affinity antibody specificities. In contrast, the second case, had stable graft function and showed only one higher affinity antibody specificity. Improved kinetic signal resolution and a larger cohort will be needed to develop this study further.

Assay protocols developed here have achieved a significant breakthrough in the ability to obtain meaningful binding kinetic data for polyclonal HLA-specific antibodies - possibly for the first time worldwide. Importantly, during this thesis, significant insights have been gained in terms of understanding the limitations in many areas of antibody and protein handling across the range of techniques associated with HLA-related analysis and areas for improvement have been illuminated.

8.2 Future directions

8.2.1 Development of an ideal biosensor platform to support transplantation

The biosensor platform used for this thesis is based on surface plasmon resonance and the interaction happens over a chip with modified alginate. The ligand (antigen) was immobilised and the analyte (antibodies) was passed over the immobilised antigen using a fluidics system. The flow through the fluidics system could be adjusted and the rate was kept low to allow the active analyte to diffuse through the flow and bind with the immobilised antigens. There are number of disadvantages of the fluidics system; due to flow the analyte binding is affected by diffusion and thus samples with lower concentration may give false low or negative signals. Secondly, the fluidic system resulted in artefact in some samples due to air aspirated during sample aspiration and also the fluidics system requires rigorous maintenance to prevent salt precipitation in the system. Thus an ideal biosensor platform is the one that is not dependent on the fluidic system; the surface used for interactions does not cause non-specific bindings and allow recovery of sample used.

Preliminary work using a novel and different biosensor platform has been investigated during a recent product demonstration. A novel biosensor technique based on the principle of BioLayer Interferometry (BLI) using the ForteBio Octet instrument was used to study protein- protein interactions (Abdiche *et al.*, 2008).

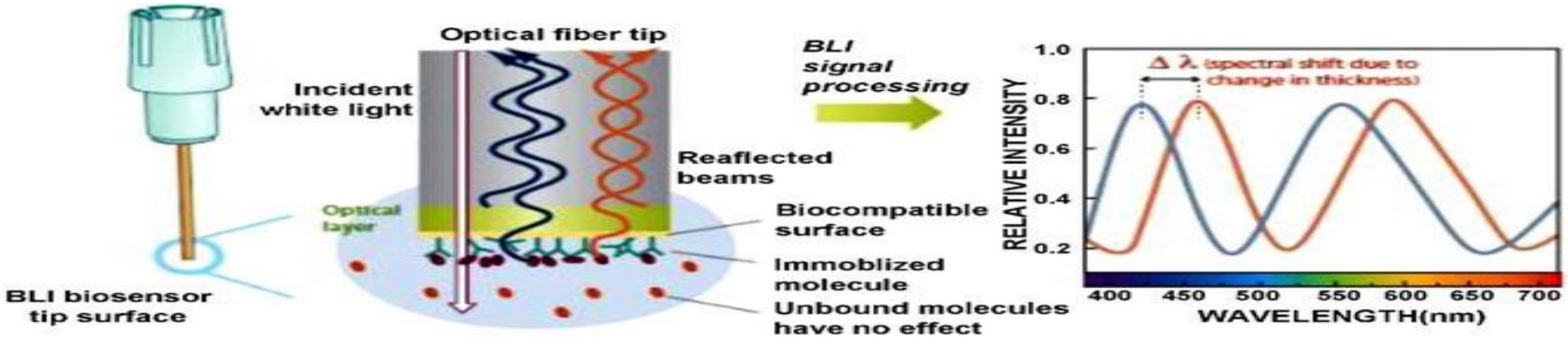


Figure 8-1 : Biosensor assay based on the BioLayer Interferometry principle

On the left side a single biosensor-tip is shown followed by a detailed illustration of the physics that allows analysis of the interference pattern of white light reflected from two discrete biosensor tip surfaces: a layer of immobilized molecules on the biosensor tip, and an internal reference layer. Any change in the number of molecules bound to the biosensor tip causes a shift in the interference pattern that can be measured in real-time. The wavelength shift (Δλ) is a direct measure of the change in thickness of the biological layer as shown on the far right panel. Unbound molecules and changes in the refractive index of the surrounding medium do not affect the optical interference pattern, leading to improved performance and greater robustness compared with SPR. Taken from (Wallner et al., 2013)

Streptavidin coated SA biosensor probes were used to immobilise biotinylated HLA-proteins and the Octet "dip-and-read" approach was used to test a range of W6/32 antibody concentrations in a simple 96 well plate format. Thus there is no microfluidic flow system and the interaction happens on the surface of the optical probe. Orbital flow is obtained by circular plate motion within the instrument to overcome the mass transport limitation. The other advantage of BLI system is that it quantifies the binding response as a direct function of distance in nanometres - obtained from determined optical wavelength) rather than arbitrary response units.

Interactions between purified W6/32 (mouse anti-human class I specificity) against HLA-A24 and HLA-A68 were studied. The HLA-antigen solution for immobilisation was prepared as 10.0 µg/ml and the concentration range of W6/32 was 3.13 to 200 nM - prepared in 1% BSA. Very good quality sensorgrams were obtained (Figure 8.2) and data seen in Table 8-1. Early impressions suggest that affinity is higher for interactions with HLA-24 compared to HLA-A68.

W6/32	Maximal response	Binding kinetics	Dissociation
HLA-24	0.97	Ka = 8.93E+04 1/Ms	4.23E-10 M
HLA-68	0.82	Ka = 7.12E+04 1/Ms	1.73E-09 M

Table 8-1 : Binding kinetics obtained from Octect platform for interaction of W6/32 and HLA-A24 and HLA-A68 proteins

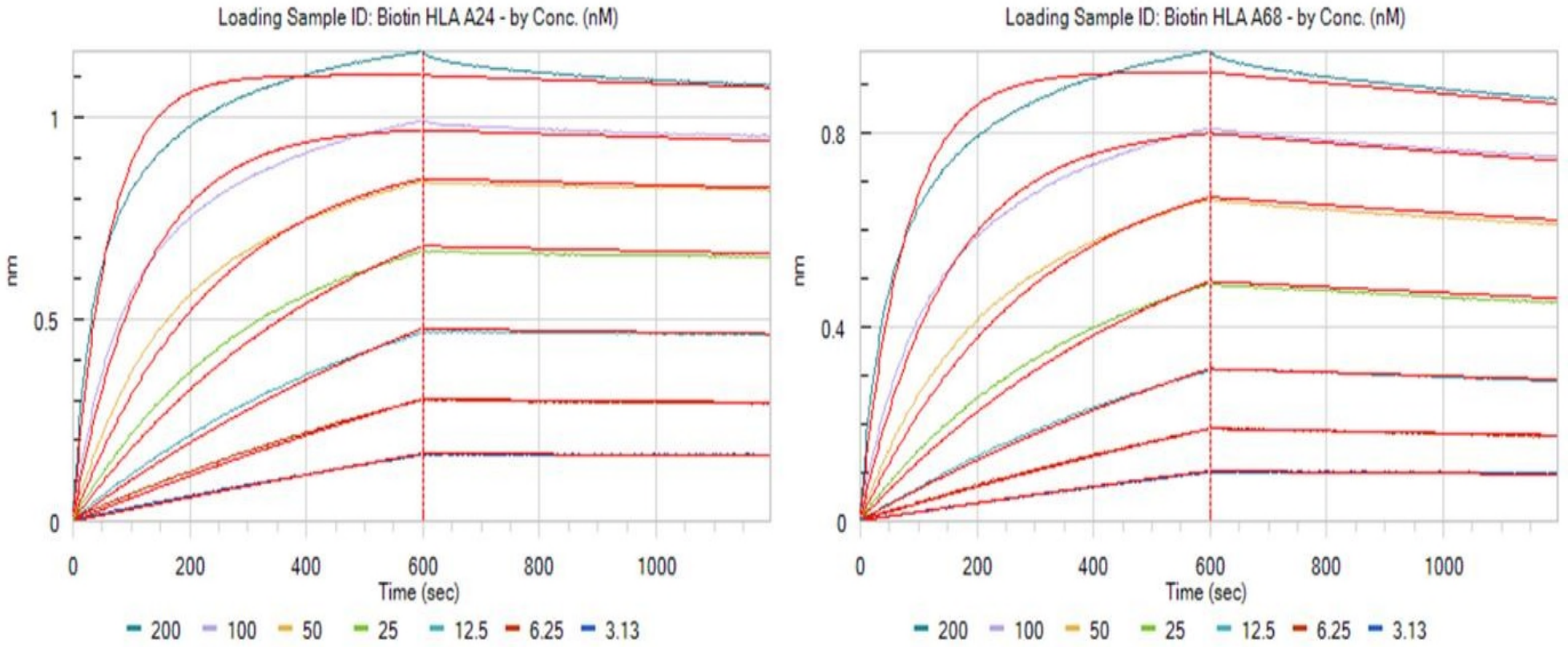


Figure 8-2 : Binding curves of interaction between W6/32 and HLA-A24 & HLA-A68 (data fitting in red lines using bivalent analyte model)

Thus successful real-time analysis of purified HLA-specific antibodies is highly feasible using the Octet BLI platform, with similar binding curves to SPR, but with greater ease. Very importantly, the dip-and-read approach here means that almost negligible amounts of antibody sample are lost. In contrast, the flow requirement of SPR means that antibody samples invariably are expended with each run and extremely difficult to recover effectively. For BLI analysis, it needs to be established whether polyclonal anti-HLA antibodies can be analysed and BLI signal fidelity also raises the exciting possibility of directly analysing serum, although it remains to be seen whether the serum samples will give rise any non-specific binding. Nonetheless, a biosensor assay based on BLI sounds extremely promising for both research work with minimal sample requirement and future clinical applications.

8.2.2 Improved mathematical modelling

One of the other major areas of work will be the development of novel mathematical models that will allow better fitting for the kinetics data generated using biosensor assays. Data from this thesis are currently being used to develop improved mathematical models tailored to the features of antibodies (Mr Harry Moyse, and Dr Neil Evans, School of Engineering, University of Warwick). As seen in Figure 8-3, early work using the Bivalent Effective Rate Constant model with spatial effects shows a superior fit compared to bivalent analyte model, with far greater potential for accurate kinetic data determination. The RSS values reduced to 1792, compared to 5991 in the earlier model.

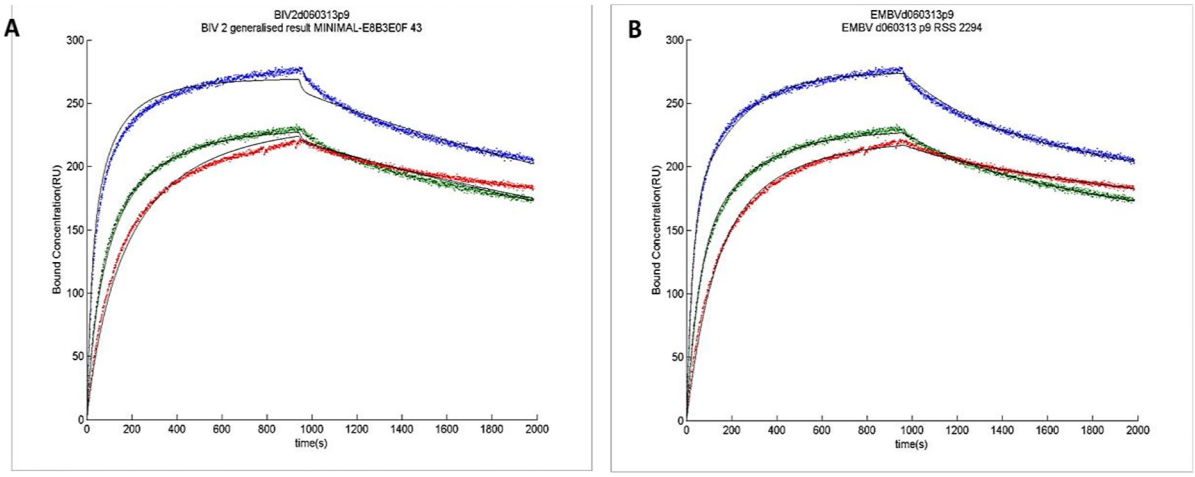


Figure 8-3 : Mathematical fitting on binding kinetics data on WK1D12 monoclonal antibody; A – Bivalent analyte model and B - Bivalent Effective Rate Constant model with spatial effects (bERCs)

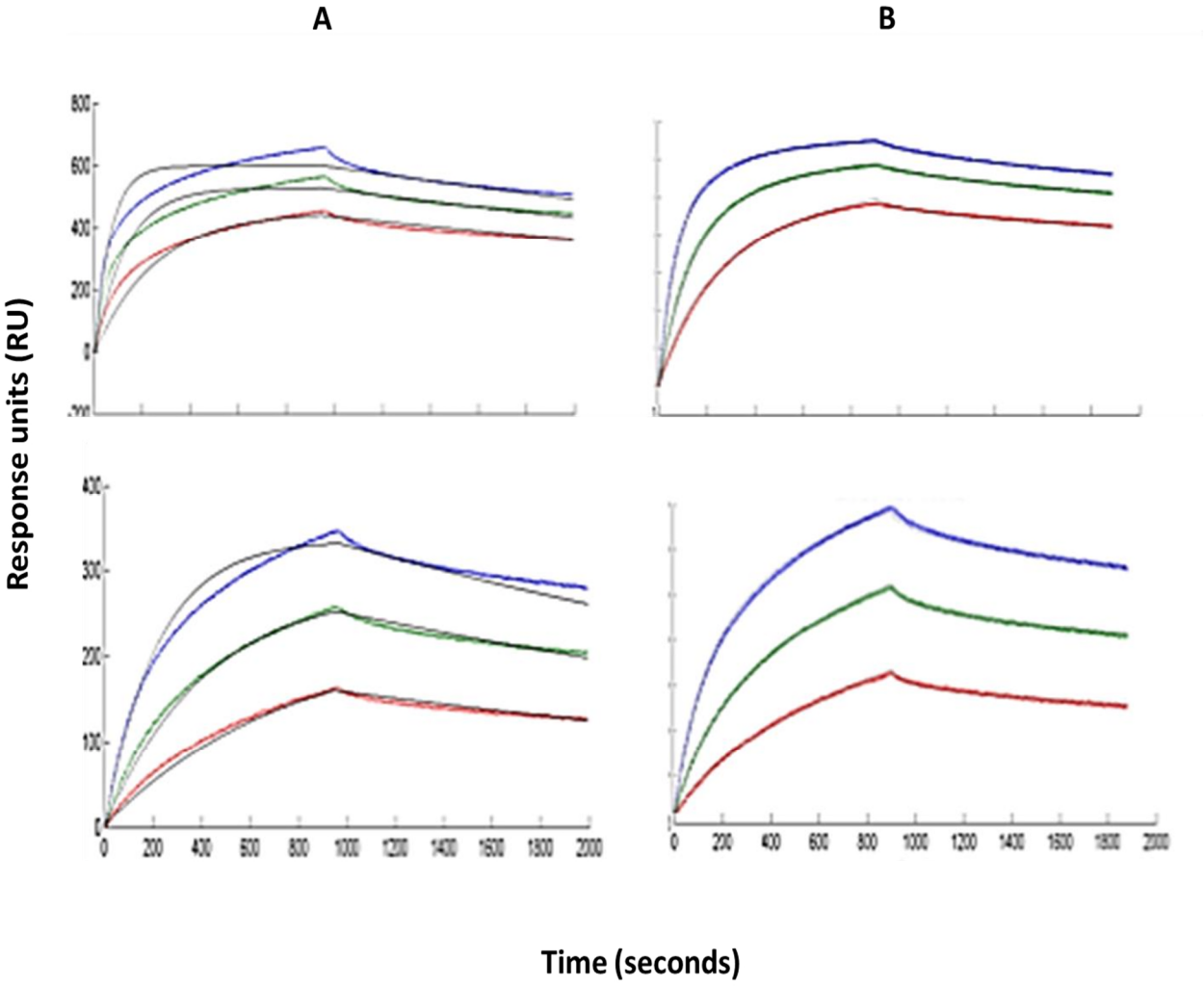


Figure 8-4 : Mathematical fitting for polyclonal HLA-specific antibodies interaction with HLA proteins using novel model (B) compared to current methods (A)

Similarly, fitting of kinetic data obtained from polyclonal samples were largely improved by applying these novel models (Figure 8-4, B) that are still under development. As seen in Figure 8-4, data fitting was better both at higher (upper panels) and lower (lower panels) concentrations of HLA-specific antibodies. These new mathematical models will undoubtedly improve accuracy when measuring HLA-specific antibody interactions with HLA proteins with potential impact on downstream clinical applications.

The SPR experiments described in this thesis relied heavily on the optimal preparation of HLA-specific antibody samples. The protocols described here were successful in allowing the study of clinical samples with a starting serum volume as low as 2 ml and has described preliminary evidence of the heterogeneity of binding parameters for different sets of HLA-specific antibodies in a cohort of highly sensitised patients.

Further studies using refined SPR or possibly BLI platforms are required to establish the range of reproducible kinetic data on larger cohort numbers and correlate these data with clinical outcomes in patients that received direct transplantation in the presence of donor specific antibodies. The presence of a clinical correlation could serve as an extremely useful biomarker for risk stratification when managing high-risk immunological organ transplantation. Similarly, binding kinetics of *de novo* DSA studied longitudinally could serve as risk predication tools for organ rejection. Three further studies are suggested to define the clinical relevance of the binding kinetics and subsequent affinity values:

1. A larger study characterising the binding kinetics of highly sensitised patients on transplant waiting lists.

2. Clinical correlation analysis between binding kinetics and adverse outcomes following direct transplantation.

3. Longitudinal measurement of binding kinetics in cases with *de novo* HLA-specific IgG responses (DSA) and relating these with adverse outcomes and success of medical intervention.

8.2.3 Wider research applications

Purified HLA-specific polyclonal antibodies are valuable tools and following work described here can now be obtained in meaningful quantities from plasma effluent of patients undergoing plasma exchange for antibody removal pre or post-transplantation. The purified IgG material will be particularly valuable for work such as assessing the impact of *defined HLA- specific antibody concentrations* on models of renal damage such as *in vitro* glomerular endothelial cell culture studies. These could examine the effects of HLA-specific antibodies on endothelial cell function, complement activation and downstream intracellular signalling pathways that could lead to cell damage. Purified HLA-specific antibodies can also be studied for glycosylation state and glycoform – a very significant field emerging in Human Immunology. Furthermore, the ability to recover purified polyclonal antibodies of this kind opens up the opportunity to study them quantitatively via a host of exciting new biophysical techniques such as thermophoresis, ion-mobility mass spectrometry and saturation transfer difference NMR spectroscopy. In addition the capturing of anti-idiotypic antibodies could be achieved by immobilising the purified HLA-specific antibodies to a solid-phase matrix via biotinylation or amine coupling and applying autologous serum to this matrix for subsequent elution.

Through establishing the performance of purification techniques such as affinity, ion exchange and size exclusion chromatography, the modification of sample handling and liquid chromatography could allow for the purification of IgG molecules from biopsy material obtained during rejection episodes. It would be remarkable to compare polyclonal HLA- specific antibodies from serum during an episode of acute antibody mediated rejection with antibodies eluted from biopsy materials.

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9 Appendix

9.1 Appendix A - Kidney Advisory Group KAG(13)34

IMPROVING ACCESS TO TRANSPLANT FOR LONG WAITING KIDNEY PATIENTS

Alex Hudson, Susan Fuggle, Robert Higgins, Rachel Johnson; Nov 2013

1. One of the objectives of the Kidney Allocation Scheme (KAS) was to reduce the number of long waiting patients on the transplant list by improving their access to transplantation. In April 2006, 15% of the kidney only transplant list had been listed for five years or longer. Within one year, the scheme had reduced this proportion to 10% although it has remained unchanged ever since.

2. A previous study presented to the Kidney Advisory Group (KAG(10)9) found that long waiting patients were predominantly highly sensitised and/or difficult to find an adequate HLA-match within the national donor pool. In 2011, the scheme was amended to allow highly sensitised patients access to Level 3 HLA-mismatched kidneys ([0 DR and 2 B] or [1 DR and 0/1 B]). This has not yet resulted in a noteworthy reduction in long-waiting patients with around 11% of the current active transplant list having waited five years or longer and 6% seven years or longer. At the last KAG meeting it was agreed that a subgroup of KAG would agree an appropriate prioritisation for such patients in the 2006 DBD Kidney Allocation Scheme.

3. Previous analysis showed that long waiting patients are predominately highly sensitised and difficult to HLA-match. Specifically, at April 2013, there were 346 patients waiting more than 7 years and 97% of them had a sensitisation of ≥ 85% (cRF%) and/or a matchability score of 8 to 10, inclusive. Further, 89% of the 346 patients waiting over 7 years had a cRF of 95-100%, with 223 (64%) being 100% sensitised.

4. Given the great difficulty in finding suitable donors for such patients it was recommended and agreed that patients waiting over 7 years for transplant should receive absolute priority for kidney transplantation (second only to any clinically urgent paediatric patients) subject to their eligibility as defined for the 2006 Kidney Allocation Scheme. That is, that no HLA mismatch 4 [(2 B mm plus 1 DR mm) or (any 2 DR mm)] transplants are allocated, and HSPs only receive a level 2 or 3 mismatch if they are local to the donor or have no unexplained (residual) sensitisation. Long waiting recipients will be ranked according to their total points score. The length of time on the waiting list that affords such priority will be regularly reviewed and may change periodically.

5. This prioritisation will be implemented as part of the DCD Kidney Allocation Scheme work in 2014. The list of patients eligible for this prioritisation will be reviewed with centres in advance to ensure that all patients remain suitable for transplant.

9.2 Appendix B - KIDNEY ADVISORY GROUP - KAG(14)45a

INCOMPATIBLE LIVING DONOR KIDNEY TRANSPLANTATION AND THE NATIONAL LIVING DONOR KIDNEY SCHEMES - RECOMMENDATIONS TO MAXIMISE TRANSPLANT ACTIVITY

1. BACKGROUND

An increasing number of donor recipient pairs who are incompatible either by ABO blood group and/or HLA wish to be considered for living donor kidney transplantation (LDKT). Options for such pairs include receiving a compatible transplant through the National Living Donor Kidney Sharing Schemes (NLDKSS) or antibody removal to facilitate incompatible LDKT. A compatible transplant is the preferred option for all recipients if available.

2. NATIONAL LIVING DONOR KIDNEY SHARING SCHEMES (NLDKSS)

The National Living Donor Kidney Sharing Schemes (NLDKSS) were established in 2007 and have subsequently been developed to maximise transplant activity and patient benefit. The NLDKSS comprise the following:

· Paired/pooled donation

· Non-directed altruistic donation

· Non-directed altruistic donor chains (introduced in January 2012).

Maximising the clinical and cost effectiveness of the NLDKSS is a key objective of the Living Donor Kidney Transplantation 2020 (LDKT 2020) Strategy. The NLDKSS currently account for 17% of annual LDKT activity and 118 non-directed altruistic donors donated into the UK programme in 2013/14.

a) General Recommendations: utilisation of the shared donor pool

The following actions are recommended with immediate effect to maximise the use of kidneys donated by both non-directed altruistic and paired/pooled donors:

i. Prioritise altruistic donor chains with all non-directed donors during donor preparation to encourage ‘opt in’ and increase the number of potential transplants per donation.

ii. If a donated kidney from either an altruistic or paired/pooled donor cannot be implanted into the intended recipient on the day of surgery, the kidney is offered through the national allocation scheme for an alternative recipient on the national transplant list.

iii. Ensure that all non-directed altruistic donor offers are accepted within 24hours by the recipient centre, as previously approved by KAG.

iv. Permit donating centres to specify dates of surgery for donations from non-directed altruistic donors to a single recipient on the national transplant list.

v. As previously approved by KAG, if multiple offers from a single nondirected altruistic donor are refused or unsuitable once a date of surgery has been scheduled, the donating centre can choose to allocate the kidney to a local recipient, to minimise disruption to the donor.

