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Review

Molecular properties of human IgG subclasses and their implications for designing therapeutic monoclonal antibodies against infectious diseases

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ABSTRACT

Monoclonal antibodies are being developed as therapeutics to complement drugs and vaccines or to fill the gap where no drugs or vaccines exist. These therapeutic antibodies (ThAb) may be especially important for infectious diseases in which there is antibiotic resistance, toxin-mediated pathogenesis, or for emerging pathogens. The unique structure of antibodies determines the specific nature of the effector function, so when developing ThAb, the desired effector functions need to be considered and integrated into the design and development processes to ensure maximum efficacy and safety. Antibody subclass is a critical consideration, but it is noteworthy that almost all ThAb that are licenced or currently in development utilise an IgG1 backbone. This review outlines the major structural properties that vary across subclasses, how these properties affect functional immunity, and discusses the various approaches used to study subclass responses to infectious diseases. We also review the factors associated with the selection of antibody subclasses when designing ThAb and highlight circumstances where different subclass properties might be beneficial when applied to particular infectious diseases. These approaches are critical to the future design of ThAb and to the study of naturally-acquired and vaccine-induced immunity.

1. Introduction

Emil von Behring's discovery of anti-toxins to diphtheria, tetanus and anthrax in 1901 eventually led to the discovery of antibodies (Gronski et al., 1991). Over a century later, the use of therapeutic antibodies (ThAb) has become one of the fastest growing areas of the pharmaceutical industry, yet ironically, the development of monoclonal ThAb against infectious diseases has been slow compared to most other fields. There remains a significant knowledge gap in identifying the roles for ThAb against specific infectious diseases, and also the exact molecular properties that are required to ensure ThAb induce the desired effector functions, but avoid unwanted responses. It is well known that the main isotypes of human immunoglobulins have unique structural features that allow them to perform specific immune effector functions (Fig. 1) (Nezlin, 1998). In this review, we focus on IgG and its subclasses and highlight the complex structure-function relationship that is critical to designing safer and more effective monoclonal ThAb (Table S1). We place this knowledge into the context of infectious diseases and highlight how studies should now evaluate the best IgG subclasses or molecular properties required for effective treatment of particular infectious diseases.

A wide repertoire of monoclonal ThAb are currently licenced with hundreds more in pre-clinical and clinical development (The Antibody Society, 2014; Wu et al., 2014). These ThAb are administered for a wide range of conditions, although the vast majority are used for cancer, autoimmune disorders and transplantation (Fig. 2A and Table S2). It is interesting to note that despite the clear role antibodies play against many infections,

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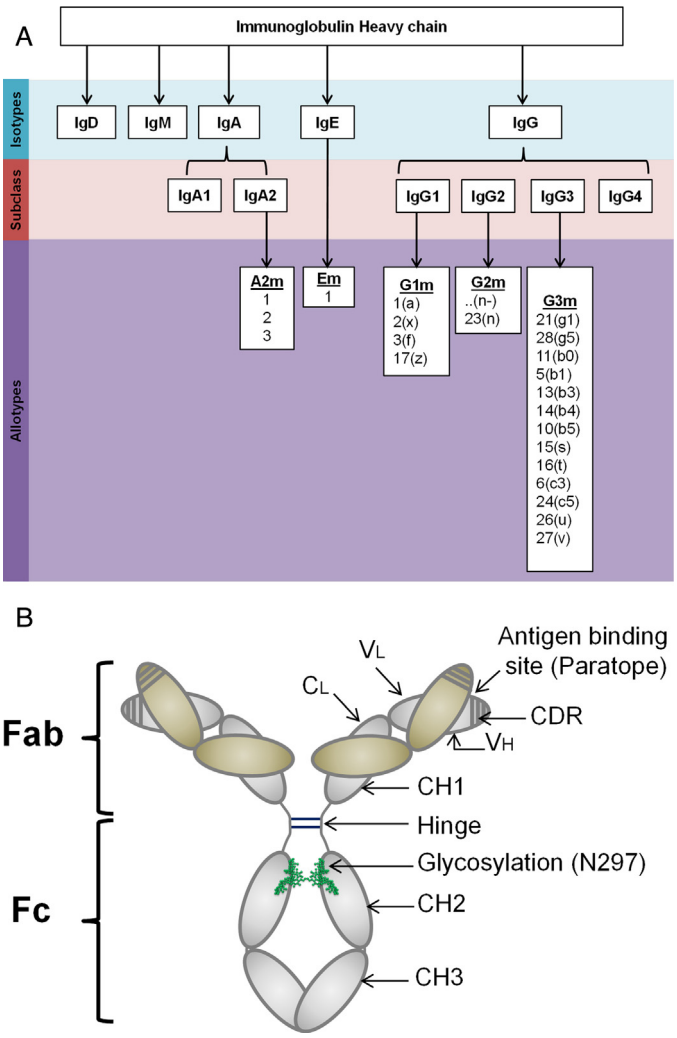

Fig. 1. Antibody structure and nomenclature. (A) The potential isotypes, subclasses and allotypes of immunoglobulins in humans. Both the letter and number code are provided for each allotype (Jefferis and Lefranc, 2009). (B) Schematic depicting the basic structure of an antibody and associated nomenclature. The light chain (shown in brown) consists of a variable (VL) and a constant (CL) domain while the heavy chain (shown in grey) consists of a variable (VH) domain and three constant domains (CH1, CH2 and CH3). Interchain disulfide bonds within the hinge region stabilise overall antibody structure. The complementarity determining regions (CDR, shown as striped lines) determine antigen specificity. The glycosylation patterns can also affect function (shown in green).

there are only two licenced monoclonal ThAb that target infectious agents (Palivizumab against human Respiratory Syncytial Virus and Raxibacumab against *Bacillus anthracis*) (Table 1). The majority of licenced ThAb are full length rather than Fab fragments (Fig. 2B). The benefits of using full length ThAb include longer serum half-life as a result of interaction with FcRn, improved effector function *via* engagement with a range of Fc receptors, and in some cases, more effective neutralisation when compared to the corresponding Fab fragment (Abboud et al., 2010; Bournazos et al., 2014; DiLillo et al., 2014; Halper-Stromberg et al., 2014). Most of the approved ThAb are either humanised or fully human IgG antibodies (Figs. 2C and S1). Murine or chimeric antibodies carry an increased risk of adverse anti-murine reactions in patients, and there has been a general move towards using a fully human scaffold in the development of potential ThAb. While using intact IgG allows for the selection of antibody IgG subclass to elicit specific effector functions, this is not reflected in the current range of licenced antibodies in which the majority are IgG1 (Fig. 2D and Table S2). This is likely due to IgG1 displaying potent effector functions, being the most predominant serum subclass, and was the backbone used in early approved ThAb. IgG2 or IgG4 has been used when a lack of specific cellular activity is desirable. Interestingly, there are no approved IgG3 ThAb, with suggestions that this may be because of (i) an increased likelihood for proteolysis due to an extensive hinge region (Carter, 2006), (ii) the many IgG3 allotypes across populations, (iii) IgG3 cannot be purified with protein A, or (iv) the reduced serum half-life of IgG3 compared to other subclasses (Table S1). In this review, we provide a concise overview of the known human IgG effector and subclass properties (Section 2) and discuss how this information could be used when designing ThAb to infectious diseases (Section 3).

2. Molecular properties of IgG subclasses relevant to therapeutic antibodies

IgG consists of two heavy chains and two light chains with the main molecular features described in Fig. 1B (Liu and May, 2012; Padlan, 1994; Ramsland and Farrugia, 2002). Within IgG, the fragment antigen binding (Fab) region contains the paratope, and can exert direct effects through binding interactions with antigen (*e.g.*, blocking a host recognition protein or inhibiting a toxin/enzyme of a pathogen). Meanwhile, the fragment crystallisable (Fc) region interacts with a variety of accessory molecules to mediate indirect effector functions such as antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP) and complement-dependent cytotoxicity (CDC) (Peipp et al., 2008) (Fig. 3A and B). These Fc mediated effector functions are especially important against infectious diseases where cellular and complement mediated responses are important for efficient pathogen clearance.

The structural and functional properties of the IgG subclasses vary, as do their response profiles to different infectious diseases, and these differences can be utilised in the development of effective ThAb (Carter, 2006; Jefferis, 2012). Although the heavy chains share greater than 90% sequence identity across IgG subclasses (Rispens and Vidarsson, 2014), there are differences in surface-exposed residues on the constant (CH1, CH2 and CH3) domains, as well as substantial variation within the hinge region (Fig.3C, D and Table S1). It is the hinge structure that confers many of the unique properties to each IgG subclass such as stability, flexibility and distances spanned by the two Fabs and the attendant Fc (Liu and May, 2012; Roux et al., 1997; Tian et al., 2014). Importantly, some areas of the Fc and the hinge that differ between IgG subclasses clearly overlap with residues known to be involved with binding to both activating and inhibitory Fcγ receptors (FcγR), the neonatal receptor for IgG (FcRn) and complement component C1q (Fig. 3A and B). The occurrence of key amino acid differences within the binding sites of these effector molecules helps explain the observed differences in the effector properties of the IgG subclasses (Table S1). This structural and molecular information is important when choosing a subclass backbone for a therapeutic antibody or introducing changes in key amino acids to tailor antibodies for a specific purpose.

2.1. Binding site on IgG-Fc for activating and inhibitory FcγRs

The Fc of IgG interacts with several cellular FcγRs to stimulate and regulate downstream effector mechanisms (Guilliams et al., 2014). There are five activating receptors, namely FcγRI (CD64), FcγRIIa (CD32a), FcγRIIc (CD32c), FcγRIIIa (CD16a) and FcγRIIIb (CD16b), and one inhibitory receptor FcγRIIb (CD32b) (Hogarth and Pietersz, 2012; Pincetic et al., 2014). IgG subclasses vary in their ability to bind to FcγR and this differential binding determines

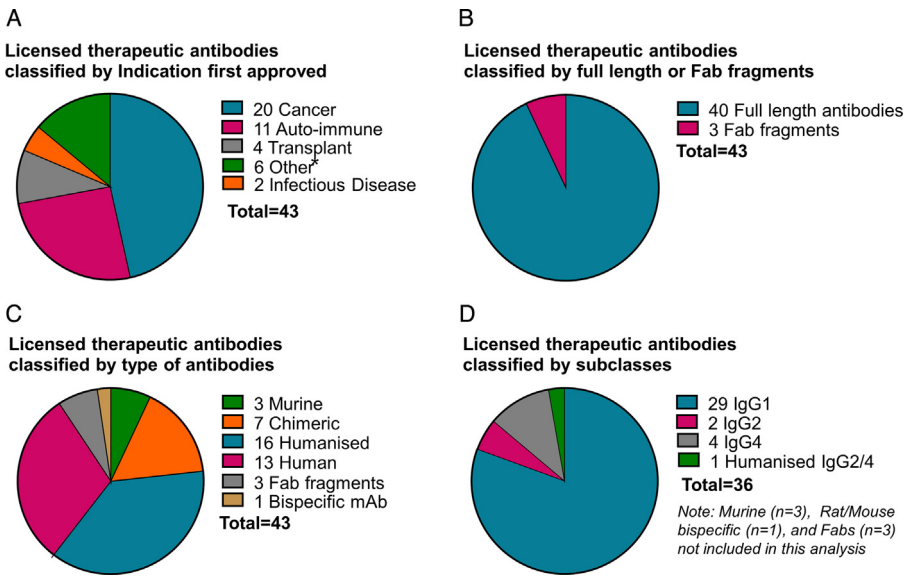

Fig. 2. The structure and targets of licenced therapeutic antibodies. The current (July 2014) list of therapeutic antibodies that have been licenced for clinical use in the USA or EU were grouped according to (A) disease indication first approved, (B) antibody structure (full length or Fab fragments), (C) antibody species type (*e.g.* murine, chimeric, humanised, fully human), and (D) antibody subclass. These figures were compiled using data from The Antibody Society (2014) and the data table used to prepare these figures is provided in the supplementary materials (Supplementary Table S2). *Panel A: 'Other' indications include prevention of blood clots, bone loss, asthma, macular degeneration, paroxysmal nocturnal hemoglobinuria and Muckle–Wells syndrome.