The following actions (b,c, and d) are proposed to commence from April 2015, with an opportunity to pilot some aspects in the January 2014 kidney donor matching run:

b) Increase the length of altruistic donor chains

This is a key priority to maximise transplant activity from a single non-directed altruistic donor. The inclusion of an additional donor recipient pair from the paired/pooled donation scheme each time an altruistic donor opts in to form a 'chain' would increase the transplant potential from 2 (currently) to 3 transplants.

c) Introduction of National Sharing Weeks for NLDKSS

i. Every participating transplant centre will be asked to commit to a two week 'sharing' period for NLDKSS surgery. During these weeks, centres will be asked to reserve routine living donor lists for 'shared' living donor transplants until:

· It is confirmed that there are no matched pairs for a centre in any given matching run.

Or Dates of surgery are agreed for matched pairs

ii. 'Sharing fortnights' will be linked with the matching run timetable and published with the annual matching run schedule via NHSBT. To enable all centres to accommodate this, a phased implementation is proposed: the sharing periods will initially be scheduled within a maximum of 10 weeks of the matching run, reducing to a maximum of 8 weeks to meet the clinical standard by April 2016. (Current average time frame from matching to scheduling surgery is 12 weeks).

d) Non-simultaneous donor surgery within the NLDKSS

i. Simultaneous donor surgery is the preferred option for both altruistic donor chains and paired/pooled donations. To enable centres to accommodate longer altruistic donor chains and, where necessary, paired pooled donations within the 8 week clinical standard, flexibility to arrange staggered donor surgery within the 'sharing fortnight' is recommended. This means that:

· Appropriate consent must be obtained from all donors and recipients prior to registration in the scheme re the option for staggered surgery

· In an altruistic donor chain, the non-directed altruistic donor must donate first in the chain

e) Increase Frequency of Kidney Donor Matching Runs (KDMRs)

i. Increasing the frequency of KDMRs from quarterly to two monthly would encourage donors and recipients to register into the NLDKSS and increase the transplant potential. If KAG approves this, the feasibility of streamlining existing paper registration and 'manual' processes will be explored first.

3. ABO BLOOD GROUP INCOMPATIBLE (ABOi) TRANSPLANTATION

The following outcomes and actions were agreed by the NHSBT Workshop for ABOi transplantation for endorsement by KAG:

i. Publication and dissemination of existing data to support the development of a model for standardised testing of haemagglutinin titre levels.

ii. Improve quality and approach to data collection through the NHSBT antibody incompatible transplantation (AAiT) registry to inform future guidelines.

iii. Encourage all laboratories to send samples to a single reference laboratory for pre-antibody removal and day of transplantation antibody quantification to reference local testing when standardised methodology is evaluated against existing local method.

iv. Ensure that ABOi LDKT is included as part of the inclusive transplant tariff and explore the feasibility of innovative therapies e.g. Eculizumab.

4. HLA INCOMPATIBLE (HLAi) TRANSPLANTATION KAG(14)45a AAiT and NLDKSS KAG paper 02-12-2014 Final 4

The following outcomes and actions were agreed by the NHSBT Workshop for HLAi transplantation for endorsement by KAG:

i. Agree definitions for high, medium and low HLAi transplantation.

ii. Agree guidelines, dependent on clinical urgency and patient choice to include:

· CDC positive crossmatch pairs are only considered for HLAi LDKT in exceptional circumstances.

· All HLAi pairs are entered into the NLDKSS

· ABOi LDKT within the NLDKSS is preferable to HLAi LDKT

iii. Improve quality and approach to data collection through the NHSBT antibody incompatible transplantation (AAiT) registry to inform evaluation of outcomes and commissioning.

iv. Ensure that HLAi LDKT is included as part of the inclusive transplant tariff and commission through evaluation of outcomes.

RECOMMENDATIONS

KAG is asked to:

1. Approve the proposals to maximise utilisation of donated kidneys within the NLDKSS as described in section 2.

2. Endorse the outcomes and actions from the NHSBT Workshop for ABOi and HLAi living donor kidney transplantation in sections 3 and 4.

9.3 Appendix C - Publications / Abstracts

9.3.1 Accepted peer-reviewed publication during the PhD studies

1. Higgins RM, ***Daga S***, Mitchell DA. Antibody-incompatible kidney transplantation in 2015 and beyond. Nephrol Dial Transplant. 2014 Dec 13. pii: gfu375. [Epub ahead of print] Review.

2. McMurtrey C, Lowe D, Buchli R, ***Daga S***, Royer D, Humphrey A, Cate S, Osborn S, Mojsilovic A, VanGundy R, Bardet W, Duty A, Mojsilovic D, Jackson K, Stastny P, Briggs D, Zehnder D, Higgins R, Hildebrand W. Profiling antibodies to class II HLA in transplant patient sera. Hum Immunol. 2014 Mar; 75(3):261-70.

3. Krishnan NS, Zehnder D, ***Daga S***, Lowe D, Lam FT, Kashi H, Tan LC, Imray C, Hamer R, Briggs D, Raymond N, Higgins RM. Behaviour of non-donor specific antibodies during rapid re-synthesis of donor specific HLA antibodies after antibody incompatible renal transplantation. PLoS One. 2013 Jul 26;8(7)

4. Higgins R, Lowe D, Hathaway M, Williams C, Lam FT, Kashi H, Tan LC, Imray C, Fletcher S, Chen K, Krishnan N, Hamer R, ***Daga S***, Edey M, Zehnder D, Briggs D. Human leukocyte antigen antibody-incompatible renal transplantation: excellent medium-term outcomes with negative cytotoxic crossmatch. Transplantation. 2011 Oct 27; 92(8):900-6.

9.3.2 Accepted for publication (in press)

1. S Shabir, J Girdlestone, B Kaul, S Chand, S Jham, ***S Daga***, H Smith, C Navarrete, L Harper, S Ball, D Briggs, R BorrowsTransitional B lymphocytes are associated with protection from kidney allograft rejection: a prospective study. American Journal of Transplantation (ref - AJT-B-14-00828.R2)

2. N Krishnan, R Coates, ***S Daga***, D Talbot, V Carter, R Higgins, D Briggs. Letter to editor regards to article 'ABO Incompatible Renal Transplantation without Antibody Removal Using Conventional Immunosuppression Alone'. Am J Transplant. 2014 Dec; 14(12):2807-13.

9.3.3 Conference paper

Daga S and Higgins R. What's Hot in Antibody Incompatible Renal Transplantation in 2013. CIN2013, 7th CONGRESS OF NEPHROLOGY IN INTERNET

9.3.4 Other publications with journal (Editor/reviewer)

1. R Higgins, D Lowe, ***S Daga***, M Hathaway, C Williams, F T Lam, H Kashi, L C Tan, C Imray, S Fletcher, N Krishnan, P Hart, D Zehnder and D Briggs. Pregnancy-induced HLA antibodies respond more vigorously after renal transplantation than antibodies induced by prior transplantation. With Human Immunology (ref HIM-D-14-00168R1)

2. N Khovanova, ***S Daga***, T Shaikhina, N Krishnan, J Jones, D Zehnder, D Mitchell, R Higgins, D Briggs, D Lowe. Subclasses analysis of donor HLA specific IgG in antibody incompatible renal transplantation reveals a significant association of IgG4 with rejection and graft failure. Transplantation

3. D Lowe, ***S Daga***, N Khovanova, D Mitchell, D Briggs, R Higgins, N Krishnan. "Meeting Report: 3rd International Transplant Conference: How Much Risk Can You Take?" International Journal of Immunogenetics. (ID is IJIG-Dec-14-0225).

9.3.5 Publications of work related to other projects

1. M Clark, R Higgins, A Gumber, D Moro, D Leech, A Szczepura, ***S Daga***, N West. A BETTER WAY TO MEASURE CHOICES' DISCRETE CHOICE EXPERIMENT AND CONJOINT ANALYSIS STUDIES IN NEPHROLOGY: A LITERATURE REVIEW. EMJ Neph. 2013;1:52-59.

9.3.6 Manuscripts in writing stage (presentation at conference /congress myself)

1. ***S Daga***, S Shabir, D Lowe, C Collins, D Zehnder, R Higgins, D Neil, R Borrows, D Briggs. Significance of de novo HLA specific antibodies following kidney transplantation

    a. As a moderator poster at WTC 2014 San Francisco USA and
    b. BTS 2014 Glasgow

2. ***Daga S***, Hussain S, Krishnan N, Lowe D, Patel P, Braitch M, Bentall A, Ball S, Mitchell D, Higgins R, Ian Skidmore, Zehnder D, Briggs D. Blood group-specific antibody class distribution in healthy cohort using novel multi-colour flow cytometer assay.

    a. Oral presentation in Second International conference on Antibody Incompatible Transplantation, Warwick UK 2012.
    b. As a moderator poster at BTS 2013 Bournemouth (presented by Prof Briggs),
    c. As a moderator poster at CEOT 2013 Arizona USA and
    d. As a moderator poster at BTS 2015 Bournemouth. (full results)

3. Lowe D, McMurtrey C, ***Daga S***, Buchli R, Krishnan N, Mitchell D, Cate S, VanGundy R, Bardet W, Briggs D, , Higgins R, Hildebrand W, Zehnder D. EFFECTIVE AND SELECTIVE HLA SPECIFIC ANTIBODY DEPLETION FROM HUMAN BLOOD USING SEPHAROSE COLUMNS CONTAINING IMMOBILISED HLA PROTEINS.

4. Clark M, Moro D, ***Daga S***, Watson V, Szczepura A, Gumber A, McCarthy K, and Higgins R. The application of labelled choice discrete choice experiments to establish patient preferences for different modes of renal dialysis.

5. Lowe D, Higgins R, Zehnder D, ***Daga S***, McMurtrey C, Buchli R, Hildebrand W, Briggs Cytotoxic capacity of serum from sensitised patients is due to HLA-specific antibody synergy

6. M L Addison, D Lowe, N Krishnan, D Briggs, ***S Daga***, R Higgins. HLA-specific antibodies in an untransfused male with a history of bare knuckle cage-fighting

9.3.7 Abstracts presented in conference (not quoted above)

From the listed abstracts, at least four first author papers will arise.

1. ***Daga S***, Lowe D, Buchli R, Mulder A, McMurtrey C, Moyse H, Krishnan N, Evans N, Claas F, Hildrbrand W, Briggs D, Higgins R, Zehnder D, Mitchell D. Measuring affinity of polyclonal HLA-specific antibodies in highly sensitised cases can serve as an additional biomarker to guide direct transplantation
    a. Accepted as oral presentation at BTS/NTV congress 2015 Bournemouth

2. ***Daga S***, Lowe D, Buchli R, Mulder A, McMurtrey C, Moyse H, Krishnan N, Evans N, Claas F, Hildrbrand W, Briggs D, Higgins R, Zehnder D, Mitchell. Differences in reactivity of HLA-specific antibodies may be explained by differences in their affinities for selected epitopes on HLA proteins
    a. Oral presentation at Third International conference on antibody, Warwick UK
    b. Accepted as oral presentation at BTS/NTV congress 2015 Bournemouth

3. Moyse H, ***Daga S***, Lowe D, Buchli R, Mulder A, McMurtrey C, Krishnan N, Claas F, Hildrbrand W, Briggs D, Higgins R, Zehnder D, Mitchell D, Evans N. New mathematical models for monoclonal antibody binding in surface plamson resonance experiments.
    a. Accepted as moderator poster presentation at BTS/NTV congress 2015 Bournemouth

4. Moyse H, ***Daga S***, Lowe D, Buchli R, Mulder A, McMurtrey C, Krishnan N, Claas F, Hildrbrand W, Briggs D, Higgins R, Zehnder D, Mitchell D, Evans N. New mathematical protocols for calculating binding kinetics of patient polyclonal antibody binding to HLA antigens via surface plamson resonance experiments.
    a. Accepted as moderator poster presentation at BTS/NTV congress 2015 Bournemouth

5. ***Sunil Daga,*** Curtis McMurtrey, David Lowe, Daniel Mitchell, David Briggs, William Hildebrand, Robert Higgins, Daniel Zehnder. RELEASE OF DONOR SPECIFIC SOLUBLE HLA FOLLOWING KIDNEY TRANSPLANTATION. Human Immunology
    a. 39th ASHI Meeting 2013, Chicago; 10/2013. (by Dr McMurtrey)
    b. BTS congress at Glasgow 2014.

6. Higgins, R. M.1; Lowe, D.2; Hattersley, J.1; Krishnan, N.1; Hamer, R.1; ***Daga, S***.3; Zehnder, D.3; Briggs, D.2. HLA Donor Specific Antibodies Modulate Rapidly after Easy in HLA Antibody Incompatible Transplantation in Responders, This does not Require Plasmapheresis, Rituximab, Bortezomib or Other Specialist Intervention. Transplantation: 27 November 2012 - Volume 94 - Issue 10S - p 1074 24th International Meeting; 11/2012

7. D. Lowe, S***. Daga***, N. Evans, H. Moyse, M. Chappell, D. Mitchell, D. Briggs, R. M. Higgins, D. Zehnder. Surface Plasmon Resonance - a Tool for Quantifying Binding Affinity of HLA Antibodies to HLA Proteins. Transplantation. 94(10S):356, November 27, 2012.; 11/2012

9.3.8 Invited talk and seminars

1. Characteristics of HLA-specific antibodies and transplantation – Renal Department, Nottingham (2013)
2. HLA-specific antibodies research – Renal Department, UHCW Coventry (2013 & 2014)
3. HLA-specific antibodies sub-class IgG - Grand round, UHCW Coventry (2014)
4. Affinity of antibodies – research methods and update – Warwickshire Immunology Group, UHCW, Coventry
5. Class distribution of Blood group specific antibodies using multi-colour flow cytometry assay – NHSBT Birmingham UK
6. Is it time to move on from Haemagglutination assay and look for newer aproaches to measure Blood group-specific antibodies, Renal Transplant Surgery department, Guys Hospital, London
7. Affinities of HLA-specific antibodies as a biomarker for risk stratification, Immunology department, University of Leiden, Netherlands

9.3.9 Relevant published paper (full text)

Nephrol Dial Transplant (2014) 0: 1–7
doi: 10.1093/ndt/gfu375

ndt
Nephrology Dialysis Transplantation

Full Review

Antibody-incompatible kidney transplantation in 2015 and beyond

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ABSTRACT

Rejection caused by donor-specific antibodies (principally ABO and HLA antibodies) has become one of the major barriers to successful long-term transplantation. This review focuses on clinical outcomes in antibody-incompatible transplantation, the current state of the science underpinning clinical observations, and how these may be translated into further novel therapies. The clinical outcomes for allografts facing donor-specific antibodies are at present determined largely by the use of agents developed in the 20th century for the treatment of T-lymphocyte-mediated cellular rejection, such as interleukin-2 agents and anti-thymocyte globulin. These treatments are partially effective, because acute antibody-mediated rejection is mediated to a considerable extent by T lymphocytes. However these treatments are essentially ineffective in chronic antibody-mediated rejection. Future therapies for the prevention and treatment of antibody-mediated rejection are likely to fall into the categories of those that reduce antibody production, extracorporeal antibody removal and disruption of the effector arms of antibody-mediated tissue damage.

Keywords: ABO, antibody, antibody-mediated rejection, HLA, kidney transplant

INTRODUCTION

Antibodies directed against transplants are becoming recognized as a critical barrier to further improvements in the access of patients to transplantation and in the survival of allografts. This review will summarize the current results of transplantation in the presence of donor-specific antibodies (DSA), and the possible research pathways that will lead to the control and prevention of such antibodies and the effective treatment of antibody-mediated rejection (AMR), both acute (AAMR) and chronic (CAMR).

The modern era of renal transplantation began in the 1960s with the introduction of azathioprine. Within a few years the importance of blood group incompatibility and HLA-specific antibodies was recognized, and their presence essentially vetoed transplantation outside experimental settings [1]. A focus on T-lymphocyte-mediated cellular rejection over the next half century has resulted in a therapeutic toolkit that has eliminated the vast majority of graft losses from this cause in adherent patients. Ultimately the therapies required to prevent T-lymphocyte-mediated rejection proved relatively simple, namely effective multipoint targeting of the interleukin-2 pathway and lymphocyte deletion therapy. An international focus from clinicians, scientists and industry on developing new treatments for antibody-mediated rejection (AMR) arguably began only a decade ago, and we are currently in an exciting era characterized by a rapid series of new discoveries about anti-graft antibodies, their mechanisms of production and action and the treatment of antibody-mediated rejection.

CURRENT CLINICAL OUTCOMES

HLA antibodies

Donor-specific HLA antibodies may be preformed or develop *de novo* after a transplant. The current status of transplantation across preformed HLA antibodies (HLAi transplantation) is that acceptable graft outcomes can be achieved in living donor transplantation, so long as the pre-transplant complement dependent cytotoxic (CDC) crossmatch is

negative [2]. However such transplants do require antibody screening and careful management. It is not enough simply to transplant across a negative CDC crossmatch without taking account of preformed HLA antibodies. In many patients with low pre-treatment levels of donor-specific HLA antibodies, successful engraftment may be achieved using standard immunosuppression of tacrolimus, mycophenolate, prednisolone and basiliximab. However, in cases with higher levels of DSA, for example where the flow cytometric (FC) crossmatch is positive, antibody removal and induction immunosuppression or therapies for antibody-mediated rejection are required [3–7].

As a generalization, current clinical outcomes seem to indicate a mortality and graft loss rate about twice that of 'antibody-compatible' transplantation in the first year, unless the CDC is positive when the graft loss rate is higher, rising to 50% at 5 years using CDC methodology where there is no enhancement with anti-human globulin [2], and 30% graft loss when the more sensitive technique using AHG is used [7]. Other adverse prognostic features that can be identified pre-transplant are DSA that are combinations of Class I and Class II, and DSA that bind the complement component C1q in microbead assays [7, 8].

Therapies used in such clinical series include antibody removal pre-transplantation (plasma exchange, plasmapheresis or immunoadsorption), cellular depleting therapies (antithymocyte globulin, rituximab, alemtuzumab), intravenous immunoglobulins and proteasome inhibitor therapy (bortezomib), but there is no consensus on which of these approaches is most effective, and randomized trials are awaited. Transplantation across preformed HLA antibodies is best performed with living donors where there is time to achieve effective antibody removal, and possibly the graft is better equipped to cope with the rigours of early post-transplant antibody assault [2–9].

The outcomes in the face of CAMR due either to *de novo* HLA antibody production or persistent preformed DSA production are less encouraging [7–11]. For example one series showed a 10-year graft survival of <60% in those with *de novo* DSA, compared with >90% for those without DSA [12]. Rejection takes the form of glomerular basement membrane damage (transplant glomerulopathy), with proteinuria and progressive graft failure, usually over 2–3 years. There is no effective therapy for this condition, though we have seen it resolve durably if the DSA levels fall. Often transplant glomerulopathy may be associated with some active cellular infiltration in the peritubular capillaries and this may be temporarily amenable to therapy, but ultimately nearly every case of transplant glomerulopathy progresses to graft failure within 3–5 years (Figure 1).

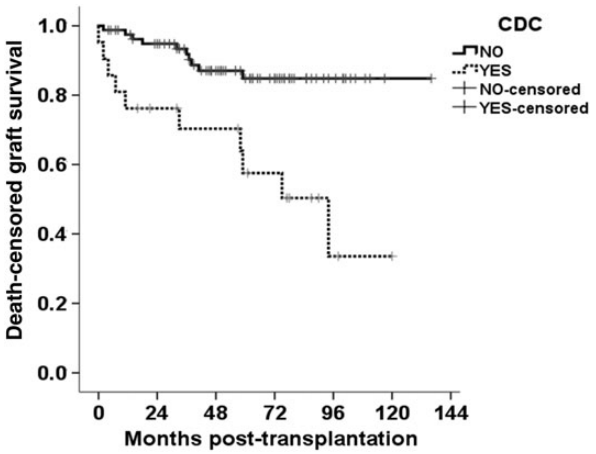

FIGURE 1: Graft survival at University Hospitals Coventry and Warwickshire in HLA antibody incompatible transplantation, for patients with pre-treatment complement dependent cytotoxic (CDC) positive crossmatch ($n = 21$) compared with those with DSA but with a negative CDC crossmatch (includes flow cytometric crossmatch positive and negative) ($n = 91$). Five- and 10-year death-censored graft survivals were 84.8% for the CDC-negative group, 57.5 and 33.6% respectively for the CDC-positive group.

ABO antibodies

Transplantation across ABO incompatibility (ABOi) generally produces excellent outcomes, and the experience in Japan over a period of decades indicates that outcomes are equivalent to ABO-compatible transplantation [13]. However, in the USA and in the UK, ABOi renal transplantation seems to have a slightly increased risk of severe AAMR which may result in graft loss, with catastrophic rejection progressing over a period of hours. This rejection may occur without any warning, and may indeed occur in those with very low pre-transplant antibody levels [14]. Further multicentre studies of this rare phenomenon (2–4% of grafts) are required.

ABOi transplantation does differ from HLAi transplantation in that chronic antibody-mediated rejection is not attributable to ABO antibodies. Biopsies may be ongoing staining for C4d on the peritubular basement membrane suggesting that there is some form of inflammation but the clinical outcome is not adversely affected. The significance of peritubular C4d differs from the same finding in HLAi transplantation, since the C4d deposition is a sign of CAMR. When CAMR is reported in ABOi grafts, this seems to be due to the concurrent presence of HLA antibodies [15].

Other antibodies

Non-HLA antibodies may be important players in AMR. Antibodies directed against the angiotensin receptor are of particular interest. These were first observed as a cause of sporadic AAMR associated with severe hypertension. Subsequent studies have extended their possible role to the causation of CAMR, and their presence has been associated with graft loss, independent of HLA antibodies [16, 17]. They may be auto- and allo-antibodies, and further work will define better the extent of their role in the causation of graft loss.

THE BIOLOGY OF ANTIBODY PRODUCTION AND REJECTION

Decades of focus on T-cell-mediated rejection and the lack of a clinical model of antibody incompatible transplantation mean that our understanding of the biology of antibody production, and the mechanisms of AMR are at a relatively early stage, but knowledge is accumulating rapidly.

Production of antibodies

Antibodies are synthesized by memory B lymphocytes and plasma cells. Both of these ultimately derive from immature cells of the B-lymphocyte lineage and receive T-cell help. It is possible that induction immunosuppression, enhanced beyond the level necessary to prevent most T-lymphocyte-mediated cellular rejection, may reduce the production of *de novo* HLA antibodies [18], and prospective and randomized studies are awaited.

In HLAi transplantation, pre-formed donor-specific HLA antibodies may have been present for many years before the transplant, and detailed monitoring of their levels in the early post-transplant period may show large changes in their levels, with both rises and falls. Figure 2 shows a case with a heterogeneous response, the rates of post-transplant increases in DSA, the pre- to peak variation and the rate of fall of DSA showing variation. The rises and falls in HLA antibody levels post-transplant are not uniform across a range of patients, and there is no clear pattern governing the responses, though HLA Class II antibodies may show greater responses than Class I, with the profiles of HLA DQ and DP similar to HLA DR [19].