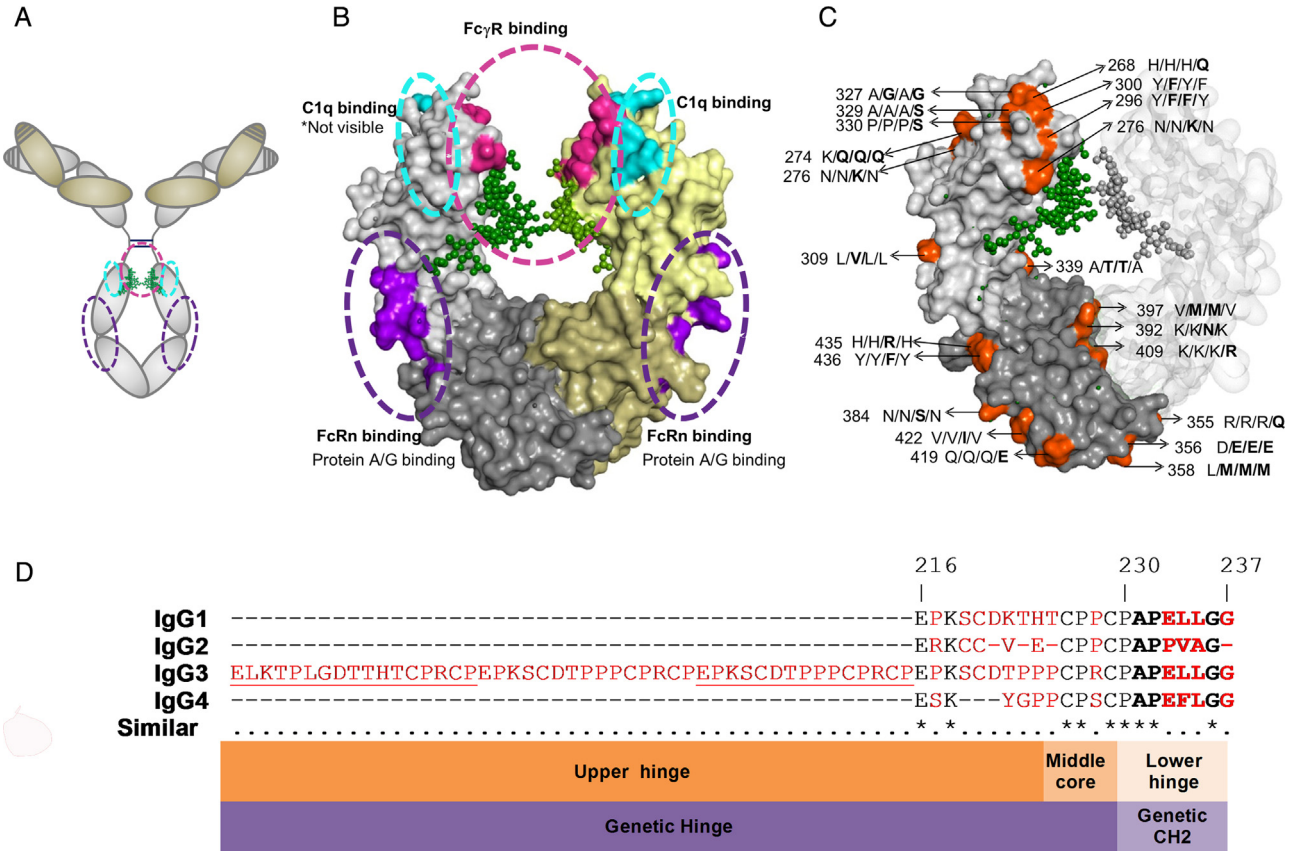

Fig. 3. Structural insights for Fc interactions and the differences between IgG subclasses. (A) A generalised schematic of an antibody with the major binding sites (Fcγ receptors (magenta), C1q (cyan) and FcRn (purple)) superimposed. (B) Crystal structure of Fc region of IgG1 (PDB: 1FC1) (Deisenhofer, 1981) with the Fcγ receptors binding, C1q binding, and FcRn binding sites highlighted. Note that there is one available binding site for Fcγ receptors and 2 potential binding sites for C1q and FcRn per IgG molecule. (C) Crystal structure of Fc region of IgG1 (PDB: 1FC1) (Deisenhofer, 1981) with all amino acid differences between the subclasses highlighted. The residues that differ from IgG1 are in bold. The allotypes used were IgG1 (G1m1,17), IgG2 (G2m..) and IgG3 (G3m5*). (D) Alignment of the hinge residues between the four subclasses. The residues are numbered using the EU numbering scheme (Kabat et al., 1991); this scheme is based on IgG1 sequences and therefore does not assign numbers for most of the long IgG3 hinge sequence. Figure B and C were constructed using Accrelys Discovery studio visualizer (Version 3.5).

Table 1
Examples of infectious diseases with ThAb that are developed or are currently in clinical trials.

Licenced

Type	Organism	Product	Type and subclass
RNA virus	Respiratory syncytial virus (RSV)	MEDI-493/SYNAGIS®/palivizumab	Humanised IgG1
Gram-positive bacteria	*Bacillus anthracis*	ABthrax®/axibacumab	Human IgG1

In development

Type	Organism	No. in development	Type and subclass
RNA viruses	West Nile Virus	1	Humanised IgG1
	Rabies virus	3	All human IgG1
	Hepatitis C virus	2	All human IgG1
	Influenza virus	3	All human 1gG1
	Human Immunodeficiency virus (HIV)	10	1 chimeric IgG1; 2 humanised IgG1; 4 human IgG1; **1 human IgG2**; 2 not described.
	Respiratory syncytial virus (RSV)	3	All humanised IgG1
DNA viruses	Cytomegalovirus (Human Herpesvirus 5)	4	3 human IgG1; 1 subclass not described.
	Hepatitis B virus	4	All human IgG1
Gram-positive bacteria	*Bacillus anthracis*	4	1 chimeric IgG1; 3 human IgG1.
	Clostridium difficile	2	Both human IgG1
	Staphylococcus spp.	4	1 chimeric IgG1; 2 humanised IgG1, 1 human IgG.
	Streptococcus mutans	1	**Murine IgG/A**
Gram-negative bacteria	*Pseudomonas aeruginosa*	2	**1 human IgM; 1 humanised pegylated Fab**
	Escherichia coli	3	2 chimeric IgG1; 1 humanised IgG1
Fungi	*Candida albicans*	1	**Human scFv Heavy chain**
	Cryptococcus neoformans	1	**Murine IgG1**

Examples selected from the following databases (Chen et al., 2011; Desoubeaux et al., 2013; Froude et al., 2011; Oleksiewicz et al., 2012; Reichert and Dewitz, 2006; The Antibody Society, 2014; Wu et al., 2014) and from the primary literature for details of the subclass. The details of these monoclonal antibodies can be found in Supplementary Table 3. All ThAb candidates that are not chimeric/humanised or human IgG1 antibodies are highlighted in bold.

their ability to elicit a range of functional responses. For example, FcγRIIIa is the major receptor involved in the activation of ADCC (Gómez Román et al., 2014) and IgG3 followed closely by IgG1 display the highest affinities for this receptor (Bruhns et al., 2009), reflecting their ability to potently induce ADCC (Table S1).