It is likely that insights can be gained from more detailed studies of the characteristics of the antibodies, for example the classes and IgG subclasses of the responses (Table 1). New antibody production is initially of the IgM class, and IgM donor-specific HLA antibodies have been associated with AAMR in the absence of equivalent IgG antibodies [20] and reported in *de novo* HLA antibody production [21]. HLA antibodies of the IgA class may also occur frequently, though their significance is not yet understood [26]. IgM antibody production may switch to IgG3 subclass, then to IgG1, IgG2 and ultimately IgG4. There is currently much interest in examining antibody subclasses in more detail [21], and we have observed some differences in subclass distribution, for example a higher incidence of IgG3 subclass in pregnancy-stimulated HLA antibodies and an association between pre-transplant IgG4 levels and subsequent AMR and graft survival [22].

One of the most remarkable observations in clinical practice is that DSA may disappear after transplantation. This has been observed in both ABO and HLA antibody-incompatible transplantation [13, 14, 19]. However, it seems likely that the mechanisms are different.

In HLA antibody-incompatible transplantation, many groups have noted that in some patients HLA antibodies may disappear after the transplant, even when sensitive microbead analysis is used. While some groups have attributed this to particular treatments administered, we have seen that this phenomenon is independent of any particular therapy over and above the standard immunosuppression of basiliximab, tacrolimus, mycophenolate and prednisolone [19]. The phenomenon does seem to be commoner when there is a vigourous antibody response post-transplant, and daily sampling shows that the fall in antibody levels occurs rapidly. This implies some form of 'active' removal of antibody from the circulation, and this is the subject of ongoing research. If it proved possible to induce the disappearance of DSA pre-transplant, that would be very useful. However,

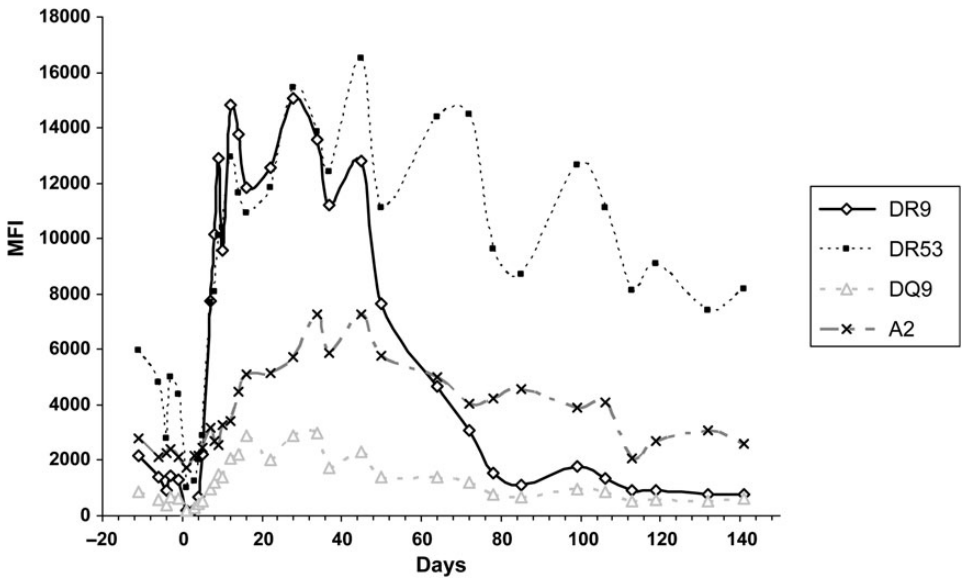

FIGURE 2: HLA and ABO antibody levels pre- and post-transplant showing variation in antibody responses within the same patient, both in terms of rate of increase, magnitude of change from pre- to peak levels, and the rates of fall. The patient received a living donor transplant from his father. DSA levels were as shown in the legend. The CDC crossmatch was positive at a titre of 1 in 16, and there was additional ABO incompatibility, donor blood group A1 and recipient B. The recipient received plasma exchange pre-transplant, and post-transplant at Days 21 and 22 only. Immunosuppression was with prednisolone, tacrolimus, mycophenolate and basiliximab, with anti-rejection treatment including ATG (Days 1–15) and eculizumab (Days 24 and 31). The graft is still functioning at 5 years post-transplant, though with proteinuria and reduced function. DSA levels were measured by microbead assay (OneLambda). MFI levels were DR9—pre 2148, peak 14821 (Day 9), late 776; DR52—pre 5982, peak 12952 (Day 9), Day 141 8162; DQ9—pre 864, peak 1468 (Day 9), late 618; A2—pre 2774, peak 7258 (Day 45), late 2612. Follow-up at 2 years post-transplant showed little change in antibody levels from the Day 140 data shown here. Haemagglutination titres to blood group A1 were, IgG, pre 1, peak 128 (Day 10), late 4; IgM, pre 4, peak 128 (Day 10), late 32.

Table 1. HLA characteristics of antibody and antigen that might affect clinical outcomes

	Comment	Reference
HLA antibody		
Concentration	Not currently possible to measure antigen-specific concentration of antibody (see Figure 3)	
Antibody binding to HLA in solid phase assay (readout a combination of avidity and concentration)	Measureable by microbead assays	[2, 4]
Class	Mostly IgG, early reports of IgM mediated AMR and occurrence of IgA	[20, 21]
Subclass	Early studies show heterogeneity in responses	[21, 22]
Affinity	Measurement under investigation	
Glycosylation	Measurement under investigation	
Cellular binding	CDC and FC crossmatches Endothelial binding	[2, 23]
Inhibitors		
Soluble HLA	Early reports of methods to quantify	
HLA-E	Early reports of correlation with clinical outcomes	[24]
Idiotypic antibodies	Hard to measure with current tools but could be important	[25]
Immune complexes		
HLA-sHLA immune complexes	Not currently measureable	

it is not clear whether this requires exposure to donor HLA (and if so in what form of delivery), as well as immunosuppression.

In ABOi transplantation, successful engraftment is often followed by the disappearance of antibody, but biopsies of the graft show ongoing C4d staining in peritubular capillaries, as if there is some persistent immunological stimulation, making it possible that some antibodies are being produced and then absorbed by the graft. It will be interesting to see what happens to ABO antibodies in patients who later lose their grafts from causes other than ABO-related rejection, and then have graft nephrectomy—will their ABO antibodies reappear or not? ABOi heart transplants in neonates may result in long-term tolerance to the transplanted ABO, but this could be a special case owing to the neonatal timing of the transplant.

Binding of antibodies to an allograft

There is much current research examining how antibody binds to antigen at a molecular level, and then how that response evolves into clinical rejection, since the simple act of antibody binding to antigen alone does not seem to cause rejection. Although the development of microbead assays have enabled far better measurement of antibody levels than was previously possible, they do not measure the concentration of antibody, even in a semi-quantitative manner, but measure a combination of concentration and affinity, as shown in Figure 3. Table 1 lists some of the antibody characteristics that

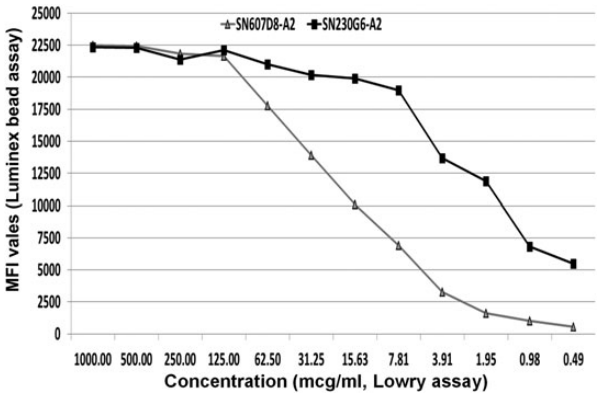

FIGURE 3: Dose response curve showing Luminex bead MFI value against concentrations of two human monoclonal HLA-A2-specific antibodies. The same concentrations of antibody may give markedly different MFI levels (e.g. <2000 and >10000, respectively, at 1.95 μg/mL).

could be associated with clinical outcomes; we are still at a preliminary stage of the understanding of which of these are important, and which could be manipulated for therapeutic benefit.

HLA is profoundly polymorphic, and the binding of antibody to an HLA molecule is determined not by the whole HLA molecule, but by small areas of polymorphism within the protein structure known as epitopes. An epitope may be determined by as little as a single amino acid substitution in a critical area of the molecule, even though the binding footprint of an antibody molecule is much larger. Different HLA molecules are defined by a series of epitopes, many of which are shared between different HLA specificities. This is why an antibody generated against one HLA allele will bind to many others [27].

A better understanding of exactly how the antibody-antigen reaction develops may allow for the development of therapies that disrupt this interaction. Hence, research groups are studying antibody affinity for antigen, the atomic structure and biophysical properties of HLA, especially in relation to glycosylation as well as the amino acid backbone.

Clinical studies of the mechanisms of rejection are hampered by the inability of current techniques usefully to measure the binding of antibody to the graft [15]. It is not clear why this is the case, but the clinical classifications of antibody-mediated rejection depend on the detection of effector responses such as complement and cells, and not on whether there is any donor-specific antibody in the graft. A test that gave an accurate readout of the levels of donor-specific antibody bound to graft endothelium would give immediate and important insights into AMR.

Complement

It is widely acknowledged that activation of the complement system is a major driver of antibody-mediated tissue damage and subsequent transplant rejection. A good indicator of this is the CDC assay that correlates positively with risk of donor organ failure [2]. The presence of the complement protein fragment C4d in renal rejection is also a 'smoking gun'

for activation of the complement cascade, which occurs downstream of C1q binding to clustered complexes of IgG and IgM antibodies on a cell surface. The antibody classes and subclasses typically found are IgG1 (HLA antibodies) and IgM (ABO antibodies), which are strong C1q binders and thus classical pathway activators. However, clinical investigation is made more difficult by the difficulties in measuring DSA in graft biopsies, and also by the observations that AAMR may occur in the absence of C4d deposition, and that C4d deposition may not reflect ongoing rejection; this could partly be due to endothelial synthesis of C4, so C4d should not necessarily be assumed to have been deposited on endothelium from circulating C4 [28].

Cellular response

The cellular aspect of acute antibody-mediated rejection is interesting, because although hyperacute rejection looks more like an innate response, with neutrophils and macrophages predominately, AAMR is characterized by a marked T-lymphocyte infiltration into the graft, visible using immunohistochemistry, and indistinguishable from T-cell-mediated cellular rejection at a molecular level [29, 30].

Response of endothelium

The predominant target of antibody-mediated rejection is vascular endothelium in the blood vessels (and glomeruli) of the allograft. Endothelial cells express ABO and HLA (classes I and II). Endothelium is not a passive victim of antibody binding and the rejection process, but has an adaptive response that may be protective to the graft. Indeed, clinical acute antibody-mediated rejection generally resolves in the presence of donor-specific antibody, and while this may be due to therapies which down-regulate the cellular responses, it is also possible that the graft becomes resistant to rejection [23, 28]. In the clinical setting, it is possible to measure vascular stress in the allograft by RNA analysis of renal biopsy material, the 'molecular microscope'. This approach can also help characterize the T- and B-cell activity within the allograft [31].

In some cases, the antibody binds to endothelium but does not cause rejection, for example in ABOi transplantation, where staining for complement C4d in the peritubular capillaries of well-functioning grafts seems to indicate accommodation of the graft to antibody. In HLAi transplantation, by contrast, longer term C4d staining is likely to indicate low grade-AMR, though in some cases there does seem to be DSA present for many years without causing rejection or C4d staining on biopsy.

NEW THERAPIES

The successful treatment and ideally prevention of antibody-mediated rejection will require the development of new therapies that have specific new actions. It is not yet clear exactly which are the ideal targets, or whether there will be a solution as conceptually simple as the targeting of interleukin-2 in T-lymphocyte-mediated rejection. Therapies under consideration can be grouped into three main categories; (i) those that target the cells responsible for antibody production; (ii) those that target mediators of antibody damage, including complement; (iii) more effective removal of antibody using extracorporeal techniques.

Targeting the cells responsible for antibody production

Once the body has been programmed to produce the antibody in the long term, the cells responsible for this can be hard to target. For example, while bone marrow ablative therapy for haematological malignancies may appear to result in deletion of the host immune system, many antibody responses induced by prior vaccination may persist. We have seen HLA antibody production persist after chemotherapy and total lymphoid irradiation conditioning for cord cell transplantation.

There are some candidate therapies in use that could prove beneficial, but have not yet been tested in randomized trials. Rituximab (CD20 monoclonal antibody) and alemtuzumab (CD52 monoclonal antibody) are both capable of killing B lymphocytes, though do not have specific action against plasma cells, and both may spare memory B lymphocytes [18, 31, 32]. In the case of alemtuzumab, any benefits in clinical use might be due to deletion of effector leucocytes rather than effects on antibody production, and its use has even been reported to be associated with increases in the production of *de novo* HLA antibody levels in previously unsensitised transplant recipients [33]. In clinical use, neither agent can completely prevent increases in preformed HLA antibody levels after transplantation, though some data suggest that *de novo* HLA antibody production after transplantation may be reduced by prior administration of anti-CD20 therapy.

Anti-thymocyte globulin has potentially a very wide action, and may be particularly effective in dealing with cells in the effector arm of the rejection process, but again does not seem to completely prevent post-transplant synthesis of preformed DSA, although in a non-randomized study, its use was associated with less production of *de novo* HLA antibodies in the first 2 years post-transplant [33].

The cells that produce antibody could be targeted by means other than cytolytic therapies, and there are some candidate therapies available. Intravenous immunoglobulins (IVIg) have been used for some time. This product has a range of actions, but may down-regulate the production of HLA antibodies, and may be combined with anti-CD20 therapy [34]. Cytokine inhibition may be another route available, and studies are currently underway to evaluate the possible benefits of inhibitors of interleukin-6 and BAFF (B-cell activating factor). BAFF is an attractive target, as studies are emerging that associate BAFF with clinical outcomes in transplantation [35].

A further approach to reducing antibody production is targeting the protein synthetic capacity of plasma cells using proteasome inhibitors. Although these agents were initially conceived selectively to target malignant plasma cells, it is possible that they may impact on metabolically active cells producing DSA post-transplant, and uncontrolled studies have shown successful transplantation in the face of active immune responses [36, 37]. The effects of bortezomib on pre-transplant antibody production have been less encouraging, but randomized

trials and the introduction of second generation proteasome inhibitors are welcomed with great interest.

Extracorporeal antibody removal

Most protocols for clinical antibody-incompatible transplantation involve extracorporeal antibody removal, at least in those with higher antibody levels. While the effects of antibody removal are partial and temporary with the current technology, it is generally believed to be beneficial [38, 39]. Randomized trials in the setting of preformed antibodies are lacking, but it does seem that removing antibodies to a level below the threshold of CDC crossmatch positivity prevents hyperacute rejection, and that plasma exchange is an effective therapy for AAMR.

Antibody removal therapies are constrained by several difficulties. These include the problem of providing long hours of therapy during times when there is a risk of bleeding; the pool of IgG outside the vascular compartment, which re-equilibrates only slowly with the vascular compartment; by rapid production of the antibody in the post-transplant period and by the inability of current therapies to selectively remove HLA antibodies, as all therapies remove total immunoglobulin, often together with other desirable blood components such as fibrinogen [39]. Similar constraints are also faced when total immunoglobulin removal methods are used in ABO-incompatible transplantation [40].

There is therefore a need for more selective therapies and modes of delivery that will allow removal of enough antibody at the times before and after transplant, and maybe soon after biopsy, and will impact on rejection at times when there is rapid antibody production (a doubling time of 12 h has been observed in some of our patients) [41]. The availability of large amounts of HLA protein has allowed the production of a selective HLA absorption column that indicates as proof of principle that HLA antibody removal therapy could be improved. Specific immunoadsorption therapy is already available to remove ABO antibodies [40, 42].

Mediators of antibody damage

Inhibition and modulation of complement activation is an attractive therapeutic route, with FDA-approved biopharmaceuticals already in clinical use for rare conditions such as hereditary angioedema (HA) and paroxysmal nocturnal haematuria (PNH). These include the drugs cinryze (C1 esterase inhibitor) for HA and eculizumab (anti-complement C5 monoclonal antibody) for PNH [43, 44]. These two agents act at different points within the complement activation cascade with potential to protect cells and tissues directly from aggressive lytic mechanisms and also broader immune activation. C1 esterase inhibitor is a protein that blocks the enzymatic function of the complement C1s component. This results in the proteolytic activation cascade being arrested upstream of powerful inflammatory and cytotoxic mechanisms such as C3/C4 opsonisation, anaphylatoxin release (a powerful leucocyte chemoattractant mechanism) and assembly of the membrane attack complex.

Eculizumab acts further downstream through functional blockade of the complement component C5, resulting in abrogated membrane attack complex formation and C5a anaphylatoxin release. Recruitment into an international randomized clinical trial of eculizumab in patients with pre-formed HLA antibodies has been completed, and results should be available in 2015. If a positive effect of this agent is shown, this will be pivotal in the future therapy of AMR, and possibly in the prevention of CAMR. If the randomized trial shows a marked reduction in AAMR rates in line with preliminary studies, there will be important questions to answer on the cost-effective application of the drug, and also whether its benefit will be extended to those with higher DSA levels who were not eligible for the current study.

SUMMARY

Antibody-incompatible transplantation has become a clinical reality in the 21st century, though graft survival rates are still suboptimal. Many patients with very high levels of preformed DSA, both HLA and ABO, are considered untransplantable with current technology. Treatment for CAMR when it leads to transplant glomerulopathy is almost completely ineffective. The understanding of the drivers of antibody production and mechanisms of AMR is progressing rapidly, and it is likely that combinations of new therapies targeting antibody production, complement and extracorporeal antibody removal will improve graft survival rates in the coming decades.

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CONFLICT OF INTEREST STATEMENT

The authors are co-researchers with Pure Transplant Solutions in production of immunoadsorption column to remove HLA antibodies.

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Profiling antibodies to class II HLA in transplant patient sera

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ABSTRACT

Immunizing events including pregnancy, transfusions, and transplantation promote strong alloantibody responses to HLA. Such alloantibodies to HLA preclude organ transplantation, foster hyperacute rejection, and contribute to chronic transplant failure. Diagnostic antibody-screening assays detect alloreactive antibodies, yet key attributes including antibody concentration and isotype remain largely unexplored. The goal here was to provide a detailed profile of allogeneic antibodies to class II HLA. Methodologically, alloantibodies were purified from sensitized patient sera using an HLA-DR11 immunoaffinity column and subsequently categorized. Antibodies to DR11 were found to fix complement, exist at a median serum concentration of 2.3 μg/mL, consist of all isotypes, and isotypes IgG2, IgM, and IgE were elevated. Because multimeric isotypes can confound diagnostic determinations of antibody concentration, IgM and IgA isotypes were removed and DR11-IgG tested alone. Despite removal of multimeric isotypes, patient-to-patient antibody concentrations did not correlate with MFI values. In conclusion, allogeneic antibody responses to DR11 are comprised of all antibody isotypes at differing proportions, these combined isotypes fix complement at nominal serum concentrations, and enhancements other than the removal of IgM and IgA multimeric isotypes may be required if MFI is to be used as a means of determining anti-HLA serum antibody concentrations in diagnostic clinical assays.

1. Introduction

Donor-specific antibodies to class I and class II human leukocyte antigens (HLA) represent a contraindication to organ transplantation. Before transplantation, sensitized patients who produce HLA-specific antibodies typically wait longer to receive a transplant. Post-transplantation, antibodies to the organ-donor's HLA contribute to hyperacute, acute, and chronic transplant rejection [1,2]. These alloantibodies mediate rejection by a number of mechanisms that include activation of the complement cascade, killing via FcγRs following innate immune cell recruitment, inflammation accompanying epithelial cell migration, and cell apoptosis [3–10]. While antibodies represent a substantial barrier to allogeneic transplants, antibody responses can differ substantially from patient-to-patient depending upon the HLA antigen in question, the route of immunization, and the immune status of the responder; substantial heterogeneity is expected among antibody responses to HLA. Indeed, variability among allogeneic immune responses has led to the observation that not all antibodies to HLA promote graft failure [11–13]. As such, a more thorough understanding of anti-HLA antibodies in transplant patients would provide new avenues for pinpointing those immunoglobulins that are truly a contraindication.

Abbreviations: cDNA, complementary DNA; BLCL, burkitt's lymphoma cell line; ELISA, enzyme-linked immunosorbent assay; TRIS, tris(hydroxymethyl)aminomethane; HPLC, high-performance liquid chromatography; QTOF, quadrupole time of flight; HCl, hydrochloric acid; DEA, diethyl amine; MFI, mean fluorescence intensity; CDC, complement dependent cytolysis; LCMS, liquid chromatography mass spectrometry; sHLA, soluble HLA; NHS, N-Hydroxysuccinimidyl; SEC, size exclusion chromatography.

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Experimentally, the phenotypic and functional evaluation of antibodies to HLA remains challenging for several reasons. First, anti-HLA antibody responses can be polyclonal, recognizing multiple epitopes on an allogeneic HLA. Second, sensitized individuals can target multiple HLA, whereby it is not clear whether one antibody response is crossreactive with various HLA or whether individual serologic responses target different HLA. Third, anti-HLA antibodies are intermingled in a complex blend of serum immunoglobulins. Clearly, the isolation of antibodies to a given HLA molecule would enable subsequent studies of anti-HLA antibody concentration, isotype, epitope specificity, and affinity. Such measurements could then be compared to transplant function/survival in order to correlate distinct humoral responses with clinical outcomes. In addition to shedding light on how antibody variables influence clinical outcomes, the ability to isolate anti-HLA antibodies would help to unravel the impact that antibody isotype, concentration, and affinity have on diagnostic bead-based assays – an area of considerable interest [11–13]. In particular, clinicians and HLA laboratory technicians share an interest in assigning an antibody titer to their HLA-sensitized patients to determine risk, a calculation that is especially effected by antibody heterogeneity.

The hypothesis tested here is that the isolation and profiling of anti-HLA immunoglobulins will demonstrate person-to-person differences in alloreactivity and lead to a better understanding of how this variability influences diagnostic tests and clinical outcomes. To test this hypothesis, appreciable quantities of native class II HLA molecules were produced and utilized to construct the first reported HLA immunoaffinity column. Patient sera containing anti-HLA immunoglobulins were then passed over this column. Antibodies specific for a particular class II HLA were retained on the column, these immunoglobulins were recovered, and these anti-HLA antibodies were profiled for concentration, isotype, cross-reactivity, complement activation, and impact on antibody screening assay outcomes. The resulting phenotypic and functional profiles represent a substantial advance in our understanding of anti-HLA antibody variability, providing new insight as to how immunoglobulin heterogeneity can influence diagnostic tests and transplant outcomes. More robust applications of this HLA antibody isolation and profiling technology are discussed, including the potential provision of an HLA-based therapeutic antibody removal tool for patients with antibodies to allogeneic HLA.