The FcγR binding region on IgG includes parts of the CH2 and hinge, with binding influenced by glycosylation at N297 (Fig. 3A and B). Specific FcγR-interacting residues on IgG have been identified through X-ray crystallography of IgG1-Fc complexes with the different FcγRs (Ferrara et al., 2011; Lu et al., 2015; Radaev et al., 2001; Ramsland et al., 2011; Shields et al., 2001; Sondermann et al., 2000). Several of these studies have shown that there are conserved residues on IgG involved in binding to all FcγRs. However, other IgG residues vary in their interaction with the different FcγRs and some of these residues differ across IgG subclasses, which helps explain the different FcγR binding profiles of the IgG subclasses (Table S1). This has provided insight into the specific residues required for IgG interaction with the receptors involved in ADCC and ADCP. The possibility of using subclass specific sequences to tailor binding to different FcγRs should be considered when developing therapeutic IgG with the desired antibody-dependent effector functions.

2.2. Complement deposition and CDC by IgG

In the classical complement pathway, IgG binds to antigen to form immune complexes, which then interact with C1 and trigger a hierarchy of interactions and enzyme reactions that can lead to the formation of the membrane attack complex and CDC, resulting in clearance of the pathogen (Gaboriaud et al., 2004; Lindorfer et al., 2014). Deposition of C1q (binding to IgG-Fc) occurs with IgG1, 2 and 3, with IgG3 being the most effective; IgG4 is unable to interact with C1q (Table S1). Interestingly, C1q deposition efficiency does not directly correlate with CDC activity, in which IgG1 is the most potent IgG subclass (Bruggemann et al., 1987). The structural basis for this difference between C1q binding and CDC by IgG subclasses remains largely unresolved. Here we briefly summarise what is known about the IgG and C1q interaction.

The binding site on IgG1 for C1q has been located to the CH2 domain and the specific residues involved in the interaction have been studied using mutagenesis of IgG1 (Idusogie et al., 2000, 2001). We show the location of the C1q binding site on a diagrammatic representation of IgG (Fig. 3A) and the crystal structure of IgG1-Fc (Fig. 3B) (Deisenhofer, 1981). Although each IgG has two binding sites for C1q on opposite sides of the Fc, only a single site needs to be involved in C1q binding (Diebolder et al., 2014; Michaelsen et al., 2006). While a crystal structure of IgG bound to C1q has not been determined, the crystal structure of C1q (globular head) has been modelled with IgG1 and suggests a conformation of C1q and IgG1 that may also involve the Fab fragment (Gaboriaud et al., 2003, 2004). A recent study using cryo-electron tomography suggests that antigen bound IgG1 can form a hexameric structure that efficiently interacts with the C1q hexamer in a manner that is reminiscent of polymeric IgM (Diebolder et al., 2014).

2.3. Binding of IgG to FcRn determines half-life and placental transport

The interaction of IgG with FcRn is important in the context of placental transport and serum half-life (Challa et al., 2014; Ward and Ober, 2009). FcRn has structural homology to major histocompatibility class I molecules (MHC I) and is also involved in antigen presentation (reviewed in Rath et al., 2014). The residues responsible for binding of IgG to FcRn were mainly elucidated using crystal structures of either rat FcRn or human FcRn (Oganesyan et al., 2014; West and Bjorkman, 2000), and are indicated on a representation of IgG (Fig. 3A) and on the 3D structure of IgG1-Fc structure (PDB ID: 1FC1; Fig. 3B) (Deisenhofer, 1981). The FcRn binding site on IgG occurs at the junction between the CH2 and CH3 domains, an area rich in histidine residues, of which H310 and H435 are thought to

act as a pH sensor in binding IgG (Burmeister et al., 1994; Martin et al., 2001; Wines et al., 2012).

Of the IgG subclasses, IgG3 has the shortest half-life of approximately 7 days (Table S1), which is largely due to an arginine at position 435 that replaces the histidine present in all other IgG subclasses (Fig. 3C). Following uptake by recycling endosomes, IgG3 binding to FcRn is competitively inhibited by IgG1, which has a higher binding affinity for FcRn; the unbound IgG3 is therefore degraded rather than returned back into circulation, explaining its shorter half-life. An allotype of IgG3 (G3m15) with H435 does not have a reduced half-life, highlighting the importance of histidine residues on overall binding affinity to FcRn (Stapleton et al., 2011). Consequently, engineering human IgG3 with H435 should lead to ThAb candidates with long *in vivo* half-lives similar to other IgG subclasses.

It is interesting to note that although FcRn is also involved in placental transport, IgG2 is often observed to be the least efficient compared to other subclasses (Table S1) (Simister, 2003). The reason for reduced IgG2 placental transport is not clearly understood, but the proportion of light chain isotypes (κ or λ) is not a factor (Einarsdottir et al., 2014). Understanding the specific receptor interactions that modulate placental transport, but not serum half-life, may be useful when engineering ThAb for use during pregnancy (Armour et al., 1999; El Mourabet et al., 2010).

2.4. Glycosylation of IgG-Fc impacts effector function

All IgG contain a conserved glycosylation site at N297 in CH2, contributing to the structural conformation of the Fc required for binding to FcγR, FcRn and C1q (Armour et al., 2003; Krapp et al., 2003) (Fig. 1B). The *N*-glycans on Fc are mainly di-antennary complex structures with core α1–6 fucosylation of the *N*-acetyl glucosamine (GlcNAc) residue attached to N297, but a large variety of glycoforms have been described in normal and recombinant IgG (Stadlmann et al., 2008; Yu et al., 2014). These differing patterns of glycosylation impart structural changes to the Fc region, which in turn affects antibody effector function (Pincetic et al., 2014). While there is some evidence that glycosylation patterns can differ between subclasses (Keusch et al., 1996; Selman et al., 2012; Vestrheim et al., 2014; Wuhrer et al., 2007), the impact on IgG effector functions has not been well characterised. IgG glycosylation is also impacted by a number of factors including age (Parekh et al., 1988; Shikata et al., 1998; Yamada et al., 1997), pregnancy (Bondt et al., 2014), inflammatory disease (Novak et al., 2005; Parekh et al., 1985; Tomana et al., 1988) and infection (Ackerman et al., 2013; Kaneko et al., 2006). We refer readers to detailed reviews on immunoglobulin glycosylation for further information (Jefferis, 2007; Yu et al., 2014).