2. Methods

2.1. Patient samples

Patient 'G' serum is a HLA-DR11antiserum with complement fixing activity (Gen-Probe, WI). Patient 1 serum was gathered from a DR11 sensitized kidney recipient (informed consent approved by UT Southwestern institutional review). Sensitized serum from patients 2 to 12 were collected according to a protocol approved by the University of Warwick institutional review board. For sensitized patients 13–14, 600 mL of double filtration plasmapheresis retentate was recovered as approved by the University of Warwick institutional review board. For Patient 13, 600 mL of retentate was diluted in 1.8 L of PBS, for patient 14 350 mL of retentate was diluted in 1.8 L HLA antibody-negative plasma.

2.2. HLA-DR11 protein production

To produce secreted HLA-DR11 (sHLA) molecules, α-chain cDNA of HLA-DRA1*01:01 and β-chain cDNA of HLA-DRB1*11:01 were modified by PCR mutagenesis to delete codons encoding the transmembrane and cytoplasmic domains. For DRA1*01:01, a 7 amino acid linker (DVGGGGG) followed by leucine-zipper ACIDp1 was added [14]. For DRB1*11:01, this linker was followed by leucine zipper BASEp1 [14]. sHLA-DRA1*01:01/DRB1*11:01 were cloned into the mammalian expression vector pcDNA3.1(–) (Life Technologies, CA). A B-LCL cell line was transfected by electroporation simultaneously with sHLA-DRB1*11:01 and DRA1*01:01. Drug resistant stable transfectants were tested for production of sHLA class II molecules by sandwich ELISA using capture antibody L243 (Leinco Technologies) and a class II detection antibody (One Lambda Inc., CA). Approximately 75 mg of sHLA-DR11 was produced in AccuSyst-Maximizer bioreactors (Biovest International) and purified using an L243 immunoafffinity column.

2.3. Mass spectrometry

10 μg of sHLA-DR11 was reduced with dithiothreitol (Sigma–Aldrich, MO) at 95 °C for 5 min and alkylated using iodoacetamide (Thermo Scientific, MA). Denatured protein was digested with trypsin (Thermo Scientific, MA), reconstituted in 30% acetic acid, and loaded onto an UltiMate 3000 HPLC system (Dionex) with a PepMap100 C18 75 μm × 15 cm, 3 μm 100 Å reverse-phase column. Peptides were analyzed on a QTOF Qstar Elite mass spectrometer (ABSciex, MA) and Mascot (Matrix Science, MA) software.

2.4. Immunoaffinity columns

A 1 mL DR11 gravity column and a 65 mL DR11 pump-flow column were prepared using NHS-activated Sepharose 4 FastFlow matrix (GE Life Sciences, NJ) at a ratio of 1 mg of sHLA-DR11 per 1 mL of matrix. A 1 cm diameter glass gravity column enclosure was used for the 1 mL column. A 65 mL Glycosorb column enclosure (Glycorex, Sweden) was utilized for the large DR11 column.

2.5. Alloantibody purification

Passing 1 mL of undiluted sera over the 1 mL gravity column purified patient antibodies. The column was washed with 7 mL of PBS pH 7.4 and eluted with 4 mL of 0.1 M glycine, pH 11. Eluate was neutralized in 1 M TRIS, pH 7.0 at a ratio of 1:5 TRIS:Eluate. For patients 13 and 14, 2.4 L and 2.1 L respectively of plasma was passed over the 65 mL column at a flow rate of 35 mL/min, washed with 1 L of PBS pH 7.4, and antibodies eluted with 240 mL of 0.1 M glycine. After each load/elution cycle, the columns were mock eluted with glycine and washed with PBS. For the L243 column test (SFig. 2), 1 mL of L243 at 200 μg/mL was absorbed by gravity and 25/200 μL flow-through fractions collected. Antibodies were eluted using 5 mL of 50 mM DEA pH 11.3. 20 fractions were collected, immediately neutralized with 1 M TRIS, and antibody content determined by OD_{280}.

2.6. Single antigen bead assays

For the single antigen bead experiments (Figs. 1 and 2) anti-HLA antibodies in the pre-column serum, flow-through, and eluate were identified using LIFECODES LSA Class II single antigen assay (Gen-Probe, WI). Briefly, bead suspension was incubated with test sample at room temperature for 30 min on an orbital shaker according to the manufacturer's ratio of beads to sample. Beads were washed and incubated with supplied LSA Conjugate Concentrate (goat anti-human IgG PE diluted ten-fold) for 30 min. Alternatively, LabScreen Class II Single Antigen Beads (Lot#009) were used to determine MFI values (One Lambda Inc., CA). Briefly bead suspension was incubated with sample and incubated at room temperature for 30 min. Beads were washed and incubated with 50 μl the detecting antibody (anti-human IgG PE secondary antibody diluted 100-fold & supplied by One Lambda) for 30 min.

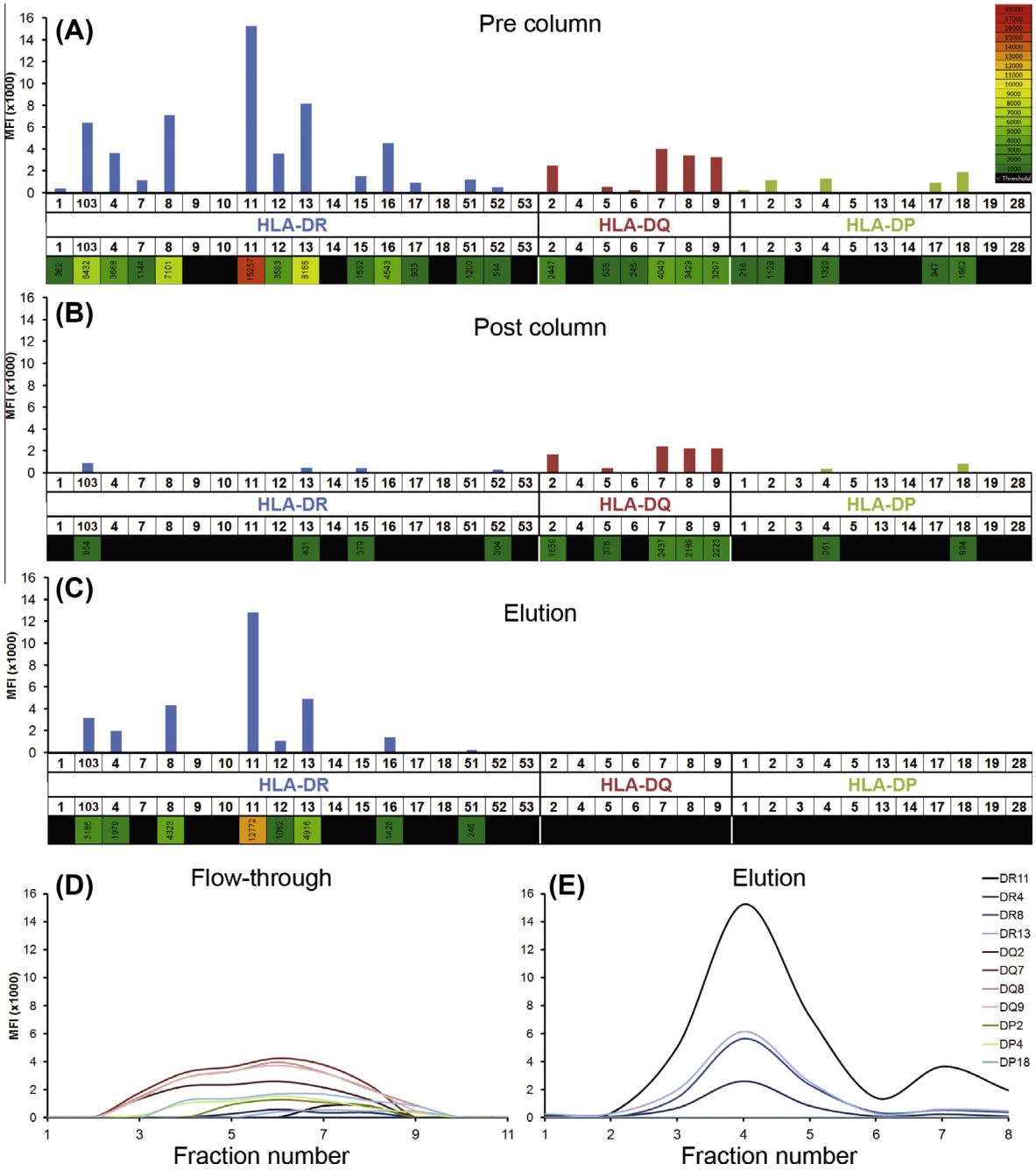

Fig. 1. Specific depletion and recovery of DR11 Alloantibodies from sensitized sera. Data in A–C, is shown as both a histogram as well a heatmap. 1 mL of DR11 sensitized sera was passed over a 1 mL sHLA-DR11 column, washed, and eluted off the column. MFI values of the sera before (A) and after (B) passage over the DR11 column. (C) MFI values of the neutralized column eluate. (D) MFI values of approximately 160 µl (3 drop) fractions of the flow-through (1 mL of sera followed by 1 mL of PBS). (E) MFI values of approximately 160 µl (3 drop) fractions of the elution. In D and E each line represents MFI values from the indicated allomorph.

After incubation with the secondary antibody the beads were washed and analyzed on a Luminex 100 analyzer. To account for technical variations in the assay, MFI values were normalized to 20,000 using the positive control beads according to the following equation: Normalized MFI = (20,000/positive control bead MFI)* MFI. A normalized value of 20,000 is the rounded average maximum positive control bead MFI.

2.7. Immunoglobulin isotyping

Antibody isotyping and quantification were completed using the Bio-Plex Pro immunoglobulin isotyping kit (Bio-Rad, CA). Briefly, 10-fold sample dilutions in 50 µl were incubated with 50 µl of bead suspension for 30 min. Beads were washed and incubated with a biotynlated secondary antibody for 30 min. Beads were then washed and incubated with streptavidin PE for 30 min. Last, beads were washed and analyzed on a Luminex 100. Sample MFI values were translated into Ig concentration using the Ig specific standard curves and the Bio-Plex Manager software.

2.8. Complement dependent cytolysis

Complement dependent cytolysis (CDC) was determined using the Lambda Cell Tray: 30 B cell panel (One Lambda Inc., CA). Lysis was performed according to manufacturer protocols. Rabbit complement was used as a source of complement. After lysis,

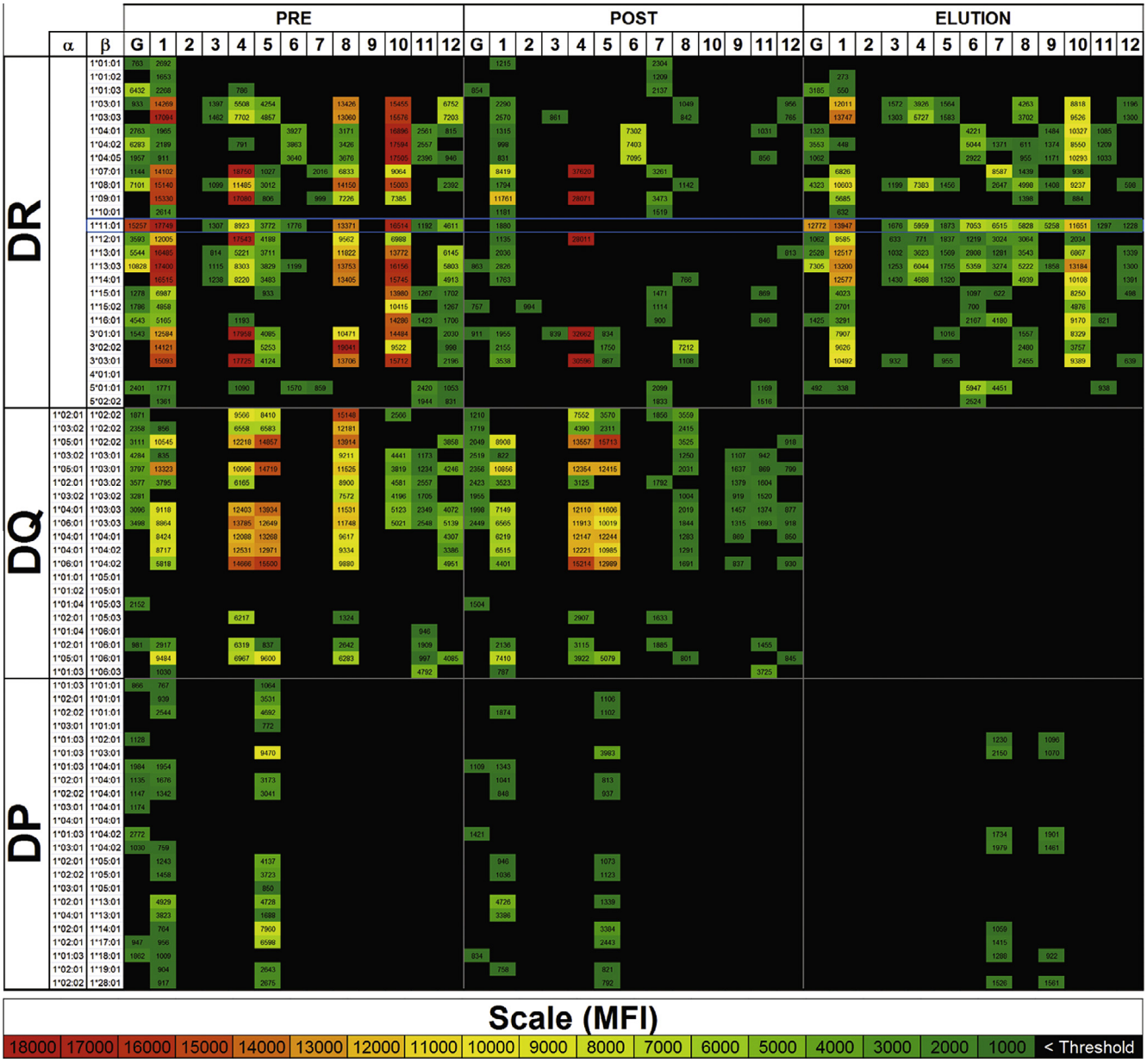

Fig. 2. Purification of DR11 specific alloantibodies from multiple patient sera. Heatmap indicating MFI values for each allomorph on the panel for the sera before the column (PRE), after the column (POST), or in the elution (ELUTION) in patients G-12. Scale of the heatmap is shown on the bottom panel. For clarity, values below the threshold are blacked out. HLA-DR11 MFI values are outlined in blue. Threshold values for PRE and POST were determined by taking the average bead MFI of negative sera plus 5 standard deviations. Threshold values for ELUTION were determined by taking the average DQ bead MFI plus 5 standard deviations.

FloroQuench dye, containing a mix of acridine orange and ethidium bromide, (One Lambda Inc., CA) was used to differentiate live from lysed cells. Live and lysed cells were analyzed using a Nikon TE200-E fluorescent microscope (Nikon, NY). Whole well images were generated using the 4x objective lens for the green (excitation: 490 nm bp 20, emission: 520 nm bp 38) and the red filter (excitation: 555 nm bp 28, emission: 617 nm bp 73). Total fluorescence was determined using MetaMorph v 7.5.5.0 and percent cell death was calculated: red fluorescence/(red fluorescence + green fluorescence).

2.9. Size exclusion chromatography

IgM and IgA multimers were separated from monomeric Ig using size exclusion chromatography. Human IgM, IgA, and IgG controls were from Sigma–Aldrich, CA. 10 μl at 1 mg/mL of human IgM, IgA, IgG, or purified alloantibodies were injected into a Paradigm MS4 HPLC (Michrom, CA) and run over a Biosep-SEC-s4000 SEC column (Phenomenex, CA) (4.6 mm ID × 300 mm length) at a flow rate of 220 μl. Antibody elution chromatograms were measured by absorbance at 215 nM of the eluting species.

2.10. Statistical analysis

All statistical analyses were performed using GraphPad Prism 5 software. Data variance was determined using a D'Agostino and Pearson omnibus normality test. On parametrically distributed data, mean and standard deviation is used to describe the data. Significant differences in mean values were determined using an unpaired *t*-test. On non-parametric data, medians and interquartile

range is used to describe the data. Significant differences in median values were determined using a Mann–Whitney test.

3. Results

3.1. HLA class II immunoaffinity columns

The isolation of anti-class II HLA antibodies requires a source of plentiful, native class II HLA. Here we prepared DNA constructs for a secreted HLA-DRA1*01:01/HLA-DRB1*11:01 alpha/beta heterodimer by replacing the transmembrane domain of the alpha and beta chains with a 7 amino acid linker followed by an ACIDp1 or BASEp1 leucine zipper domain respectively (SFig. 1A) [14]. This approach was implemented so that (1) the lack of a transmembrane domain would make the class II complex soluble whereby transfected B lympoblastoid cell lines would continually secrete the desired alpha/beta/peptide complex, and (2) the leucine zipper domain would bring and keep the HLA-DRA1*01:01/HLA-DRB1*11:01 heterodimer together in solution.

To confirm that the secreted class II HLA molecules purified from tissue culture harvests were indeed HLA-DRA1*01:01/HLA-DRB1*11:01 heterodimers, the purified class II protein was digested with trypsin and subjected to liquid chromatography mass spectrometry (LCMS) analysis. In a BLAST analysis, the only protein sequences detected were derived from the transfected sHLA-DR11 alpha and beta chains of the class II complexes produced and isolated here (SFig. 1B).

Pure sHLA-DR11 was covalently coupled to Sepharose-4 Fast Flow to create an immunoaffinity column. In order to confirm the serologic activity of secreted class II HLA was maintained following its coupling to a column, the anti-HLA-DR monoclonal antibody L243 that recognizes intact class II HLA proteins was passed over the HLA affinity column. Fractions of 200 μl were collected during the L243 loading process (flow through) and L243 antibodies that bound to the class II HLA on the column were then eluted from the column intact. A total of 200 μg of L243 was passed over the HLA-DR11 column, 170.7 μg (78%) of which was recovered: 122.9 μg (72%) bound to the class II HLA column and was recovered in the eluate while 47.8 μg (28%) passed through the column and was recovered in the flow through (SFig. 2A). Recovered L243 antibody maintained its activity and specificity in an HLA single antigen bead assay (SAB) (SFig. 2B). These data demonstrate that a sHLA-DR11 column can be used to recover anti-HLA antibodies that are intact and suitable for use in immunoassays.

3.2. Affinity purification of alloantibodies

Anti-sera cytotoxic to only DR11 expressing cells purchased from Genprobe GTI Diagnostics were passed over the column. This DR11 serum was crossreactive with DR, DQ and DP specificities (Fig. 1A). Following passage through the DR11 column, DR reactive antibodies bound to the column while DP and DQ specificities flowed through (Fig. 1B, D). Antibodies to DR11 eluted from the column did not react to DQ or DP (Fig. 1C, E). The recognition of several HLA-DR by the purified antibodies suggests that amino acids 70D 73A and 37Y 38 V of the class II beta chain are likely serologic epitopes for these sera.

In addition to the GTI serum, a panel of DR11 sensitized patient sera was passed over the column (Table 1). All patient sera were positive for DR11 activity by either SAB, flow crossmatch, or both (Table 1). For each of these twelve patients, antibodies in the pre-column sera, post-column flow through, and antibody eluate were compared using an HLA single antigen bead assay (Fig. 2). The column completely depleted DR11 specificities from the sera of 11 patients with the exception being patient 1 who exhibited an extremely high titer of antibodies to DR11. The DR11 antibodies recovered from the column were positive on SAB with the exception of the antibodies recovered from patient 2. Eluted antibodies reacted with multiple DR whereby patterns of DR crossreactivity were consistent with known serologic epitopes [15]. Antibodies purified from patients 7 and 9 reacted with DR and DP via a shared DRB1–57D 58E and DPB1–55D 56E epitope [16–18]. There was no evidence of DQ activity in the recovered/purified antibodies (Fig. 2) and the DR11serologic epitopes recognized by patient antibodies could be defined on the basis of crossreactivity with other DR/DP molecules.

3.3. Alloantibody serum concentration

The class II HLA column removed most of the DR11 reactivity in the 13 patient samples tested. When the total antibody recovered from the column was assessed, and column loss was adjusted to approximately 25%, the serum concentration of DR11 alloantibodies ranged between 0.76 and 26.1 μg/mL with a median concentration of 2.3 μg/mL. The average bulk serum Ig concentration was

Table 1
Patient sample information.

Sample	Age*	DR1	DR1	DR345	DQ	DQ	Previous Tx	Probable DR sensitising events	DR11 IgG FXM	DR11 IgM FXM	DR11 SAB
GTI	Unk						Unk	Unknown	N.T.	N.T.	+
1	50						1	First graft DR4 DR11 mismatch	N.T.	N.T.	+
2	61	7	13	52, 53	2	7	0	No known sentitizing event	+	+	–
3	29	1	7	53	2	5	3	First graft no DR mismatch. Four pregnancies; partner type unknown	–	–	+
4	42	1	4	53	5	8	1	First graft DR7 mismatch	–	–	+
5	43	1	4	53	5	8	1	First graft type unknown	+	–	+
6	22	9	17	52, 53	2	9	1	First graft DR4 mismatch	–	–	+
7	65	4	17	52, 53	2	7	0	Two pregnancies; partner DR7	+	–	–
8	34	10	15	51			1	First graft type unknown	+	+	+
9	43	13	13	52			0	Two pregnancies; partner type unknown	+	–	–
10	47	1			5		0	No known sentitizing event	–	–	+
11	61	1	17		2	5	1	First graft DR7 mismatch	+	–	+
12	48	1	7		2	5	1	First graft DR17 mismatch	+	–	+
13†	75	9	17	52,53	2	9	0	Blood transfusion; type unknown	+	–	+
14†	62	4	17	52,53	2	7	1	First graft DR11 mismatch	+	–	+

* At time of blood draw.
† Patients used in large-scale antibody purification expriments.

48.6 mg/mL in this cohort: between 0.002% and 0.054% of total serum Ig were DR11 reactive.

3.4. Alloantibody isotype profile

DR11 column-isolated antibodies were profiled for their immunoglobulin isotype. All isotypes were detected in all patient sera, including IgG, IgM, IgA, and IgE (Fig. 3A). Antibody ratios differed from patient to patient: Patient 1 had substantial IgG1 while >70% of patient 2 antibodies were IgM. In comparison to bulk Ig, a pattern emerged whereby isotype IgG1 was underrepresented and IgG2 was overrepresented. IgG3 and 4 levels were unchanged as compared to total serum IgG (Fig. 3B). Taken together, the IgG isotypes were under represented and the IgM isotype compensated for this drop as IgM accounted for 31% of the anti-DR11 Ig. The percentage of IgA in the eluate was similar to that observed in bulk serum IgA while the percentage of DR11 IgE alloantibodies were significantly higher than levels found in sera. In summary, every isotype was represented among the eluted DR11 alloantibodies, IgG2, IgM, and IgE are enriched, and IgG1 is underrepresented.