2.5. Allotypes of IgG subclasses

Allotypic variation (polymorphisms) in IgG heavy chains (Gm) has the potential to influence both natural or vaccine induced IgG responses. The number of allotypes for each IgG subclass varies: IgG1 has 4, IgG2 has 2, IgG3 has 13 and IgG4 has none (Fig. 1A) (Lefranc and Lefranc, 2012). These IgG allotypes have been occasionally associated with enhanced protection against certain diseases (Ambrosino et al., 1985; Granoff et al., 1988; Migot-Nabias et al., 2011; Pandey et al., 2008, 2010). Importantly, it has been suggested that the allotype of a ThAb may contribute to therapy resistance associated with anti-ThAb immune responses (Pandey and Li, 2013). To date, only a few studies have clearly investigated the effects of allotype mismatch in the development of anti-ThAb responses. In these studies, there was no significant association between IgG allotype and the occurrence of anti-monoclonal antibody responses (Bartelds et al., 2010; Magdelaine-Beuzelin et al., 2009). However, further research is required before allotype contributions to anti-ThAb responses can be safely dismissed, especially since relatively few allotypes have been tested in the clinic.

2.6. IgG subclass and Fc engineering

Fc engineering has been important for developing therapeutic antibodies with potent and specific activity, therefore reducing both dosage and potential side-effects. An understanding of the differences across the IgG subclasses has been used in Fc engineering and studies have shown that introducing specific residues from one subclass to another can transform certain effector functions, while retaining others (Armour et al., 1999, 2003; Hessell et al., 2007; Redpath et al., 1998; Vafa et al., 2014). Examples include engineering IgG2 with residues from IgG4 to ablate effector functions (An et al., 2009) or engineering IgG3 with residues from other subclasses to increase half-life (Stapleton et al., 2011). This approach is complemented by other methods such as evaluation of alanine mutants, high throughput computational screening methods or resolving the structure of mutated Fc domains (Doerner et al., 2014; Lazar et al., 2006; Liu et al., 2014; Mimoto et al., 2014; Oganesyan et al., 2008; Shields et al., 2001; Stavenhagen et al., 2007). These Fc engineering approaches are pertinent in the context of infectious diseases, as specific antibody effector function is often critical in efficient pathogen clearance. The antibody Fc has also been modified to remove sites of bacterial proteolysis, which is thought to be a mode of immune evasion by certain pathogens (Kinder et al., 2013). The broad scope and possibilities for Fc engineering have been reviewed in detail elsewhere (Carter, 2011; Kaneko and Niwa, 2011; Peipp et al., 2008; Ying et al., 2014).

3. Therapeutic antibodies to infectious diseases

The use of ThAb for infectious diseases can involve the passive transfer of antibodies for pre/post exposure prophylaxis or for treatment. ThAb may be derived from pooled human sera or recombinant monoclonal antibodies. In this review, we focus on the use of monoclonal antibodies, but briefly discuss serum therapies and polyclonal antibody preparations as these have been used to treat infectious diseases (Section 3.1). We discuss the relevance of IgG subclasses in the context of infectious diseases (Section 3.2) and highlight the current pipeline of monoclonal ThAb to antigens of infectious organisms (Section 3.3).

3.1. Therapeutic antibodies against infectious diseases

Commonly used polyclonal ThAb preparations consist of purified immunoglobulin fractions from hundreds to thousands of donors and are delivered intravenously (IVIG), intramuscularly (IMIG) or subcutaneously (SCIG) (Stiehm, 2013; Wootla et al., 2014). We focus on the use of pooled IgG for specific infectious conditions, although it has also been used as a transfusion for patients with primary immunodeficiency diseases (Gouilleux-Gruart et al., 2013) or inflammatory or autoimmune diseases (Schwab and Nimmerjahn, 2013; Wootla et al., 2014). The main infectious diseases where pooled IgG is used are tetanus, hepatitis A, hepatitis B, measles, rubella, rabies, varicella, Respiratory Syncytial virus and Cytomegalovirus (Gonik, 2011; Hemming, 2001). Disease specific products are sometimes enriched by pooling antibodies from individuals with high titres to these disease antigens (Young and Cripps, 2013).

The polyclonal nature of pooled IgG enables the targeting of multiple epitopes which is advantageous in treatment of certain infectious diseases (Gonik, 2011; Gust, 2012; Hemming, 2001). However, this does create challenges with standardisation across

batches, increased costs, the potential for contamination, and the lack of efficacy due to dilution of functional antibodies (Hemming, 2001; Stiehm, 2013; Young and Cripps, 2013). An alternative approach is to use monoclonal antibodies that target relevant epitopes and that are designed with subclass backbones, which mediate the desired effector response. Monoclonal antibodies have the additional benefit of being standardised, can be mass-produced and have a low risk of contamination (Gust, 2012; Hemming, 2001). Therapeutic monoclonal antibodies might be particularly useful for applications such as dengue fever, where there are currently no licenced antiviral drugs or vaccines, and treatment is predominantly supportive. Monoclonal antibodies would be favourable as they could be selected to target neutralising epitopes common to all four dengue virus serotypes. Furthermore, concerns about increased pathogenesis driven by antibody dependent enhancement (mediated by Fc:FcγR interactions) could be overcome by monoclonal antibodies with IgG4 backbones or mutations to remove binding to FcγRs (Chan et al., 2013).