3.5. Alloantibodies to DR11 fix complement

Purified DR11 alloantibodies were tested for their ability to fix complement. Four different DR11 expressing B-cell lines served as target cells. The commercial serum 'sample G' had only DR11 specific complement fixation activity (Fig. 4A). After column absorption, complement-fixing activity was recovered in the eluted antibodies with no cytolysis in the flow-through: Column purified DR11 alloantibodies fix complement. Next, alloantibodies from the 12 patients were column purified and tested for complement-fixing activity (Fig. 4B). Antibodies from 10 patients exhibited cell lysis >40%, including the predominantly IgM antibodies of patient 2. Patient 5 had little cytolytic activity even with an isotype profile similar to other patients, and patient 1 had no CDC activity despite a prevalence of IgG1 alloantibodies – an

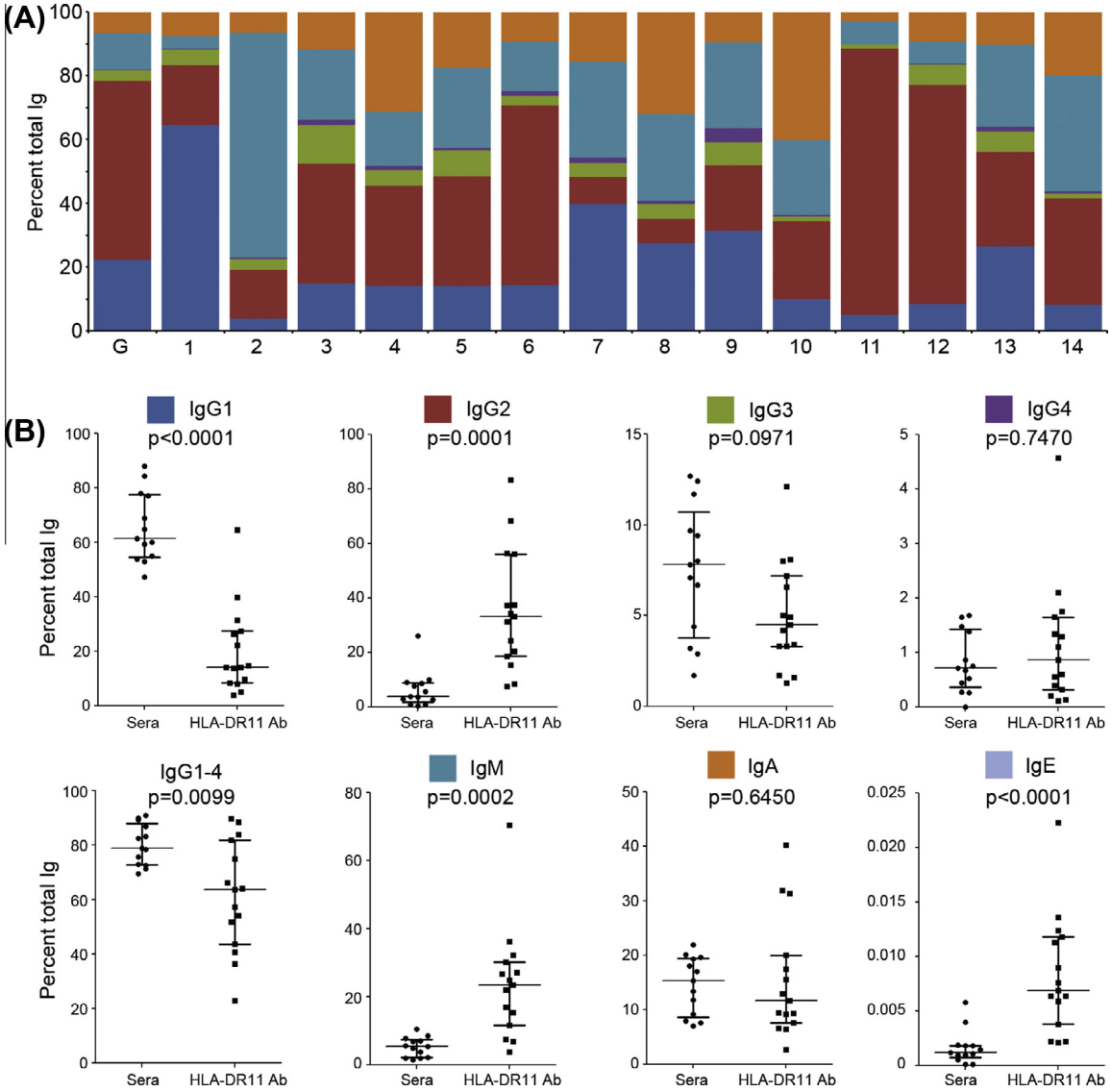

Fig. 3. Isotype profiles of the purified DR11 specific alloantibodies. (A) Isotype profiles of the purified antibodies from all of the patients in the study. Seven different isotypes were analyzed: IgG1 (blue), IgG2 (red), IgG3 (green), IgG4 (purple), IgM (teal), IgA (orange), and IgE (light blue). (B) Proportion of indicated isotype in purified HLA-DR11 antibodies compared to bulk serum antibodies. Line represents the median value and bars show the interquartile range. *P* values are shown as the result of a Mann–Whitney *t*-test.

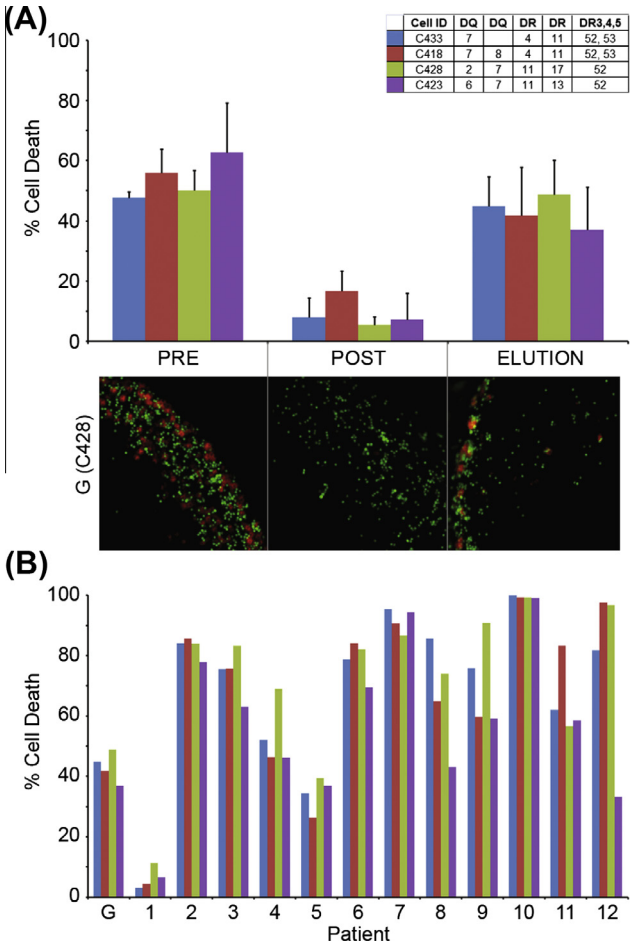

Fig. 4. CDC activity of purified DR11 specific alloantibodies. (A) Sera from patient 'G' was passed over the column and the eluted antibodies were collected. These three samples (PRE, POST, ELUTION) were then added to 4 different HLA-DR11 positive B-cells (C433 blue, C418 red, C428 green, C432 purple) in the presence of complement. Cell death was measured according to the materials and methods and is shown in the histogram. A representative image of the assay (cell line C428) is shown below the histogram. Class II haplotype of each cell line is shown in the table inlay. (B) Complement dependent cell death of the eluted antibodies from patients G-12.

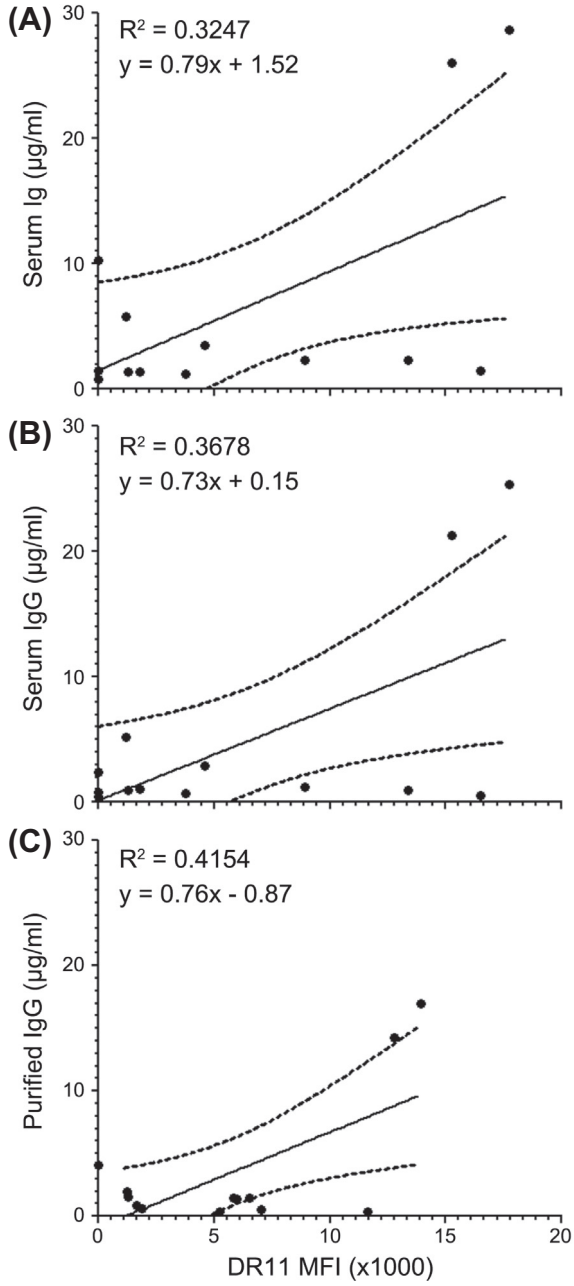

Fig. 5. Correlation of DR11 alloantibody concentration and MFI values. Serum MFI values of 13 different patients plotted against the total serum Ig (A). or IgG1–4 (B). Purified antibody MFI values from the same patients plotted against the IgG1–4 concentration (C). Data was fit to linear model and shown with the 95% confidence bands. R^2 values for each line are shown.

isotype known to fix complement. Thus, DR11 alloantibodies consistently fixed complement and this was likely due to the large proportions of IgM.

3.6. MFI and antibody concentration

HLA column purification enabled the determination of DR11 alloantibody concentration in DR11 patient sera. Hence, we were next able to assess the correlation of MFI values and serum antibody concentration. A comparison of serum antibody concentrations and MFI values in patients 'G' and 1–12 Fig. 5A indicates a variable but significant ($R^2 = 0.3247$) linear correlation. When plotted in terms of serum IgG concentration (having subtracted IgA, M, & E), this correlation remained ($R^2 = 0.3678$) (Fig. 5B). Antibody purification further reduced MFI versus IgG variability ($R^2 = 0.4154$) (Fig. 5C). However, antibody concentrations for many patients fell outside the 95% confidence level, including three patients with low antibody concentrations yet consistently high MFI values. Thus, a high degree of variation makes it difficult to assign serum antibody concentrations using MFI values.

3.7. Multimeric immunoglobulins and MFI

Multiple factors are positioned to influence the behavior of antibodies in a diagnostic test: Different isotypes, antibody affinity, and the recognition of different epitope specificities. The removal of IgM multimers by either DTT reduction or size exclusion has been reported to provide more meaningful determinations of IgG concentration [19], and we hypothesized that MFI values would more accurately reflect patient-to-patient IgG concentrations were IgM and IgA multimers removed. Milligrams of DR11 reactive alloantibody were purified from patients 13 and 14 (the patients with large quantities of available sample; see materials and methods), and the IgM and IgA were separated from IgG by size-exclusion

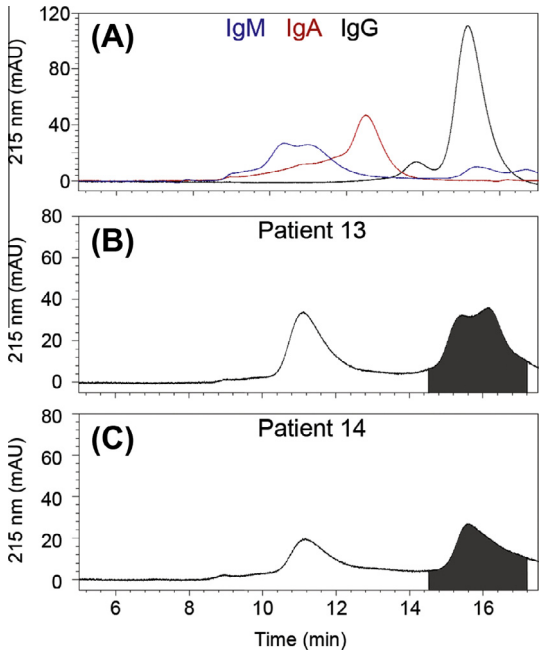

Fig. 6. SEC–HPLC of purified DR11 alloantibodies. 10 μg of purified human IgM (blue), IgA (red), and IgG (black) were run individually over a size-exclusion column (A). 10 μg of neat DR11 alloantibodies from patients 13 (B), 14 (C) was run over a size-exclusion column. The collected Ig monomeric fraction is shaded in grey.

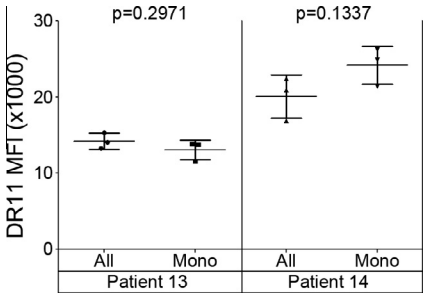

Fig. 7. MFI values of total Ig or monomeric Ig. HLA-DR11 MFI values of the different antibody preparations. For patients 13 and 14 MFI values were determined for native antibodies (all), monomeric antibodies (Mono), saturating concentration of 200 μg/mL. *P* values are shown as the result of unpaired *t*-test.

chromatography (Fig. 6), and purified monomeric DR11 reactive antibodies were >88% IgG (SFig. 3). These IgG preparations were adjusted to 20 μg/mL and tested by SAB assay. As a note, these purified samples had CV values ranging from 7.4% to 10.0%, and because these CV values are well within those reported for this MFI range [20], a statistical comparison within three replicates was completed. Patient 13 had an average MFI value of 10,660 and patient 14 had an MFI of 15,075; a significant ($p = 0.0285$) difference of 4415 MFI remained between these equilibrated samples. Moreover, the removal of considerable IgM and IgA did not significantly change the DR11 MFI values (Fig. 7). Thus, multimeric IgM and IgA had little effect on MFI values within a patient and multimer removal did not make MFI values comparable between patients.

4. Discussion

Donor specific anti-HLA antibodies represent a pre-transplant contraindication and a post-transplant risk for graft loss. While it is clear that antibodies to HLA mediate graft rejection and loss, not all anti-HLA antibodies are detrimental to allografts [11–13]. Substantial heterogeneity exists between antibody responses, and there is a great interest in distinguishing pathogenic anti-HLA antibodies from those that are not a threat to the transplanted organ. To date, the antibodies that warrant clinical intervention remain ambiguous from those responses that do not impact clinical outcomes. Here, we developed an HLA-DR11 immunoaffinity column to purify HLA alloantibodies and, once purified, we profiled these antibodies. This ability to isolate anti-HLA antibodies is positioned to augment both clinical and basic scientific endeavors to unravel the complex nature of humoral responses to HLA.

Through the recognition of epitopes that are shared by multiple HLA allomorphs, alloantibodies to HLA cross-react with several HLA antigens [15,21–23]. Here, purified DR11 alloantibodies were cross-reactive with numerous DR and some DP molecules. In many cases, the broad cross-reactivity of these purified alloantibodies made it difficult to define the epitopes recognized without sequential absorption and/or blocking experiments. Nonetheless, several serologic epitopes were readily apparent when the DR11 column purified antibodies were tested. For example, the cross-reactivity of sera from patients 7 and 10 with DR11 and multiple DP (HLA-DP2, 3, 04:02, 6, 9, 10, 14, 16, 17, 18, 28) is likely due to a shared 57D 58E epitope. Epitope 57D 58E is defined by an Asp at position 57 and a Glu at position 58 on the beta chain of DR11, with the exact same amino acids found at positions 55 and 56 on the DP beta chain [16–18]. Another commonly observed class II HLA epitope is 10YST, defined by residues Y10, S11, T12, and S13 that are conserved on DR11, DR17, DR18, DR13, and DR14. Interestingly, these amino acids are located underneath the peptide groove, suggesting that DR11 alloantibodies can recognize an epitope or epitopes outside/underneath the binding groove. A similar HLA-DR epitope has been reported by Muro et.al. [24]. Thus, the anti-DR11 antibodies purified in this study were cross-reactive with other allomorphs in a way that is consistent with known epitopes.

Multiple IgG subtypes, IgA, and IgM can target HLA alloantigens [25–30]. Such antibody heterogeneity is understandable when one considers the different routes of sensitization (pregnancy, previous transplant, transfusions, etc.) as well as the many different HLA specificities that can serve as immunogens [30]. Here we fixed the specificity by characterizing only anti-HLA-DR11 alloantibodies and, by purifying microgram quantities of DR11 reactive antibodies, we were able to elucidate HLA reactive isotypes representing as little as 0.001% of the total Ig. In each patient *every* isotype was detectable, including low abundance IgE antibodies. Because these patients were sensitized via a variety of antigenic exposures, no single alloantibody isotype was consistently under or over represented when compared to bulk serum antibodies: there was a large degree of variation among patients. Nonetheless, the IgG1 isotype, which has a high affinity for Fc receptors (FcR), was underrepresented throughout the patient panel, suggesting that IgG-mediated ADCC may play a minor role in the class II alloresponse. IgG subtypes 1 and 3 have a high affinity for C1q, and the lack of these alloantibodies should result in low CDC activity. However, most patients had a relatively large proportion of IgM, an isotype that exhibits the highest affinity for C1q; IgM may compensate for any dearth of IgG-mediated C1q interaction. Indeed, when tested for CDC activity, most patient's purified alloantibodies lysed >50% of the DR11 expressing cells. A high proportion of IgM class II specific antibodies may provide ample CDC activity when IgG1 and IgG3 proportions are low.

The profiling of purified DR11 alloantibodies demonstrates that heterogencous alloantibody mixtures as a whole fix complement. The one exception, patient 1, merits some discussion because this patient's purified Abs were predominantly IgG1, an isotype that readily fixes complement. Patient one's results highlight previous

observations showing that CDC activity is mediated by multiple factors. For example, Dequesnoy et.al. have shown that the recognition of an HLA epitope and its surrounding regions (or paratopes) by multiple CDR loops of an alloantibody will influence the ability of that antibody to bind C1q and fix complement [22]: An antibody's avidity increases as more CDR loops of the antibody interact with a particular allomorph, increased avidity leads to a change in antibody conformation, and antibodies in an optimal conformation more readily bind C1q. Here we found a counter-intuitive example whereby patient (patient 1) had nearly 70% IgG1 yet had little complement fixing activity. It is possible that patient one's IgG1 alloantibodies did not optimally interact with DR11 or that various alloantibodies in the mixture were competing for epitopes and thereby inhibiting each others ability to optimally bind DR11. While mechanistic studies will be required to definitively address this case, these data demonstrate that isotype alone is not *sufficient* for complement fixation and that factors such as avidity may influence complement activation.

As a clinical diagnostic, single antigen bead assays are widely used to screen for HLA-specific antibodies in patient sera. Such assays are very effective at determining reactivity to a given HLA allotype, but it remains difficult to determine antibody concentration or functional relevance via gradations in MFI. Several explanations might explain a lack of correlation between MFI and antibody concentration, and here we examined how changes to the antibody milieu impact correlations of MFI and IgG concentration. Interference of IgM is thought to effect SAB assay MFI values [19], and we postulated that a lack of consistency between MFI and IgG measurement might be due to IgM and IgA multimeric alloantibodies interfering with the detection of IgG. However, the removal of multimeric antibodies by physical separation failed to align MFI values and IgG levels in the patients tested. These data suggest that multimeric antibodies have a modest influence on MFI values and that variables including antibody affinity and epitope specificity must also influence MFI indications of IgG concentration. Future studies that test purified antibodies by surface plasmon resonance may best assess the correlation of antibody affinity and diagnostic MFI.

HLA proteins have proven their worth in diagnostic assays but they have not been tested as a therapeutic. Given the data presented here, one could envision the development of an HLA antibody absorption device for the depletion of humoral immune responses to HLA, leaving otherwise beneficial humoral immunity intact. Today, antibody reduction therapies such as plasma exchange deplete bulk antibodies to facilitate transplants for recipients who are otherwise serologically incompatible. By adding the element of HLA specificity to these existing reduction therapies it might be possible to remove only the deleterious anti-HLA antibodies in pre and post-transplant scenarios. Antigen-specific antibody depletion columns are used to remove antibodies to blood group A and B antigens, and in this report we show that liters of patient plasma can be processed over columns equivalent in proportion to the Glycosorb and Immunosorba columns used for blood group desensitization [31–36]. More than 20 mg of DR11 reactive antibody were isolated in both patients 13 and 14, significantly reducing MFI values in the plasma flow through (data not shown). While an HLA absorption device remains distant from the clinic, the columns tested here demonstrate that complement-fixing antibodies to HLA can be absorbed from patient samples. In addition to serving as diagnostic tools, HLA proteins may be developed as future transplant therapies.

In summary, milligram quantities of native class II HLA proteins were produced and coupled to a column support for use in HLA antibody purification. The recovered DR11 reactive antibodies were functionally active and cross-reactive with DR and DP allomorphs. Detectable amounts of every antibody isotype were present in each patient and the IgG2, IgM, and IgE isotypes were enriched. Although IgG1 and IgG3 levels were not elevated, the HLA alloantibody mixtures remained active in complement fixation assays. Testing of the purified alloantibodies with HLA SAB confirmed the lack or association between MFI values and antibody concentration, an inconsistency not remedied by the removal of multimeric antibody isotypes. Future column studies with other class II and class I allormorphs will be needed to better elucidate the character, reactivity, and quantity of alloantibodies and to better define those antibodies that promote transplant rejection.

Disclosure

The authors of this manuscript have conflicts of interest to disclose as described by the American Journal of Transplantation. Rico Buchli and Rodney VanGundy are employees of Pure protein LLC. Curtis McMurtrey is a paid consultant for pure protein LLC. William Hildebrand is the chief scientist of pure protein LLC. Pure protein sells the soluble HLA, used in the manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.humimm.2013.11.015.

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Behaviour of Non-Donor Specific Antibodies during Rapid Re-Synthesis of Donor Specific HLA Antibodies after Antibody Incompatible Renal Transplantation

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Abstract

Background: HLA directed antibodies play an important role in acute and chronic allograft rejection. During viral infection of a patient with HLA antibodies, the HLA antibody levels may rise even though there is no new immunization with antigen. However it is not known whether the converse occurs, and whether changes on non-donor specific antibodies are associated with any outcomes following HLA antibody incompatible renal transplantation.

Methods: 55 patients, 31 women and 24 men, who underwent HLAi renal transplant in our center from September 2005 to September 2010 were included in the studies. We analysed the data using two different approaches, based on; i) DSA levels and ii) rejection episode post transplant. HLA antibody levels were measured during the early post transplant period and corresponding CMV, VZV and Anti-HBs IgG antibody levels and blood group IgG, IgM and IgA antibodies were quantified.

Results: Despite a significant DSA antibody rise no significant non-donor specific HLA antibody, viral or blood group antibody rise was found. In rejection episode analyses, multiple logistic regression modelling showed that change in the DSA was significantly associated with rejection ($p = 0.002$), even when adjusted for other antibody levels. No other antibody levels were predictive of rejection. Increase in DSA from pre treatment to a post transplant peak of 1000 was equivalent to an increased chance of rejection with an odds ratio of 1.47 (1.08, 2.00).