3.2. Relevance of IgG subclasses in infectious diseases

The differential interactions of the four IgG subclasses with various immune effector molecules (see Section 2) suggest subclass-specific roles during natural infection. This is reflected in the characteristic subclass profiles seen in individuals after exposure to infectious agents. In this setting, typical subclass responses can differ according to the pathogen, the antigen, or even the epitope (discussed further in Section 4.1). The importance of IgG subclasses to infectious diseases is also supported by studies of individuals that are deficient in certain IgG subclasses. Selective IgG subclass deficiencies are among the four most common paediatric primary immune-deficiencies (Stiehm, 2007; Zhao and Hammarström, 2009). The most common clinically observed syndromes are those in which IgG2 deficiency is associated with increased risk of infections caused by encapsulated bacteria, and IgG3 deficiency is associated with recurrent respiratory infections (Jefferis and Kumararatne, 1990; Meyts et al., 2006; Umetsu et al., 1985; Visitsunthorn et al., 2011 and further reviewed in Vidarsson et al., 2014). However, these studies describe association and not necessarily causation, and may be confounded by certain IgG subclass deficiencies that occur in combination with other immunodeficiencies (Buckley, 2002; Pan and Hammarstrom, 2000). Accordingly, IgG subclass responses may affect immunity to pathogens and this should be further investigated to progress ThAb development for particular infectious diseases.

3.3. Monoclonal ThAb to infectious diseases in development

Currently, there are only two monoclonal ThAb licenced for use against infectious diseases, with approximately 36 in preclinical and clinical stages of development (Table 1) (Desoubeaux et al., 2013; Oleksiewicz et al., 2012; Reichert and Dewitz, 2006; ter Meulen, 2007; Wu et al., 2014). These ThAb target approximately 14 different species of viruses and bacteria, while only two target fungi and there are none that target parasitic organisms. Those in development target similar organisms as existing pooled human IgG products, reflecting the move from blood-based products to recombinant monoclonal antibodies. Furthermore, combinations of monoclonal antibodies to the same organism are being assessed to increase targeting efficacy and to prevent escape mutants that may cause resistance (Table S3). Interestingly, almost all of the ThAb in development are built upon a human IgG1 backbone with an absence of most other IgG subclasses (Table 1).

The use of monoclonal ThAb can be applied to a wide range of pathogenic and clinical settings (Table 2). This includes: (1) organism-specific settings to target spores, toxins or biofilms; (2) clinical settings in which there may be nosocomial/iatrogenic outbreaks, drug resistance, pandemic outbreaks, bioterrorism attacks, emerging infectious diseases, and use in high risk host groups or in severe disease; (3) when there is a lack of other treatment options available; and (4) as adjunct therapies that have anti-inflammatory or immune-modulatory roles. (This could include ThAb against TNF-α and other immune mediators. in humans and are not discussed further in this review.) Some of these applications are illustrated by the use of the licenced ThAb against *B. anthracis* toxin. *B. anthracis* is a Gram-positive spore-forming, toxin-producing bacterium that poses a bioterrorism threat. ThAb against *B. anthracis* might be especially useful because a ThAb has a longer half-life than antibiotics and the antibiotics only target the bacteria, not the toxin, and require a 60-day course which poses significant challenges for compliance. Furthermore, vaccination requires repeat doses and approximately 4 weeks to induce an effective antibody titre (Chen et al., 2011).

Table 2
Situations where ThAb against infectious diseases would be useful.

Situation	Example
Organism specific	
Pathogenesis through spores	*Bacillus anthracis*
Pathogenesis through toxin	*Escherichia coli (Shiga toxin)*
Formation of biofilms	*Pseudomonas aeruginosa*
Clinical setting	
Nosocomial/iatrogenic settings	*Clostridium difficile*
Drug resistance	*Staphylococcus aureus (VRSA)*
Epidemic/pandemic outbreaks	Ebola virus
Bioterrorism setting	*Bacillus anthracis*
Emerging diseases	Nipah or Hendra virus
In young children	Respiratory Syncytial Virus
In immunocompromised individuals	Cytomegalovirus retinitis in HIV patients
Infections in premature babies	*Staphylococcus aureus*
Prophylaxis in high risk individuals	Influenza virus
Infections in transplant patients	Hepatitis C
In mother to child transmission of a disease	HIV virus
Adjunct therapy for treatment of severe disease	*Plasmodium falciparum*
Lack of other treatments	
Vaccine/drug not available	Marburg virus
Increase the therapeutic window of antibiotics	*Bacillus anthracis*
Post exposure prophylaxis	Rabies virus
Currently using polyclonal IVIG	Enterovirus

Clearly, ThAb do not have an application for all infectious diseases. In instances where there is a potential therapeutic benefit, the relative advantages and disadvantages of ThAb need to be considered and weighed up against alterative therapeutic options (Table 3) (Casadevall et al., 2004). In many instances the factors that are most likely to limit applications for ThAb include: (1) specific characterisation of monoclonal Ab with respect to epitope targets and functional effector responses; (2) the costs of development and production; (3) the need for parenteral administration; and (4) the ability to deliver sufficient levels of ThAb for maximal effectiveness. The selection of IgG subclass in developing ThAb is often under-evaluated, but can have major implications for ensuring that functional responses are obtained, adverse effects are minimised, and antibody half-life is optimised.

4. IgG subclass selection for ThAb against infectious diseases

4.1. Subclasses and the natural immune response

Exposure to most microorganisms will lead to the induction of an antibody response, some of which will mediate clinical

Table 3
Advantages and disadvantages of developing ThAb against infectious diseases.[a]

Advantages	Disadvantages
• High specificity • Does not affect normal flora • Low toxicity • Targeted biological effector function which may include neutralisation, ADCC, ADCP, or CDC • Variety of isotype and subclass options to develop ThAb with desired functions • Act synergistically with antimicrobials • Rapid provision of passive immunity compared to longer periods required for vaccine-induced responses • Conjugating toxins or therapeutic molecules to ThAb to target these responses more precisely	• Requires detailed knowledge of the micro-organism's pathogenesis, protective epitopes and effective immune response • May not recognise viral escape mutants[b] or organisms with polymorphic antigens • Cost of production, storage and delivery and the risk of contamination • Requires systemic administration (parenteral, subcutaneous, IV or intra-muscular) • Requirement to administer therapeutic levels of ThAb • ThAb must be administered early in infection for maximal efficacy

ADCC: antibody-dependent cellular cytotoxicity; ADCP: antibody-dependent cellular phagocytosis; CDC: complement-dependent cytotoxicity.
[a] Table summarised using readings from Casadevall et al. (2004).
[b] ThAb cocktails with different monoclonals might overcome this.

protection, but others are non-functional and merely reflect exposure. In both instances, these naturally acquired responses will often be highly skewed with respect to isotype and subclass. Understanding the characteristics of these naturally acquired responses is the first step in selecting the appropriate subclass for an effective ThAb. The IgG subclass bias that occurs for different microorganisms has been demonstrated by IgG responses to bacterial polysaccharides, which indicate a predominance of IgG2, with reduced amounts of IgG1 and IgG3 (Chudwin et al., 1987; Islam et al., 1995). In contrast, antibody responses against HIV, Ebola virus, *Coxiella burnetii* (Q fever), and *Plasmodium falciparum* (malaria), have been shown to be mainly IgG1 and IgG3 (Camacho et al., 1995; Leroy et al., 2001; Raux et al., 2000; Richards et al., 2010), whereas responses to *Schistosoma mansoni* are predominantly IgG4 (Boctor and Peter, 1990). Additionally, differences in subclass responses have been observed to vary according to age and gender (Perez-Perez et al., 2010; Simon et al., 2013), and can differ for different antigens from the same pathogen, or for different domains of a single antigen (Richards et al., 2010; Stanisic et al., 2009). This knowledge of naturally acquired subclass responses can then be used in functional assays to determine their likely significance in inducing protective immunity.

4.2. Functional assessment of subclasses isolated from natural immune responses

Discriminating between antibodies that reflect exposure and those that mediate functional protection can be challenging, but the use of pathogen-specific *in vitro* functional assays can be informative. Most microorganism-specific fields rely on only a handful of well-accepted functional assays (*e.g.* neutralisation assays for influenza or growth inhibition assays for malaria), and there is a need for a much wider range of functional assays to be developed and used to systematically assess the function of antibodies. These assays will then need to be correlated with clinical outcomes to determine whether the measured responses mediate *in vivo* protection. Well-validated functional assays can then be used to assess the significance of IgG subclass by purifying out the subclass specific antibodies. For example, an early study compared purified subclass antibodies in Herpes Simplex virus neutralisation assays, determining that IgG3 and IgG4 had the greatest ability to neutralise the virus even though they were not the predominant subclass (Mathiesen et al., 1988). Similar studies for HIV purified IgG1 and IgG3 and compared the ability of antibodies to neutralise HIV virus when isolated from individual samples or when pooled from multiple individuals (Cavacini et al., 2003; Scharf et al., 2001). Interestingly, these studies found that IgG3 was more effective at neutralising HIV-1 than IgG1 when pooled samples were used, but this was not observed for individual samples, indicating that epitope specificity also played an important role in neutralisation. A further study of responses against human enterovirus 71 used purified IgG subclass specific fractions from pooled immunoglobulins and found that IgG1 and IgG2 fractions were the most effective at neutralisation, and that IgG3 led to enhanced infection (Cao et al., 2013). These studies highlight the potential value of purifying out specific IgG subclasses from the sera of naturally infected individuals and testing these antibodies in functional assays to better understand their contribution to clinical protection or to disease pathogenesis. Such studies also need to be complemented by experiments in which subclass switch variants are generated and assessed in functional assays.

4.3. Subclass switch variants

Subclass switch variants utilise the same variable domains, but are expressed on different subclass heavy chain backbones. Therefore, subclass switch variants are monoclonal antibodies that bind the same epitope, allowing the contribution of the different subclasses to be evaluated in pathogen-specific *in vitro* functional assays and *in vivo* models to determine differences in effector function.

Subclass switch variants have been assessed using either human or murine subclasses for: (1) viruses including HIV (Cavacini et al., 1995; Kunert et al., 2000; Liu et al., 2003; Miranda et al., 2007), Yellow Fever Virus (Schlesinger and Chapman, 1995; Schlesinger et al., 1993), West Nile Virus (Mehlhop et al., 2007), and Varicella Zoster Virus (Lloyd-Evans and Gilmour, 2000); (2) bacteria including *Staphylococcus aureus* (Brown et al., 2009; Kelly-Quintos et al., 2006; Varshney et al., 2014), group A *Streptococcus* (Cooper et al., 1991, 1993), *B. anthracis* (Abboud et al., 2010; Hovenden et al., 2013), *Pseudomonas aeruginosa* (Pollack et al., 1995; Schreiber et al., 1993), and *Escherichia coli* (Akiyoshi et al., 2010; Oishi et al., 1992; Pelkonen and Pluschke, 1989); (3) fungi including *Cryptococcus neoformans* (Sanford et al., 1990; Yuan et al., 1995); and (4) parasites including *P. falciparum* (Lazarou et al., 2009). These studies of subclass switch variants have utilised human, mouse and rat antibodies; a summary of human switch class variant studies is shown (Table 4). In some instances, the studies have indicated clear differences between subclasses in mediating HIV neutralisation (Cavacini et al., 1995; Miranda et al., 2007), opsonic phagocytosis of *S. aureus* (Kelly-Quintos et al., 2006), and in functional studies for *P. aeruginosa* (Eichler et al., 1989; Schreiber et al., 1993), *E. coli* (Oishi et al., 1992), Yellow Fever Virus (Schlesinger et al., 1993), Varicella Zoster Virus (Lloyd-Evans and Gilmour, 2000) and *Cryptococcus* (Yuan et al., 1995, 1998). In other instances, subclass switching did not appear to have a major effect on the outcome of functional assays (Bachmann et al., 1997; Brown et al., 2009; Cavacini et al., 1995; Kelly-Quintos et al., 2006; Kunert et al., 2000; Liu et al., 2003; Sanford et al., 1990). This suggests that the subclass of ThAb may not always be crucial and that other factors such as antibody levels may also be important (Bachmann et al., 1997). A complexity in assessing subclass switch variants is that the subclasses for mice and other animals are not directly equivalent to that of humans due to the diversification of IgG into subclasses occurring after speciation (Butler, 2006). Therefore, observed differences in subclass specific function obtained in animal models cannot be

Table 4
Examples of studies using human IgG subclass switch variants.