Conclusion: In spite of increases or decreases in the DSA levels, there were no changes in the viral or the blood group antibodies in these patients. Thus the DSA rise is specific in contrast to the viral, blood group or third party antibodies post transplantation. Increases in the DSA post transplant in comparison to pre-treatment are strongly associated with occurrence of rejection.

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Introduction

Antibodies are major factors not only in the human immune response against bacteria and viruses but also for allograft rejection and transplant kidney survival. The determinants of the levels of antibodies are not fully understood. Until recently it has been difficult to study the characteristics of human leukocyte antigen (HLA) antibodies after transplantation in the face of preformed HLA antibodies, first because the methods used to measure antibody levels were neither sensitive nor specific, and secondly because the results of such transplants were poor. It is now possible to follow the levels of HLA antibodies closely after renal transplantation.

There has always been a theoretical concern that infections can trigger rejection episodes and increase HLA antibodies. A recent study has shown that there is a strong association between the development of infection and increases in both breadth and strength of HLA antibodies [1]. The increase in the breadth of HLA antibodies was mainly due to expansion of reactivity among other antigens of a cross-reactive group (CREGs). Other studies have shown that in transplant kidney biopsies of acutely rejecting patients with viral infections the presence of plasma cell infiltrates and C4d deposition [2,3]. The relationship between infection and rise in HLA antibodies is thought to be secondary to the presence of a robust memory B-cell response to the release of pro-inflammatory cytokines.

It is of interest to find out if rises in DSA levels with or without rejection is associated with rise in viral and blood group antibodies. The response of blood group and viral antibodies in pre-sensitized patients to a renal allograft is not fully understood. Changes in the

levels of these antibodies soon after transplantation might illuminate the relationship between DSA and these antibodies and there may be insights into the processes determining the production and elimination of HLA antibodies [4].

With regards to blood group antibody levels after blood group incompatible transplantation, studies have shown a reduction in the levels of blood group antibodies in the long term, to undetectable levels in many patients. Higher levels of antibodies were associated with short-and long term dysfunction in some patients [5]. The rapid disappearance of blood group antibodies soon after transplantation in many patients with good functioning grafts contrasts with the reports in HLA antibody incompatible transplantation [6,7,8]. Looking at blood group antibody levels after HLA antibody incompatible transplantation would confirm whether the changes observed after blood group incompatible transplantation are specific to that setting, or also occur when there is a marked humoral response to HLA after transplantation.

The aims of this study were to examine in detail the wider humoral response during a period of intense re-synthesis of HLA antibodies after renal transplantation. The choice of antibodies studied meant we studied antibodies that had been stimulated by infection, immunization, and 'natural' antibodies.

Methods

The study was approved by the West Midlands Research Ethics committee, U.K. Patients sensitized to HLA antigens were selected after obtaining consent for our program of antibody incompatible transplantation if they had current reactivity with donor specific HLA antigens measured by cytotoxic crossmatch (CDC), flow crossmatch (FC), or by microbead assay. We analysed 55 such patients, 31 women and 24 men, who underwent HLAi renal transplant at our center from September 2005 to September 2010. Pre-transplant, patients were treated with five alternate day sessions of double filtration plasmapheresis, the aim being to achieve a negative flow crossmatch at the time of surgery. In some cases with low starting levels of DSA, fewer sessions of plasmapheresis were administered. In some cases with high starting levels of DSA, more sessions of plasmapheresis were administered, and/or the transplant was performed in the presence of positive crossmatch. The number of plasmapheresis sessions administered varied between two to seven, with the majority getting five sessions. Patients who had blood group antibody incompatibility or who died in the early post-transplant period were excluded from our study.

Serum samples for antibody analysis were done at four time points, namely pre-treatment, at peak DSA post transplant, at rejection and late sample which was around six weeks to three months post transplant. Peak DSA was defined as the highest level of DSA within the first six weeks post transplant. As samples were collected from patients on a daily basis, the peak time point was chosen on retrospective analysis. Third party antibodies (TPA) were defined as HLA antibodies in the recipient, which were not specific for epitopes expressed on the donor antigens. Though there were many potential TPA's, the one which was predominant in that individual patient, was studied.

Immunosuppression

Imunosuppression consisted of mycophenolate mofetil 1000 mg bd started five days before transplant, with dose reduced if white cell count fell below 4.0×10^9/l. Tacrolimus was started three days before transplant at a dose of 0.15 mg/kg/day in divided doses, with a target trough level of 10–15 μg/l in the first month. Prednisolone 20 mg od was started at the time of surgery, and

Table 1. Patient Characteristics.

	Groups N = 55			Rejection status N = 52		
	Peak DSA higher - Group 1 (n = 34)	Peak DSA lower - Group 2 (n = 21)	P value	Rejection(n = 26)	No Rejection(n = 26)	P value
Male	13 21	11 10	**0.3041**	10 16	12 14	**0.5745**
Female						
Age (mean)	44.8	43.8	**T = 0.31, p = 0.761**	45.5	42.0	**T = −1.07 p = 0.287**
Number of previous transplants	**0 1 2 3** 12 17 3 2	6 11 2 2		9 13 1 3	8 13 4 1	NS
DR mismatch	**0 1 2** 6 23 5	7 12 2		4 16 6	9 16 1	NS
Change in DSA with reference to rejection Negative Positive				6 20	17 9	**0.0021**
Change in TPA with reference to rejection Negative Positive				17 9	15 11	NS
Rejection with reference to peak DSA Yes No	23 9	3 17				**0.0001**

DSA- Donor specific antibody; TPA – Third party antibody.
doi:10.1371/journal.pone.0068663.t001

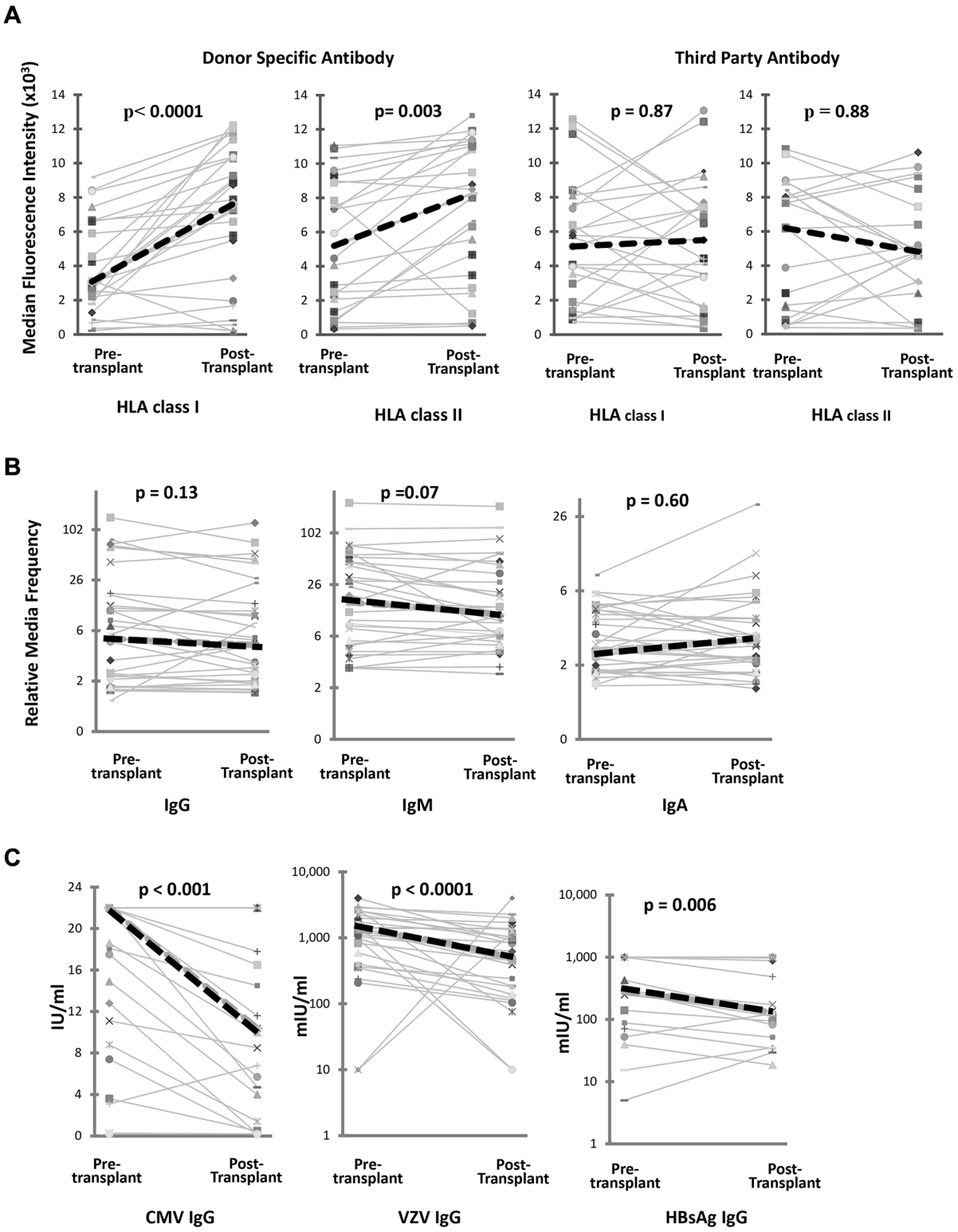

Figure 1. Antibody response in patients with an acute rise in donor specific HLA antibody after HLA antibody incompatible renal transplant. This shows the changes in **A)** the donor kidney specific antibody (DSA) for HLA class I & II and third party class I & II, **B)** IgG, IgM and IgA blood group antibodies and **C)** viral antibody levels in patients with significantly higher post-transplant peak DSA levels compared to pre-transplant levels. There was no rise in third party HLA antibodies or blood group antibodies. The viral antibodies showed a significant fall in serum antibody levels; cytomegalovirus (CMV) IgG ($p<0.001$), varicella zoster virus (VZV) IgG ($p<0.0001$) and Hepatitis B surface antigen (HBsAg) IgG antibody ($p=0.006$). Only patients with measurable viral antibody levels pre-transplant were included. Graphs show individual patients (solid thin lines). Mean values are illustrated with the thick dashed line.
doi:10.1371/journal.pone.0068663.g001

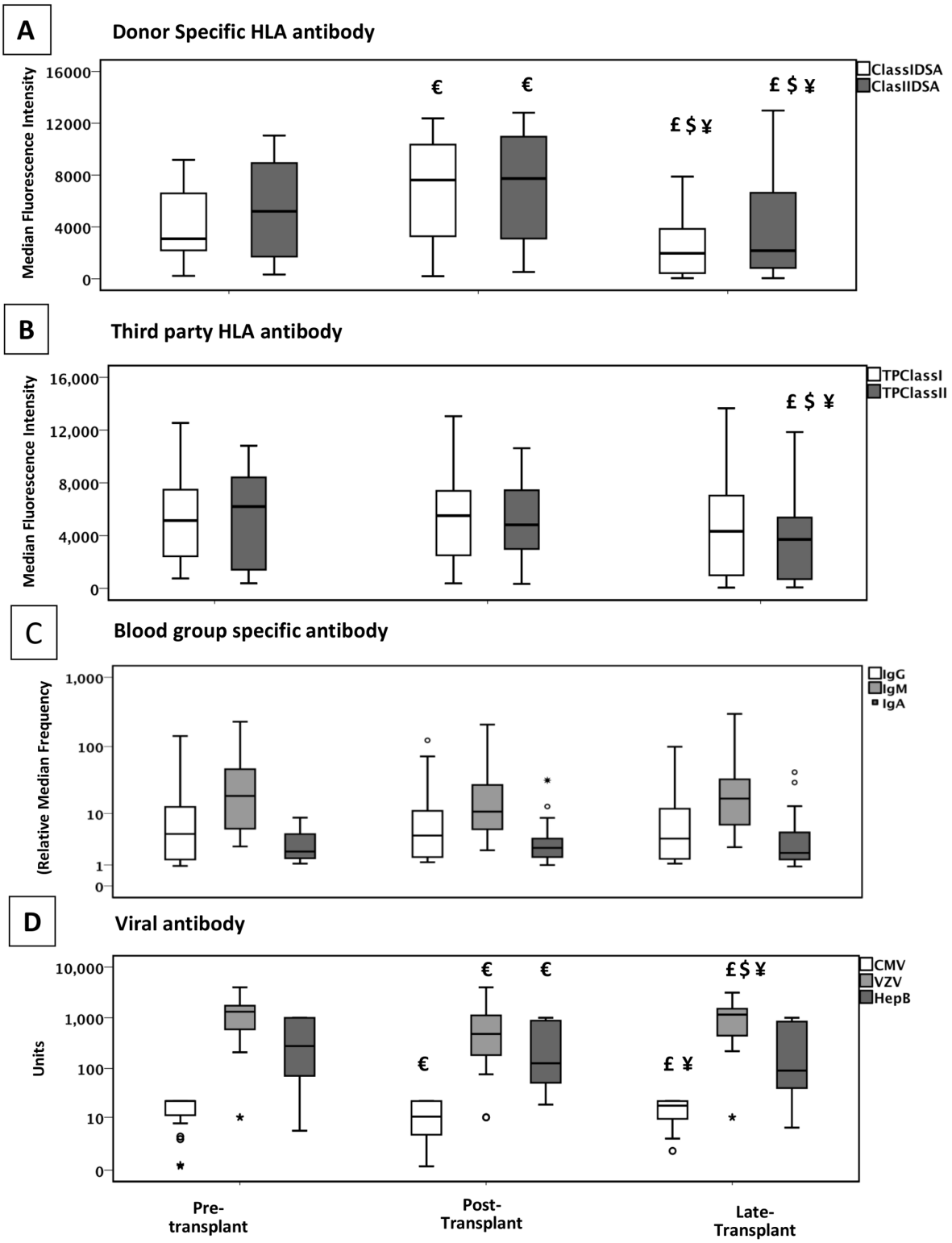

Figure 2. Antibody response during the first few weeks after HLA antibody incompatible renal transplant in patients with an acute rise in donor specific HLA antibody. This shows the changes in **A)** the donor kidney specific antibody (DSA) for HLA class I and II, **B)** third party class I and II, **C)** blood group antibodies and **D)** viral antibody levels in patients with significantly higher post-transplant peak DSA levels compared to pre-transplant levels over the first couple of months. Over a longer observation period there was no significant change in third party HLA antibody, IgG, IgM or IgA blood group antibody, cytomegalovirus (CMV) IgG, varicella zoster virus (VZV) IgG and Hepatitis B surface antigen (HBsAg) IgG antibody observed. Only patients with measurable viral antibody levels pre-transplant were included. Box plot shows the statistical significant changes in the groups €- $p<0.05$ pre-transplant vs. post-transplant; £ - $p<0.05$ post-transplant vs. late; $ - $p<0.05$ pre-transplant vs. late and ¥ - $p<0.05$ overall trend.
doi:10.1371/journal.pone.0068663.g002

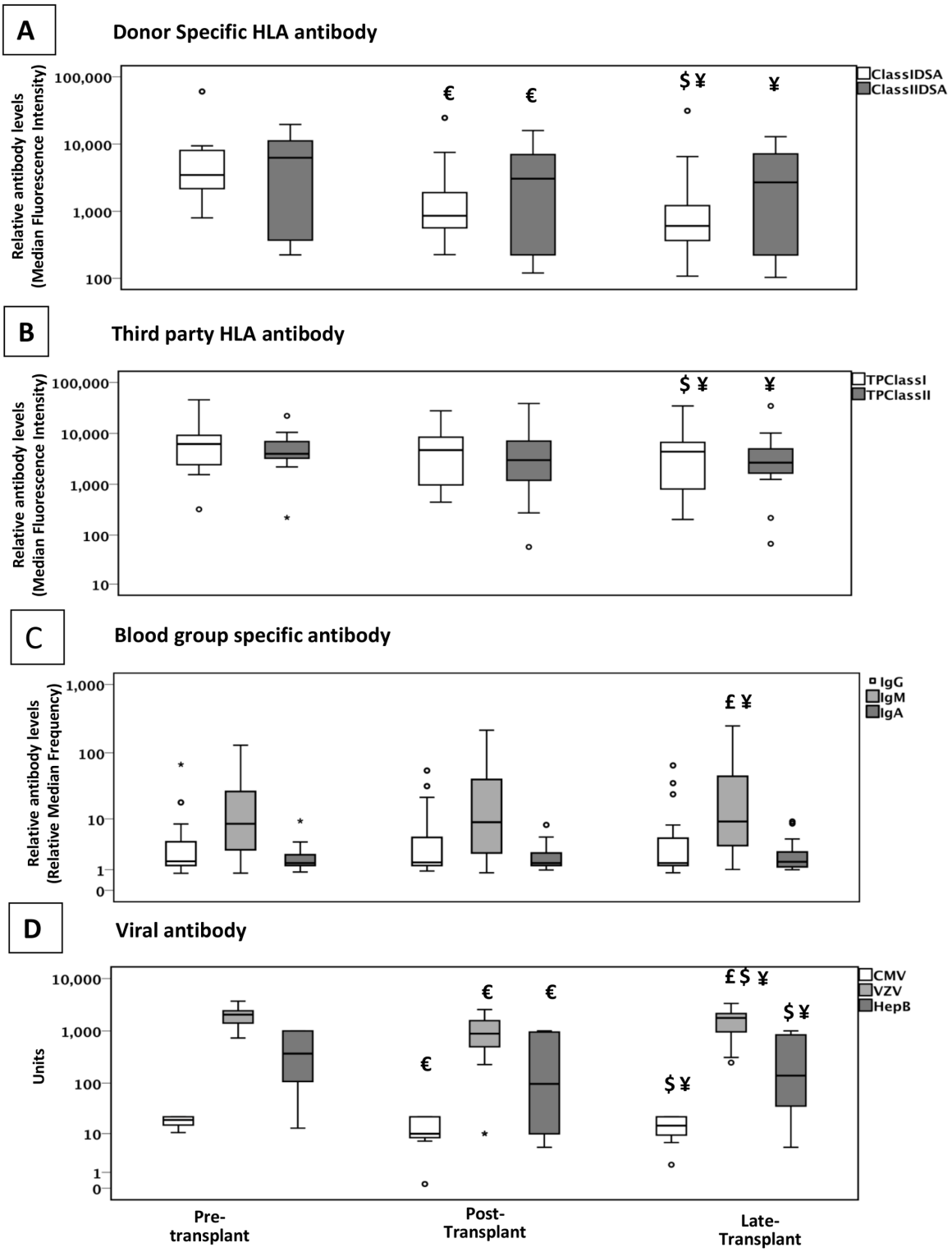

Figure 3. Antibody response during the first few weeks after HLA antibody incompatible renal transplant in patients with an acute fall in donor specific HLA antibody. This shows the changes in **A)** the donor kidney specific antibody (DSA) for HLA class I and II, **B)** third party class I and II, **C)** blood group and **D)** viral antibody levels in patients with significantly lower post-transplant peak DSA levels compared to pre-transplant levels over the first couple of months. Over a longer observation period there was no significant change in third party HLA antibody, IgG, IgM or IgA blood group antibody, cytomegalovirus (CMV) IgG, varicella zoster virus (VZV) IgG and Hepatitis B surface antigen (HBsAg) IgG antibody observed. Only patients with measurable viral antibody levels pre-transplant were included. Box plot shows the statistical significant changes in the groups **€**- $p<0.05$ pre-transplant vs. post-transplant; **£** - $p<0.05$ post-transplant vs. late; **$** - $p<0.05$ pre-transplant vs. late and **¥** - $p<0.05$ overall trend.

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methylprednisolone 500 mg was given as a single intravenous dose during the transplant operation. Two doses of basiliximab 20 mg were given, at days zero and four. The protocol was the same as stated in our previous publications [9,10].

Rejection

Rejection was diagnosed by renal biopsy if the renal function deteriorated, or clinically if there was rapid onset oliguria with a rise in both creatinine and in DSA levels. Biopsies were when clinically indicated and these were independently analysed by pathologists. Diagnosis of antibody mediated rejection, cellular rejection or mixed were made according to Banff classification of transplant biopsies [11,12]. Rejection was treated with three days high dose methylprednisolone and OKT3 early on in the series or ATG later on. Rituximab was not given to any of our patients; two patients received IVIG one month post transplant and four patients received post-transplant plasmapheresis.

Microbead Assays

The main DSA, cumulative DSA and the third party HLA Abs both HLA Class I and Class II specific antibodies were analysed using microbead assay manufactured by One Lambda Inc (Canoga Park, CA, USA), analysed on the Luminex platform (XMap 200, Austin, TX, USA) as used in similar studies previously [13]. Raw mean fluorescence intensity (MFI) values were used to follow antibody levels. All assays were performed using serum/bead volume ratios and one thousand MFI was used as the cut off for positive and negative beads according to the manufacturer's instructions.

Flowcytometric estimation of blood group antibodies. Plasma samples were analysed using flowcytometry for estimating IgG, IgM and IgA blood group antibodies against reagent cells. We have used the method previously published by us [14].

Quantification of viral antibodies using LIAISON® analyzer. Using the LIAISON ® analyzer (DiaSorin S.p.A, Saluggia, Italy), Cytomegalovirus (CMV), Varicella Zoster (VZV) and Anti Hepatitis B Surface antigen (Anti-HBsAg) IgG antibodies were quantified from the corresponding serum samples according to manufactures instructions. The LIAISON® viral antibody test is a fully automated two-step direct sandwich immunoassay for in *vitro* quantitative determination of antibodies to the specific viral antigen, based on chemiluminescent technology, to be run on the LIAISON. The method for quantitative determination of specific IgG to viral antigen is an indirect chemiluminesence immunoassay (CLIA) [15].

CMV screening and prophylaxis. Routine CMV screening is done one, three and six months post transplant in all patients using quantitative PCR (Argene PCR, Biomerieux, CMV R- gene quantification assay) with the cut-off for positivity being 400 CMV copies/ml of plasma. As per protocol all CMV IgG negative (R-) recipients who receive CMV IgG positive (D+) kidneys and any recipient receiving ATG or OKT3 will get prophylaxis with Valganciclovir for three months. Primary CMV infection is defined as CMV viremia post transplant in a sero-negative recipient. Secondary infection is CMV viremia in a sero positive patient post transplant.

Statistical Analysis

For comparison of baseline characteristics between groups, including rejection status defined, the chi-squared test was used for categorical variables. Means and their differences were compared using the t-test and Wilcoxon non-parametric test as appropriate and the level of significance was set at $P<0.05$.

To investigate the influence of antibody levels and changes in levels from pre-transplant to post transplant peak on the risk of rejection, allowing for the effects of other antibodies and potentially confounding variables, multiple logistic regression modelling was used. In all logistic models, age, sex, DR mismatch and number of previous transplants were retained as potentially confounding. The combined DSA (classes I & II) antibodies levels, pre-transplant, post transplant peak and change (peak – pre) were investigated as the main explanatory factor for rejection. Pre-transplant, peak and changes in other antibodies were included in models, to examine their influence on the DSA antibody effects.

IBM SPSS software version –19 was used to compare the antibody levels between the groups. The overall trend over time was analysed using Kendall test.

Results

We analysed the data using two different approaches; i) based on DSA levels and ii) based on rejection episode post transplant. Since the primary study aim was to see if non-HLA antibody levels increased along with the HLA antibodies, the patients were divided into 2 groups; 1) Group 1 - Patients who had higher post transplant peak DSA than pre-treatment levels and 2) Group 2 - those that had lower post transplant peak DSA than pre-treatment levels. Patient characteristics are summarized in Table 1. There were 34 patients in Group 1 and 21 in Group 2. The mean age was similar in both the groups (38.9 and 40.9). The mean and the range (given in parentheses) pre-treatment class I & II DSA were 3875 (220, 9181) and 5317 (331, 11055) in Group 1 and 7847 (799, 60605) and 7281 (315, 19652) in Group 2 respectively. And the mean peak class I & II DSA post transplant were 7024 (193, 12383) and 7195 (514, 12811) in Group 1 and 4449 (120, 15914) and 5757 (447, 27756) in Group 2 respectively. Two patients received IVIG one month post transplant and four patients received post-transplant plasmapheresis. Analysis was repeated excluding these patient samples and there was no difference in the results.

Though some of the patients were negative for CMV IgG, anti-HBsAg or VZV IgG pre- transplant, they were included in the study. This is because we measured the actual values to obtain a continuous data to see if there was a rise in the level of these antibodies along with the DSA's or not. If the patients were negative at pre-treatment and stayed negative throughout the four time points, they were excluded from the analysis, as it was not possible to differentiate between a lack of response and a lack of prior immunization. Thus in Group 1, 25 patient samples were analysed for CMV; 32 for VZV and 23 for anti- HBsAg. Similarly in Group 2, 13 patient samples were analysed for CMV, 20 for VZV and eight for anti-Hbs.

When measuring the viral antibodies, if the samples had reached the saturation of the assay, they were retested in 1 in 10 dilutions. There was no increase detected in the viral antibody level even on dilutions. Five patients had CMV viremia post transplant. Two of them had primary infection with concomitant change of CMV antibodies from negative pre-treatment to positive in late samples. Out of the three who had secondary infection one had no change in antibodies whereas the other two showed a slight increase in the late samples. Analysis was repeated excluding these patient samples and there was no difference in the results.

Thirty two patient samples from group 1 and 20 from group 2 were analysed for blood group antibodies and rejection outcome. There were three patients in total who were excluded for analysis of blood group antibodies from the two groups, as they had received both blood group incompatible and HLA incompatible

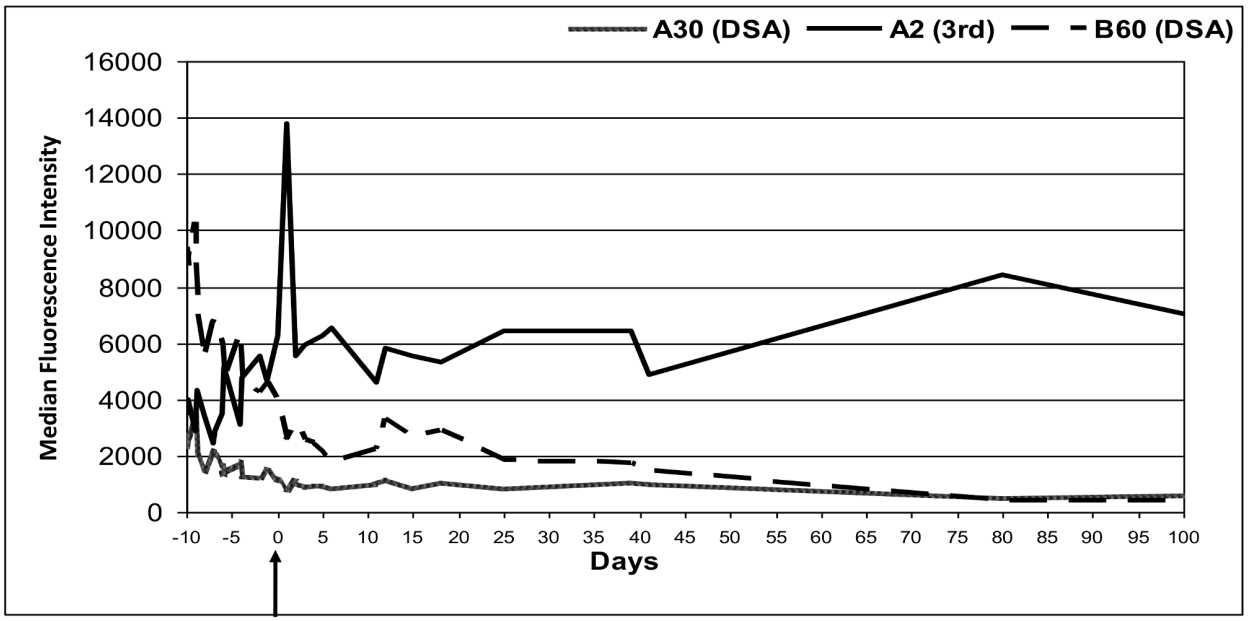

Figure 4. Patient with rise in third party HLA antibody after an HLA antibody incompatible renal transplant. Exceptionally a change in third party HLA antibodies was not noted which was not explained by the current understanding of epitope sharing of the third party HLA with donor specific HLA. In this example donor-specific antibodies to HLA A30 and B60 were going down post transplant, but the third party antibody HLA A2 was increasing. Though the HLA A2 is known to share epitopes with HLA A30, the behaviour of these two antibodies was very different to each other. Also, the patient did not receive any blood products after the transplant.
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kidney transplantation. Twenty three out of 32 patients had an episode of rejection in group 1 as opposed to three out of 20 in group 2, which was statistically significant ($p = 0.0001$). Fourteen patients had antibody mediated rejection, five had cellular rejection, two had mixed and five were treated as rejection clinically though the biopsies did not show evidence of rejection.

There was a statistically significant increase in the DSA levels between pre-treatment and peak level post transplant in class I and

Table 2. Results of multiple logistic regression modelling, comparing the occurrence of rejection in relation to DSA.

	Baseline pre-transplant DSA		Changes in DSA(Peak to Pre)		Changes in DSA(Peak to Pre)	
	Model 1		Model 2		Model 3	
	OR (95% CI)	P-value	OR (95% CI)	P-value	OR (95% CI)	P-value
Any DSA pre-Tx (1000s)#	1.08 (0.93, 1.25)	**0.2989**	1.30 (1.06, 1.58)	**0.0116**	1.47 (1.08, 2.00)	**0.015**
Sex (F vs. M)	1.32 (0.22, 7.95)	**0.7613**	0.58 (0.08, 4.54)	**0.6067**	0.45 (0.03, 6.56)	**0.5593**
Age	1.05 (0.98, 1.13)	**0.178**	1.07 (0.98, 1.18)	**0.141**	1.05 (0.96, 1.14)	**0.2964**
Previous Tx (Yes vs. No)	2.49 (0.36, 17.03)	**0.3532**	2.57 (0.27, 24.4)	**0.4111**	1.00(0.07, 15.08)	**0.997**
DR mismatch (0 vs 1 or 2)	3.78 (0.65, 21.93)	**0.138**	7.48 (0.85, 65.7)	**0.0696**	29.34 (1.96, 440.0)	**0.0145**
Any TPA pre-Tx.	1.00 (0.98, 1.02)	**0.8166**	1.00 (0.98, 1.02)	**0.9205**	1.00 (0.97, 1.03)	**0.7664**
VZV pre-Tx.	0.44 (0.20, 0.96)	**0.038**	0.37 (0.16, 0.84)	**0.0172**	2.32 (0.67, 8.05)	**0.1853**
CMV pre-Tx.	0.95 (0.87, 1.03)	**0.1869**	0.93 (0.85, 1.02)	**0.1031**	0.81 (0.66, 1.01)	**0.0623**
antiHBsAg pre-Tx.	1.00 (1.00, 1.00)	**0.3648**	1.00 (1.00, 1.00)	**0.2416**	1.02 (1.00, 1.03)	**0.0346**
IgA pre-Tx.	0.80 (0.51, 1.27)	**0.349**	0.69 (0.43, 1.11)	**0.1249**	0.92 (0.62, 1.37)	**0.689**
IgG pre-Tx.	1.02 (0.98, 1.06)	**0.4639**	1.02 (0.98. 1.07)	**0.3514**	1.04 (0.97. 1.11)	**0.2683**
IgM pre-Tx.	1.02 (0.99, 1.05)	**0.2334**	1.02 (0.99, 1.06)	**0.1917**	0.90 (0.80, 1.01)	**0.0773**

Model 1 is for DSA baseline level adjusted for age, sex, DR mismatch (0 vs. 1 or 2), previous transplant (Tx) (Yes vs. No), and baseline levels of TPA, CMV, VZV, IgA, IgG and IgM antibodies.
Model 2 is for change in DSA level (peak – pre-transplant), adjusted for age, sex, DR mismatch (0 vs. 1 or 2), previous Tx (Yes vs. No) and baseline levels of TPA, CMV, VZV, IgA, IgG and IgM antibodies.
Model 3 is for change in DSA level (peak – pre-transplant), adjusted for age, sex, DR mismatch (0 vs. 1 or 2), previous Tx (Yes vs. No) and changes in all antibody levels (peak – pre-transplant levels of TPA, CMV, VZV, IgA, IgG and IgM antibodies).
#In all models, DSA pre-transplant and change levels have been expressed in 1000s, so the OR presented are for an increase of 1000 DSA units.
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class II antibodies in group 1. This was associated with a simultaneous statistically significant decrease in the viral titres, but no change in blood group antibodies. With regards to the third party antibodies, though class I showed an increasing trend this was not statistically significant. Figure 1 shows the changes in the DSA class I and II, third party class I and II, viral and blood group antibody levels in group I patients, comparing pre-treatment levels to peak DSA levels.

In group 2, there was a statistically significant fall in the DSA levels between pre-treatment and peak post transplant. The fall in the viral titres was also significant, but there was no change in the blood group antibody levels. Figures 2 and 3 show the trend of the different antibodies over time points of pre-treatment, peak post transplant and late samples in group 1 and group 2 respectively.

Though most of the rise or fall in class I third party antibodies could be attributed to shared epitopes with the DSA, some third party antibodies behaved completely different to the DSA. This cannot be explained by the current understanding of epitope sharing of the third party HLA with donor specific HLA. Figure 4 shows a patient wherein the DSAs HLA A30 and B60 were going down post transplant, but the third party antibody HLA A2 was rising and the patient did not receive any blood products after the transplant. In four other patients there was similar dissociation between donor specific and third party HLA antibody levels.

The second sets of analyses were done on 52 patients (excluding three as alluded to above) with reference to rejection as the outcome. Multiple logistic regression modelling was used to compare the occurrence of rejection with factors age, gender, DR mismatch, number of previous transplants, and DSA, TPA, viral and blood group antibodies (Table 2). Baseline levels of all variables including DSA were not predictive of rejection. This was true both in the crude model with no adjustments and in the ones following adjustment for age, sex previous transplant, DR mismatch and other baseline antibody levels. No other variables except change in the DSA were significantly associated with rejection ($p = 0.01$) consistently in all models with or without adjustment (all data not shown). An increase in DSA of 1000 units from pre treatment to a post transplant peak was equivalent to an increased odds of rejection of 30%, 1.30 (1.06, 1.58) if the adjustment was made for other antibodies at baseline. The odds increased to 50%, 1.47(1.08, 2.00) if it was adjusted for changes in the level of all other antibodies from pre to peak post transplant. Though VZV, anti Hbs and DR mismatch showed significance on isolated models, the effect was not constant.

Discussion

The particular stimulus to this study was the observation that HLA antibodies may rise after an infection or blood transfusion, even though the HLA antigen is not being directly represented to the subject [1]. This raised important questions about the specificity of the humoral immune response. Hypotheses to explain the increase in HLA antibody levels during a viral infection include cross reactivity between epitopes on HLA molecules and viruses, immune up regulation secondary to a polyclonal antibody response to a single antigen, or an increase in non-self HLA expression on any non-self cells in the host expressing HLA [16]. One way interrogate these hypotheses was to look at the reverse situation in clinical practice, ie, during a response when the subject is synthesising HLA antibodies rapidly, do other antibody levels change?

This study has examined in detail, for the first time, the levels of HLA and several non-HLA antibodies after HLA antibody incompatible transplantation. The study has shown several novel findings. First, although HLA antibodies may rise during a viral infection, viral antibodies did not rise during an intense HLA antibody response. Second, there were some unexplained antibody responses to non-donor HLA antibodies, not explained by the current understanding in epitope sharing. On regrouping the patients with reference to rejection as the outcome and studying the relationship of all the variables, it was evident that the increase in the DSA peak post transplant in comparison to the pre-treatment level was the only factor significantly associated with occurrence of rejection.

Viral and blood group antibodies did not rise during intense re-synthesis of HLA antibodies, indicating specificity in the immune response and the lack of a bystander effect between plasma cell clones. Studies have shown that vaccination expands both specific and bystander memory T cells but antibody production remained vaccine specific [17]. Some other studies have shown that influenza vaccine in stable kidney transplant patients is not associated with the risk of acute rejection or increase in DSA levels [18,19]. In contrast, other studies have shown vaccination to potentiate allograft rejection [20]. This is thought to occur due to non-specific immune activation and induction of cross-reactive immunity, resulting in enhanced humoral or cellular responses against the donor antigens [21,22,23]. A recent study from Switzerland showed that multiple doses of influenza vaccine may lead to the production of anti-HLA antibodies in a significant proportion of kidney transplant recipients [24].

We compared quantitative antibody analysis of latent viral antigens at different time points, to see if there was a rise in these antibodies due to non-antigen specific stimulation i.e. bystander activation of memory cells due to antigenically unrelated activation. This study was not designed to look at the dominant type of immune response (humoral and cellular) generated for controlling specific viral infections. A recent study looked at the longitudinal quantitative analysis of antibody titres specific for various viral antigens including varicella-zoster virus for a period of up to 26 years [25]. They showed that in spite of vaccinations, viral infections, and reactivation the antibody changes were very specific, thus ruling out bystander activation as a cause of antibody production.

Zachary and associates have previously shown that in HLA incompatible renal transplantation, the viral antibodies detected by ELISA did not change in relation to plasmapheresis [26]. It could be that the immunosuppressive medications could have profoundly decreased the humoral immune responsiveness [27]. However, in these patients the concomitant administration of cytomegalovirus immune globulin (CMVIg) with high levels of CMV antibodies meant that the samples used for testing were some weeks after the transplant. The timing of testing was based on calculated time for clearance of the CMVIg originated antibodies. This is the first study that has been able to examine the memory immune response early after transplantation, particularly during an intense donor-specific humoral response.

In our study patients received pre transplant plasmapheresis to remove DSA prior to transplantation. Also some patients received OKT3 or ATG for treatment of humoral and steroid resistant rejection post transplant. This could have modified all antibody levels post transplant. However, more than half of the study patients (29/52) had a significant rise in DSA levels post transplant, out of whom 18 patients received ATG/OKT3 for treatment of rejection. Thus it can be said that in spite of the overall immunosuppression there was an exquisite rise in the HLA antibodies and hence did not affect the analyses.

With regards to TPAs, many of these did follow the donor specific antibody levels, and this could be explained by epitope

sharing. Because of the extent of possible epitope sharing it was not easy to exclude epitope sharing as the reason for change in non-donor specific HLA antibody levels in nearly all cases. However, in five cases there was evidence of a response in non-donor specific HLA antibody levels that was not explicable by any possible epitope sharing. It is possible that this observation is due to shared epitopes not previously described or another form of shared antigenicity such as denaturing of antigen on the microbeads. This requires further investigation with techniques such as absorption studies using donor specific HLA protein.

There are some shortcomings in this study. Samples were collected prospectively for studies on antibodies on a daily basis in the early post transplant period, so that changes in antibody levels were closely tracked. However, the inevitable heterogeneity in a clinical series means that patients did receive different treatments. Also, a more qualitative analysis like mapping of viral epitopes and the change over time, would aid in a better understanding of the infection-alloimmunity connection. Unfortunately this was outside the scope of this study. Further studies are needed in this area to increase our understanding about the immunological response.

Conclusions

During a period of intense re-synthesis of donor specific HLA antibodies, it was possible to follow in detail the levels of non-donor specific HLA antibodies, and other antibodies previously stimulated by infection, immunization, or 'natural' blood group antibodies. Our results showed that the immune response was generally specific for donor HLA in contrast to the viral, blood group or third party antigens post transplantation and the increase in the DSA post transplant in comparison to pre-treatment is strongly associated with occurrence of rejection.

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Author Contributions

Conceived and designed the experiments: NSK DZ SD RMH DL FTL HK LT CI RH NR DB. Performed the experiments: NSK. Analyzed the data: NSK RMH DZ SD NR. Contributed reagents/materials/analysis tools: NSK DZ SD RMH. Wrote the paper: NSK DZ RMH.

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Human Leukocyte Antigen Antibody-Incompatible Renal Transplantation: Excellent Medium-Term Outcomes With Negative Cytotoxic Crossmatch

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Background. Human leukocyte antigen (HLA) antibody-incompatible renal transplantation has been increasingly performed since 2000 but with few data on the medium-term outcomes.
Methods. Between 2003 and 2011, 84 patients received renal transplants with a pretreatment donor-specific antibody (DSA) level of more than 500 in a microbead assay. Seventeen patients had positive complement-dependent cytotoxic (CDC) crossmatch (XM), 44 had negative CDC XM and positive flow cytometric XM, and 23 had DSA detectable by microbead only. We also reviewed 28 patients with HLA antibodies but no DSA at transplant. DSAs were removed with plasmapheresis pretransplant, and patients did not routinely receive antithymocyte globulin posttransplant.
Results. Mean follow-up posttransplantation was 39.6 (range 2–91) months. Patient survival after the first year was 93.8%. Death-censored graft survival at 1, 3, and 5 years was 97.5%, 94.2%, and 80.4%, respectively, in all DSA+ve patients, worse at 5 years in the CDC+ve than in the CDC−ve/DSA+ve group at 45.6% and 88.6%, respectively ($P<0.03$). Five-year graft survival in the DSA−ve group was 82.1%. Rejection occurred in 53.1% of DSA+ve patients in the first year compared with 22% in the DSA−ve patients ($P<0.003$).
Conclusions. HLA antibody-incompatible renal transplantation had a high success rate if the CDC XM was negative. Further work is required to predict which CDC+ve XM grafts will be successful and to treat slowly progressive graft damage because of DSA in the first few years after transplantation.

Keywords: HLA antibodies, Plasmapheresis, Antibody-incompatible transplantation, Antibody-mediated rejection, Antithymocyte globulin.

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Donor-specific human leukocyte antigen (HLA) antibodies are being increasingly recognized as a major problem in renal transplantation. In the United Kingdom, approximately 30% of those waiting for a deceased donor transplant have HLA antibodies, and more than 200 potential living donor transplants per annum are complicated during work by the discovery of donor-specific HLA antibodies (DSA). Each year in the United Kingdom, more than 400 transplants fail after the first year posttransplant, and in the majority of these, DSAs probably play a part.

Treatments for acute and chronic HLA antibody-mediated damage are not yet fully effective. The main advances that have occurred in the past two decades are the ability to identify DSA with a sensitive microbead assay and to transplant with some early success across all but the highest levels of DSA (*1–3*).

Although national consensus meetings and national guidelines for best practice in HLA antibody-incompatible transplantation have been produced (*4*, *5*), national registry data have only recently been collected (*6*), and there is only one fully published randomized controlled trial in this field

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(*7*). Thus, the optimal treatment protocols and the outcomes of this type of transplant are not fully defined.

Several centers have identified retrospectively the patients who were transplanted across DSA but who were not part of a program of antibody-incompatible transplantation. Transplants with complement-dependent cytotoxic (CDC) crossmatch (XM−ve)/DSA+ve pretransplant had inferior outcome (*8–11*). Even transplants with negative CDC and flow cytometric (FC) XM and DSA detectable by microbead only have been reported to have an inferior outcome (*12*).

Single centers using who have transplanted across DSA with prospective identification of DSA and interventions including antibody removal have reported better outcomes. However, there was a reduced rate of graft survival in those with high DSA levels, and transplant glomerulopathy developed in some transplants over a longer follow-up (*13–19*).

In our center, HLA antibody-incompatible transplants have been performed since 2003, and these transplants were reviewed to determine the factors affecting rates of acute antibody-mediated rejection (AMR), graft and patient survival, and the longer term development of proteinuria and chronic antibody-mediated graft damage.

RESULTS

Between June 2003 and February 2011, 84 patients received transplantation across pretreatment DSA, mean follow-up was 39.6 (range 2–91) months. The total numbers with follow-up for 1, 3, and 5 years were 75, 48, and 17, respectively. Those at risk for events of death at 1, 3, and 5 years were 70, 43, and 12, respectively, and those at risk for events of graft loss after censoring for death at 1, 3, and 5 years were 67, 41, and 8, respectively. With increasing DSA levels, there was a higher proportion of patients with regrafts, more patients who started treatment for established renal failure as children, and more patients on longer time on renal replacement therapy (Table 1). The DSA-ve were more likely to be from deceased donors, with better HLA matching.

Pretransplant DSA and Plasmapheresis

The relationship between XM status and Luminex levels is shown in Figure 1. The numbers of sessions of plasmapheresis given pretransplant are listed in Table 1, together with the numbers of patients still positive at that level of antibody testing immediately pretransplant.

Patient Survival and Other Complications

Actuarial patient survival was 93.8% at 1, 3, and 5 years in the DSA+ve patients. Of the five deaths, four were FC XM +ve patients: a 66-year-old woman with chronic dialysis hypotension developed small bowel infarction 3 days posttransplant (had received double filtration plasmapheresis [DFPP]); a 25-year-old woman developed postoperative pulmonary edema and capillary leak syndrome (had received DFPP and alemtuzumab); a 61-year-old woman developed pneumonia at 2 weeks (had received DFPP and antithymocyte globulin [ATG]); and a 44-year-old woman developed cytomegalovirus infection at 11 months (had received DFPP and ATG). One patient whose DSAs were only microbead+ve died: a 47-year-old man who developed ascending biliary sepsis in the early postoperative period (had received DFPP and ATG). Two DSA−ve patients died: a 41-year-old diabetic man from hypoglycemia at 4 months and a woman aged 50 years at transplant from lung cancer at 5 years.

Infectious complications are listed in Table 2. Only three patients with cytomegalovirus infection required intravenous ganciclovir. There were a number of other complications associated with recipient comorbidity, either because of previous transplant surgery or complications associated with long-term renal failure. Ten DSA+ve patients, apart from those who died, required unplanned admission to critical care for circulatory or respiratory support.

Early Acute AMR

Figure 2 shows rejection-free survival according to the pretreatment DSA level. Early rejection episodes were almost exclusively compatible with AMR, showing glomerulitis and peritubular capillaritis with little tubulits. The findings in many of these patients have recently been published in detail (*20*).

In the DSA+ve patients, acute AMR was more frequent in women who received transplants from a partner or child than in other transplants, 13 of 18 (72%) vs. 34 of 66 (52%), respectively ($P<0.01$).

Of 38 episodes of early AMR, one graft failed in the first 3 months and another at just over 3 months, giving a rejection reversal rate of 94.7%. The early failure was a transplant with positive CDC XM due to donor-specific HLA DR4, whose CDC XM was not fully reversed by DFPP and the graft never functioned. The failure at just over 3 months was another case with positive CDC XM, who developed a glomerular thrombotic microangiopathy after early AMR.