Type	Organism	Monoclonal	Target	Subclass variants	References
RNA viruses	HIV	F105	HIV envelope: gp120 CD4 binding site	IgG1; IgG3	Cavacini et al. (1995)
		F425B4e8	HIV envelope: gp120 V3 loop	IgG1; IgG2; IgG3	Liu et al. (2003)
		2F5	HIV envelope: gp41	IgG1; IgG3	Kunert et al. (2000)
		F240	HIV envelope: gp41	IgG1; IgG3; IgG4	Miranda et al. (2007)
	Flavivirus	E16	West Nile Virus: Domain III	IgG1; IgG2; IgG3; IgG4	Mehlhop et al. (2007)
Gram-positive bacteria	*Staphylococcus aureus*	F598	Poly *N*-acetyl-glucosamine (PNAG)	IgG1; IgG2	Kelly-Quintos et al. (2006)
		F628	Poly *N*-acetyl-glucosamine (PNAG)	IgG1; IgG2	Kelly-Quintos et al. (2006)
		F630	Poly *N*-acetyl-glucosamine (PNAG)	IgG1; IgG2	Kelly-Quintos et al. (2006)
Gram-negative bacteria	*Pseudomonas aeruginosa*	1E1/2E12	Serogroup O6 Lipopolysaccharide	IgG1; IgG2; IgG3; IgG4	Preston et al. (1998)
	Escherichia coli (STEC)	5C12	Shiga toxin 2	IgG1; IgG2; IgG3; IgG4	Akiyoshi et al. (2010)
Parasite	*Plasmodium falciparum* (Malaria)	12.10; 12.8	Merozoite Surface Protein-1 (MSP-1)	IgG1; IgG3	Lazarou et al. (2009)

HIV: Human Immunodeficiency virus; gp: glycoprotein; CD4: cluster of differentiation 4; V3: Variable loop 3.

directly inferred for humans (Mestas and Hughes, 2004). For example, murine IgG1 is a prominent response for many pathogens and most murine monoclonal antibodies are IgG1, but murine IgG1 does not engage activating FcγRs or fix complement in mice (Bruhns, 2012). However, interactions and effector mechanisms determined for human IgG subclasses and their cognate effector systems (human FcγRs, FcRn and C1q) are indeed relevant for the development of ThAb to treat infectious diseases.

4.3.1. *The use of murine models to guide development of therapeutic antibodies*

Despite the difficulty in directly transferring the findings from studies of murine subclass switch variants to humans, such studies can be useful in the development of ThAb by increasing an understanding of protective immune mechanisms and for their application in *in vivo* animal models. Examples include murine studies that have indicated that ThAb need to include FcγR function to effectively induce neutralisation of anthrax toxin (Abboud et al., 2010) and that inducing opsonic phagocytosis is more important than complement-mediated activity to protect against *C. neoformans* (Shapiro et al., 2002; Yuan et al., 1997).

4.3.2. *Subclass-specific function varies according to the characteristics of the epitope*

Identifying target epitopes that mediate functional immunity is clearly a key component in developing a ThAb. Interestingly, the impact of antibody subclass on such functional epitopes may vary dramatically between epitopes, even within the same organism. For instance, human IgG3 monoclonal antibodies that target gp120 have been shown to have higher HIV neutralising ability than IgG1 counterparts, but no such subclass difference was found for monoclonal antibodies targeting gp41 (Cavacini et al., 1995; Kunert et al., 2000; Liu et al., 2003; Miranda et al., 2007) (Table 4). The relationship between the specific target epitope and the subclass of antibodies is also likely to depend on the density of epitopes, and the flexibility of different antibody subclasses to interact with those epitopes. For instance, studies using subclass switch variants against *P. aeruginosa* (Pollack et al., 1995; Preston et al., 1998) and group A *Streptococcus* (Cooper et al., 1991, 1993, 1994) suggest that antigen density greatly influences the ability of some antibody subclasses to bind effectively, thus highlighting the importance of assessing subclass responses using assays that reflect native antigen conformation and density. There is also evidence that the functional effect of different subclasses may vary according to the strain of the organisms (Cavacini et al., 1995; Cooper et al., 1993; Miranda et al., 2007; Pollack et al., 1995; Yuan et al., 1995), indicating that it is also important to assess a variety of strains when developing ThAb (Preston et al., 1998).

4.3.3. *Assessing subclass responses using functional* in vitro *assays*

As mentioned previously, there is a need for a wider array of functional assays that can be used to systematically assess the role of IgG subclasses for many infectious diseases. The use of such functional assays needs to be tempered by the validation of these assays as indicators of actual *in vivo* protection. Studies into murine subclass responses against *C. neoformans* have demonstrated such discrepancies between *in vitro* functional assays and *in vivo* models (Sanford et al., 1990; Schlageter and Kozel, 1990; Yuan et al., 1998). Where possible, functional assays should reflect the likely mechanism of *in vivo* antibody mediated activity. For example, *in vivo* protection against *E. coli* is associated with complement mediated clearance; therefore ThAb should be assessed using functional assays that reflect this mechanism. However, *P. aeruginosa* is able to resist complement mediated lysis, and therefore a complement assay may not accurately predict *in vivo* protection (Oishi et al., 1992; Pollack et al., 1995). Well-validated functional assays can also be used to determine whether different IgG subclasses have the potential to interfere with the action of other IgG subclasses, *i.e.* blocking antibodies. Similarly, functional assays can be used to assess the ability of some antibodies to have a detrimental effect by enhancing infection, as observed with dengue virus, West Nile Virus, HIV, and fungi including *Candida albicans* and *C. neoformans* (Mehlhop et al., 2007; Weber and Oxenius, 2014).

5. Concluding remarks

There are over 40 licenced ThAb but only two that target pathogens associated with infectious disease. Almost all the licenced ThAb, as well as those in clinical development, have a human IgG1 Fc domain. The diversity of subclass responses against natural infections and the unique structural and functional characteristics of the four human IgG subclasses underscores the importance of considering subclass properties when developing and testing ThAb, especially against infectious diseases. Although there are approximately 36 new ThAb against infectious diseases in preclinical development, translational research in this field has been slow compared to development in cancer and autoimmunity. There are a multitude of potential settings for the application of ThAb against infectious diseases and the pros and cons should be carefully assessed for each therapeutic setting. Knowledge of the subclass profile against the pathogen and antigen of interest would be beneficial. This assessment should include the study of naturally acquired antibody responses, serum-derived antibodies and subclass switch variants in relevant *in vitro* functional assays, and *in vivo* studies where appropriate. Such studies have indicated that individual IgG subclasses may have different functional effects against different pathogens, with no subclass being singularly

important in mediating protection from disease. Furthermore, the nature of the target epitope is extremely important for effective functional responses, and the epitope density or pathogen strain used in the assay may profoundly impact on results; it is therefore important to test a range of these factors. Finally, the knowledge obtained from IgG subclass switch studies, combined with a molecular understanding of IgG subclass properties will allow for the engineering and development of highly effective pathogen specific monoclonal ThAb.

Conflict of interest

The authors have declared that no competing interests exist.

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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.molimm.2015.03.255

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