Treatment for AMR evolved during the series. Initially, DFPP, intravenous immunoglobulin (IVIg), and rituximab were used, but these were replaced by cellular depletion therapies using OKT3 or ATG. Eleven patients were treated with OKT3 for acute AMR. Four were CDC XM+ve before treatment, five were FC+ve, and two were microbead+ve only. Three patients were positive for HLA class 1 DSA only and the other nine for various combinations of classes 1 and 2 DSA. There were no deaths. Three of these grafts failed at 3, 15, and 32 months. The other eight grafts are functioning at follow-up of 38 to 60 months.

Longer Term Graft Survival

Figure 2 shows death-censored graft survival and proteinuria-free survival according to pretreatment DSA levels assessed by cellular crossmatching, and Figure 3 shows graft survival according to DSA measured by microbead. Of the 15 patients with proteinuria, 5 have not yet progressed to serious graft dysfunction; in 1 patient, the proteinuria may have been related to recurrent urinary tract infections and in the other 4 patients, proteinuria declined and renal function improved or stabilized as DSA levels slowly declined in the first 3 years posttransplant. All graft losses have been because of rejection or transplant glomerulopathy, except one CDC+ve graft with severe early rejection who had mitral valve replacement at 33 months posttransplant followed by graft failure.

Other Complications

Table 2 shows other major complications. Two DSA+ve patients developed malignancy. A 20-year-old man treated

TABLE 1. Patient characteristics

	CDC + ve	Flow + ve	Microbead + ve	DSA − ve	*P*
N	17	44	23	28	
Male:female	11:6	11:33	8:15	13:15	<0.005
Age, mean (SD), yr	37.5 (11.5)	43.4 (11.2)	46.3 (14.1)	44.2 (14.2)	NS
Graft number, n (%)					
1	5 (29)	13 (30)	13 (57)	17 (61)	<0.005
2	10 (60)	24 (54)	7 (30)	10 (36)	
3	1 (5.5)	5 (11)	1 (4)	1 (3)	
4	1 (5.5)	2 (5)	2 (9)	0	
Years renal replacement therapy, mean (SD)	14 (8.8)	11.7 (7.0)	9.3 (9.7)	7.4 (6.3)	<0.003[a]
Number started RRT age <17 yr, n (%)	6 (35)	8 (18)	3 (14)	1 (4)	<0.001
Number with significant comorbidity, n (%)	9 (53)	23 (52)	6 (26)	8 (29)	<0.005
HLA mismatch, mean (SD)	3.3 (1.3)	2.6 (1.2)	3.0 (1.1)	1.5 (1.4)	<0.005[b]
DR mismatch, mean (SD)	1.1 (0.6)	0.8 (0.6)	1.1 (0.7)	0.25 (0.44)	<0.005[b]
Living donor relation					
Sibling	4	14	4	3	
Partner/child to mother	3	9	6	0	
Other living related	6	16	6	5	
Other living unrelated	3	2	4	6	
Deceased donor, n (%)	1 (6)	3 (7)	3 (13)	14 (50)	<0.01
HLA DSA main specificity					
Class1	4	28	16	0	<0.01[c]
DR	7	5	3	0	
DP/DQ/DRB3–4	6	11	4	0	
Class 1 and any class 2	12	16	8	0	<0.01[c]
Class 1 and DR	10	13	5	0	<0.01[c]
HLA DSA mean no.	3 (1.4)	2.2 (1.2)	1.4 (0.7)	0	<0.05[c]
Luminex single highest, mean (SD)	8461 (2542)	5502 (3632)	1514 (1211)	—	<0.01[c]
Luminex total, mean (SD)	18,423 (8397)	8006 (6395)	1740 (1275)	—	
RMF pretreatment	22.9 (11.4)	7.6 (5.5)	2.3 (1.5)	—	<0.01[c]
RMF pretransplant, mean (SD)	15.6 (11.0)	5.5 (4.9)	2.0 (1.9)	—	<0.01[c]
No sessions plasmapheresis, mean (SD)	5.9 (2.8)	3.8 (2.0)	1.2 (1.6)	0	<0.01[c]
Crossmatch negative after plasmapheresis[d]	9/16	19/37	2/9	—	

[a] Significance for CDC+ve against all other groups and FC+ve or microbead+ve against DSA−ve.
[b] All DSA+ve grafts vs. DSA−ve grafts.
[c] DSA−ve cases not included.
[d] Number (%) of patients receiving plasmapheresis whose crossmatch was a level lower after plasmapheresis, i.e., CDC+ve who became CDC−ve but could be either FC+ve or FC−ve; FC+ve became FC−ve; microbead+ve became microbead−ve, <500 U. The aim with plasmapheresis in microbead+ve cases was to reduce the microbead level to below 2000 U, not to below 500 U.

CDC, complement-dependent cytotoxic; DSA, donor-specific antibody; SD, standard deviation; RRT, renal replacement therapy; HLA, human leukocyte antigen; RMF, relative median fluorescence; FC, flow cytometric; NS, not significant.

with OKT3 developed testicular cancer at 6 months post-transplant, resolved 4 years later after chemotherapy and radiotherapy. A woman aged 42 years at transplant with no early rejection and no ATG or OKT3 developed a squamous cell carcinoma of the skin at 2 years, treated with radiotherapy, and at 3 years developed a squamous cell carcinoma of the rectum and is currently receiving radiotherapy with a view to surgical resection.

DISCUSSION

This series of HLA antibody-incompatible renal transplants is large enough to show the impact of different DSA levels on clinical outcomes without having to use historical controls. We have shown that intensive monitoring and interventions in CDC−ve/DSA+ve transplants produced excellent graft survival with no graft losses from rejection in the first 6 months after transplantation. However, outcomes in positive CDC XM transplants or those with microbead DSA more than 10,000 were less good. Perhaps because intensive immunosuppression was given on demand routinely, mortality was acceptable.

Pretransplant

DSA measured by Luminex showed considerable overlap between the CDC+ve and FC+ve XM groups. Further work is required to examine these HLA antibodies in more

detail. Issues may include variation in sensitivity of the beads between specificities or isotypes, or saturation of microbeads at high levels of antibody (in this study, we present only analysis with "neat" serum). The presence of HLA antibodies inhibitory to the microbead assay, which may be of IgM class, may also be an issue as we have observed marked changes in Luminex activity after treatment of serum with dithiothreitol (DTT), as previously described (*21*).

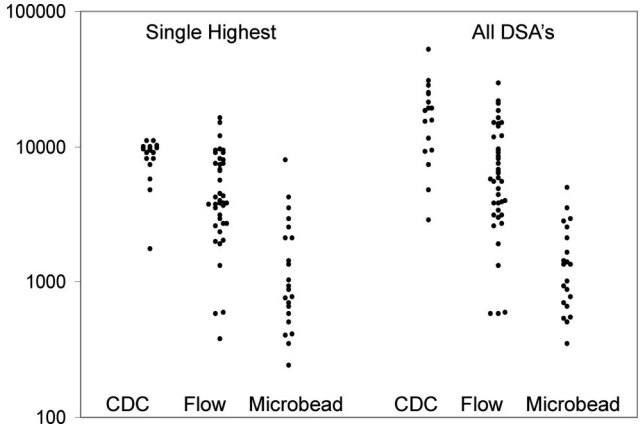

FIGURE 1. Relationship between Luminex levels and XM status. (*Left*) Single highest DSA, (*right*) sum of all DSA's. Microbead analysis performed with serum at standard dilution only. XM, crossmatch; DSA, donor-specific antibody.

Many of the patients in this program had significant comorbidity, and the comorbidity count increased with increasing levels of DSA. To achieve successful early engraftment and simultaneously to minimize recipient morbidity and mortality, the intensity of treatment was kept to a minimum. In the pretransplant period, plasmapheresis was felt to be the key intervention, and we used this as intensively as possible. The choice of DFPP enabled us to double the plasma volumes treated per session compared with plasma exchange (*22*). Despite this, many patients were transplanted across persistent DSA with early rejection, as we have previously discussed. However, we were able to reduce the intensity of other agents pretransplant, such as cellular depleting therapies.

Risk of Rejection

In the presence of DSA at transplant, approximately 50% of the cases in this series experienced early acute AMR. The rejection rates in 119 patients transplanted across HLA antibody barriers at the Mayo Clinic have recently been reported (*14*). For patients with a total DSA level of median fluorescence intensity (MFI) more than 10,000 as measured by microbead, or a positive anti-human globulin-enhanced CDC XM, they reported an early AMR rate of 50%. However, with lower DSA levels, their rate was approximately 20%, which is lower than in this series. The difference may be be-

TABLE 2. Outcomes after transplantation

	CDC + ve	Flow + ve	Microbead + ve	DSA − ve	*P*
N	17	44	23	28	
Died, n (%)	0	4 (9)	1 (4)	2 (7)	NS[a]
Graft failure, n (%)	6 (35)	1 (2)	3 (13)	2 (7)	0.03[a]
Number with acute rejection, n (%)	11 (65)	26 (59)	10 (44)	7 (25)	<0.01[a]
Rejection in female recipients with child or partner donor, n (%)	3/3 (100)	7/9 (78)	3/6 (50)	0/1	<0.01
Rejection in transplants from other donors, n (%)	8/14 (57)	19/35 (54)	7/17 (42)	7/27 (26)	
Dialysis dependent during rejection, n (% of patients with rejection)	4 (36)	7 (27)	2 (20)	0	<0.01
Rejection treatment, n (% of patients with rejection)					
Methylprednisolone	10 (91)	25 (96)	8 (80)	7 (100)	
PP	5 (45)	5 (19)	2 (20)	0	
ATG	2 (18)	13 (50)	4 (40)	1 (14)	
OKT3	4 (36)	2 (8)	2 (20)	0	
IVIg	3 (27)	1 (4)	0	0	
Eculizumab	0	1 (4)	0	0	
Complications					
Serious bacterial infection	3	11	3	5	
CMV	1	5	5	3	
BK	1	3	1	0	
Other, including fungal	1	2	1	1	
Malignancy	1	0	1	1	
eGFR in functioning grafts, mL/min/1.73 m^2, mean (SD)					
12 mo	53.8 (16.9)	57.2 (25)	73.0 (39.1)	67.4 (30.6)	NS
36 mo	45.4 (18.9)	54.2 (28.7)	46.9 (19.2)	56.7 (12.2)	NS

Complications are for the first year after transplantation, except malignancy, which is to latest follow-up.

[a] Significance calculated from Kaplan-Meier analysis, see Figures.

CDC complement-dependent cytotoxic; DSA, donor-specific antibody; PP, plasmapheresis; ATG, antithymocyte globulin; IVIg, intravenous immunoglobulin; CMV, cytomegalovirus; BK, BK virus; eGFR, estimated glomerular filtration rate; NS, not significant.

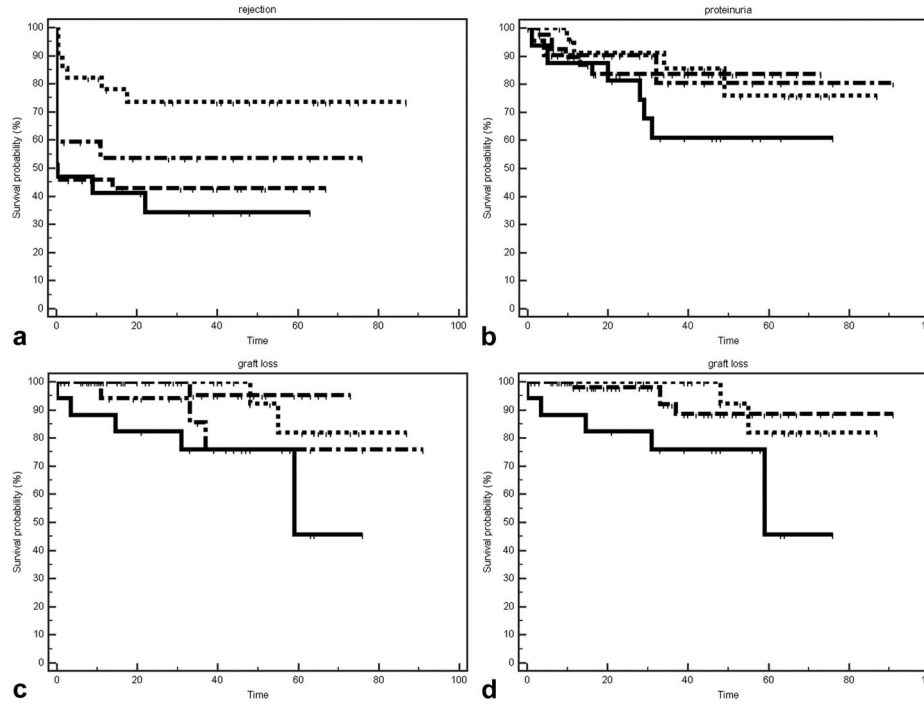

FIGURE 2. Death-censored outcomes according to pretreatment levels of cellular crossmatch. *Solid line*, positive CDC crossmatch; *dashed line*, positive FC crossmatch, negative CDC crossmatch; *dashed/dotted line*, positive microbead DSA, negative crossmatch; *dotted line*, negative DSA. (a) Rejection-free survival, *P* less than 0.03; (b) proteinuria-free survival; (c) graft survival *P* less than 0.03; and (d) graft survival, *P* less than 0.03. The FC XM+ve and microbead+ve/XM−ve groups are combined in the dashed line. CDC, complement-dependent cytotoxic; FC, flow cytometric; DSA, donor-specific antibody; XM, crossmatch.

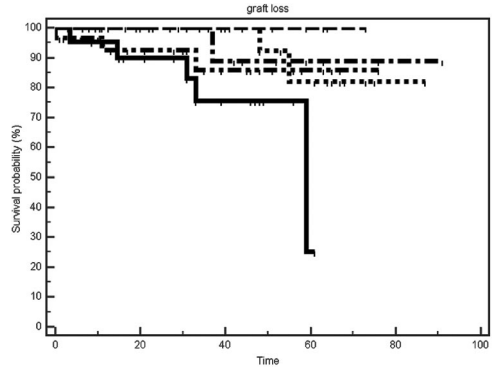

FIGURE 3. Death-censored graft survival according to pretreatment microbead DSA level, *P* less than 0.05. Microbead DSA less than 500, *dotted line*; 500 to 1000, *dashed line interrupted by two dots*; 1000 to 5000, *dashed line interrupted by single dots*; and 5000 to 10,000, *dashed line*; more than 10,000, *solid line*. DSA, donor-specific antibody.

cause routine ATG induction was administered. Given the excellent outcomes of prompt treatment of AMR with ATG, we continue to use ATG only on demand, meaning that nearly half of our patients are not exposed to this augmented immunosuppression.

The risk of rejection was high in women receiving transplants from partners or children. This is presumably a consequence of the presentation of the same antigens to which they have been sensitized. Further work is required to examine the contributions to risk of the behavior of the DSA (e.g., affinity or resynthesis rates), or an associated T-cell response (some T cells may recognize different epitopes on HLA to antibodies, so transplanting across exactly the sensitizing HLA antigen may expose the graft to a specific T cell and a specific antibody challenge).

Treatment of Rejection

We have previously shown in this series of transplants that glomerular leukocyte infiltration preceding AMR may be initiated within 30 min of graft reperfusion even at low DSA levels (*20*). Over the next few days, there may be progressive cellular infiltration of glomeruli and peritubular capillaries with T lymphocytes, monocytes, and neutrophils, but few B lymphocytes. Staining for C4d may be negative for the first few days. DSA levels may rise sharply to peak levels at approximately 12 days posttransplant (*23*), then the graft will function in the presence of DSA, and then DSA levels may fall rapidly in the second and third weeks after transplantation.

This understanding led us to focus on early administration of cellular depletion therapy for the treatment of AMR, either T-cell depletion alone using OKT3 or polyclonal ATG. Either of these strategies achieved early reversal of rejection in more than 95% of cases.

Proteinuria

It was encouraging that no new proteinuria developed in our patients after 3 years, perhaps suggesting that the DSA present pretransplantation will cause problems either fairly early after transplantation or not at all. However, the numbers of patients with long-term follow-up was small, and proteinuria and transplant glomerulopathy continued to develop after 3 years in the larger Mayo Clinic series (*13, 14*). More detailed follow-up is indicated.

Graft Loss

The CDC XM was the best predictor of outcome in our series. Unfortunately, within the CDC+ve group, there was no consistent difference between those with good outcome and those with failure or progressive dysfunction. For example, one case with good outcome had positive CDC XM with class 1 HLA, though this became negative after DFPP. We

would not transplant across a positive CDC XM for class 1 or DR that could not be rendered negative by DFPP, but we transplanted successfully across positive CDC XM in the operating theater for HLA DQ and DP and DRB3–4.

The 5-year death-censored graft survival in the United Kingdom for all first deceased donor transplants is 82% (95% CI: 81–83) and for first living donor transplants is 89% (95% CI: 87%–90%; NHS BT data, accessed March 2011 at www.transplant.org.uk). Outcomes in our CDC−ve grafts were equivalent, indicating that transplantation across HLA antibodies can be achieved successfully with an intensive treatment program.

Without prospective identification of DSA and appropriate therapy, results seem to be less good. The Royal London Hospital showed that CDC−ve/DSA+ve status was associated with a relative risk of graft failure at 5 years of 6.5 compared with those with no HLA antibodies and non-donor-reactive HLA antibodies. Graft survival was 62% in the CDC−ve/DSA+ve group and 79% in the DSA−ve group (*8*). The St. Louis Hospital, Paris, reported 5-year death-censored graft survival of 71.2% with CDC−ve/DSA+ve, compared with 89.2% in nonsensitized patients and 92.5% sensitized with no DSA (*9*). The University of Wisconsin showed, using alemtuzumab induction, increased rejection was observed in CDC−ve/DSA+ve transplants, and there was reduced graft survival in those with DSA against HLA DR at MFI more than 1000 (54.3% vs. 86.5%) (*10*).

Other recent single-center reports from HLA antibody-incompatible programs show outcomes in DSA+ve patients with FC+ve or CDC+ve crossmatches. The Cedars Sinai Medical Centre used IVIgs and rituximab pretransplant in a series of 76 transplants and reported overall 2-year patient and nondeath-censored graft survival rates of 95% and 84%, respectively; these were divided between living and deceased donors at 100%/90% and 91%/80%, respectively (*24*). Risk of AMR was associated with the level of DSA. The University of Maryland reported the outcomes in a series of 41 patients, in whom 1- and 5-year graft survival was 89.9% and 69.4%, respectively. These patients were FC+ve, though it is not clear how many were CDC+ve as well (*15*).

Despite the excellent early outcomes in our CDC−ve transplants, a number of grafts were lost from rejection and transplant glomerulopathy between 9 and 36 months posttransplant. Numerically the number of losses was greater in the microbead+ve group than the FC+ve group, suggesting that the starting level of DSA was less important in these patients than the evolution of DSA posttransplant and/or the response of the graft to that DSA.

Mortality

Mortality after HLA antibody-incompatible transplantation is a concern, especially as the patients with highest DSA levels require more intense treatment and also tend to have spent more time on renal replacement therapy and have more comorbidities (Table 1). Four of the five deaths in this series were in patients who had received DFPP and cellular depletion therapies. We try to avoid combinations of these therapies, normally using DFPP only pretransplant and administering cellular depletion therapies only on demand. Mortality is also critically dependent on patient selection, which is difficult to define in single-center studies. Larger studies and registry data may cast more light on how patient selection and choice of immunosuppression affect graft outcome and mortality.

Summary

This series indicates that good outcomes can be achieved in HLA antibody-incompatible renal transplantation using a strategy of careful monitoring and "on demand" intervention with leukocyte depletion therapy. Longer term outcomes were satisfactory in CDC−ve transplants although there were graft losses at 9 to 36 months, even in patients with low starting levels of DSA. Further work is required to improve the early and long-term outcomes in HLA antibody-incompatible renal transplantation.

MATERIALS AND METHODS

Patients

Patients were included if transplanted across a total pretreatment DSA level of at least MFI 500 as measured by microbeads. ABO-incompatible transplants were excluded. Patients transplanted between 2003 and 2011 who had a panel reactive antibody level of more than 10% but no DSA-detectable pretransplant were used as a comparison group. The study was approved by the Coventry Research Ethics Committee.

Double Filtration Plasmapheresis

DFPP was performed using an HF-440 (Infomed, Geneva, Switzerland) machine with a Plasmacure plasma separator (Kuraray Medical Inc., Okayama, Japan) and Evaflux 2A plasma fractionator (Kawasimi Laboratories, Tokyo, Japan), as previously described (*22*).

IVIgs (Sandoglobulin, CSL Behring, West Sussex, United Kingdom) were used in a few patients at the start of the program, either as a single dose after the course of pretransplant DFPP or during an episode of rejection, but subsequently were not used.

Immunosuppression and Rejection

Immunosuppression consisted of mycophenolate mofetil 500 or 1000 mg twice daily started 10 days before transplant, with dose reduced if white cell count fell below 4.0×10^9/L. Tacrolimus was started 4 days before transplant at a dose of 0.15 mg/kg/day in divided doses, with a target trough level of 10 to 15 μg/L in the first month. Prednisolone 20 mg once daily was started at the time of surgery, and methylprednisolone 500 mg was given as a single intravenous dose during the transplant operation. Two doses of basiliximab 20 mg were given at days 0 and 4.

Posttransplant serum samples for antibody analysis were taken daily for the first 2 weeks and then three times a week for the next 2 weeks. Rejection was diagnosed by renal biopsy if the renal function deteriorated or clinically if there was rapid onset oliguria with a rise in both creatinine and in DSA levels. Rejection was treated with high-dose methylprednisolone and daily plasmapheresis for 3 days if DSA levels were raised, until patient 37 when posttransplant plasmapheresis was phased out, and with OKT3 (muromomab-CD3) (Orthoclone) or rabbit (ATG) (Genzyme, Cambridge, MA) if rejection was steroid resistant. Some patients felt to be at high risk of early rejection were started on ATG shortly after transplant if there was oliguria.

Crossmatching and HLA Antibody Detection

Cellular crossmatching was performed on B cells and T cells separated from peripheral blood (living donors) and either peripheral blood or spleen (deceased donors). For CDC crossmatching, 2 μL serum+1 μL cells (2×10^6/mL) were incubated for 60 min at room temperature with and without DTT. Five microliters complement (rabbit serum) was added and incubated for 60 min at room temperature. Cytotoxicity was visualized using acridine orange/ethidium bromide cocktail. Anti-human globulin enhancement was not used.

FC crossmatching and microbead testing were performed as previously described (*23*, *25*). For the FC XM, the readout was the ratio of the median

channel fluorescence of the test sample over that for a negative control AB serum as relative median fluorescence. The threshold for a positive XM was set at a relative median fluorescence of 4.0 for primary grafts and 2.5 for regrafts.

HLA classes I- and II-specific antibodies were identified using microbead assay manufactured by One Lambda Inc. (Canoga Park, CA) analyzed on the Luminex platform (XMap 200; Luminex, Austin, TX). Phenotype-coated beads were used until 2009, and single-antigen beads have been used to retest most of the prior samples to confirm specificities.

A positive cellular XM was only considered to be due to DSA if the microbead assay confirmed the presence of a DSA. Uncertainty in the interpretation of the assays was resolved with auto-crossmatching and/or further testing of the microbead samples using reduction of IgM antibodies with DTT or dilutions of the serum samples.

Statistical Analysis

Kaplan Meier survival analysis was performed using Med Calc (Mariakerke, Belgium), and chi-squared and *t* testing using Excel (Microsoft, Redmond, WA).

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