INVESTIGATING THE IMMUNOGENICITY OF THERAPEUTIC PROTEINS: PROTEIN AGGREGATION AND HOST CELL PROTEIN IMPURITIES

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Biology, Medicine and Health

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Abstract

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The development of anti-drug antibodies (ADA) against therapeutic proteins can impact upon drug safety and efficacy. This is a major challenge in the development of biotherapeutics. Various factors have the potential to contribute to protein immunogenicity and the production of ADA. Protein aggregation is one of these factors, though the mechanisms underlying aggregate immunogenicity are poorly understood. In this thesis the effect of protein aggregation on immunogenicity has been investigated.

The thermal and/or mechanical stresses required in order to achieve subvisible aggregates of three test proteins were determined. Stressed preparations of proteins were characterised using a suite of biophysical techniques, including dynamic light scattering and circular dichroism. The immunogenic potential of subvisible aggregates of a humanised single chain variable fragment (scFv) and ovalbumin (OVA) was studied following intraperitoneal exposure in BALB/c strain mice. Monomeric proteins induced a T helper (Th) 2 dominant immune response, but when aggregated, the responses gained a Th1 phenotype, with a significant increase in the antigen-specific IgG2a antibody response. Cytokine profiles in supernatants taken from splenocyte-dendritic cell co-cultures were also consistent with aggregated preparations of OVA inducing a Th1-type response.

Host cell protein (HCP) impurities can also contribute to immunogenicity. Mass spectrometry analysis of an scFv preparation identified the presence of the *Escherichia coli* (*E.coli)* heat shock protein DnaK, amongst other HCP, as an impurity. Protein preparations free from DnaK were spiked with recombinant *E.coli* DnaK to mimic the HCP impurity. The effect of DnaK on the immunogenicity of aggregated and monomeric scFv preparations was then investigated. BALB/c mice were immunised with monomeric and aggregated preparations, with and without *E.coli* DnaK at 0.1% by mass. Aggregation alone resulted in an enhanced IgG2a antibody response, and the presence of DnaK increased this further. Comparable investigations were also conducted using mouse albumin; here an increase in immunogenicity was observed with protein aggregation, and the presence of DnaK was found to increase the IgG2a response.

Collectively, the evidence presented in this thesis shows that aggregation can impact upon the magnitude and characterof induced immune responses, and that subvisible aggregation promotes a Th1 immune skewing. Additionally, *E.coli* HCP DnaK enhances protein aggregate immunogenicity, which indicates that heat shock proteins, as a class of HCP, could have an adjuvant-like effect on biotherapeutic aggregates.

Declaration

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Abbreviations

ADA	anti-drug antibody
AFM	atomic force microscopy
ANOVA	analysis of variance
APC	antigen presenting cell
AUC	analytical ultracentrifugation
BcR	B cell receptor
BM	bone marrow
BSA	bovine serum albumin
CD	circular dichroism
CDR	complementarity determining region
ConA	Concanavalin A
CV	column volume
DC	dendritic cell
DEAE	diethylaminoethanol
DLS	dynamic light scattering
E.coli	*Escherichia coli*
EDQM	European Directorate for the Quality of Medicine
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
FACS	fluorescence-activated cell sorting
FCS	foetal calf serum
FDA	Food and Drug Administration
FFF	field flow fractionation
FITC	fluorescein isothiocyanate
FTIR	fourier transform infrared spectroscopy
GH	growth hormone

GM-CSF	granulocyte/macrophage-colony stimulating factor
HCP	host cell protein
HDX	hydrogen/deuterium exchange
HLA	human leukocyte antigen
HRP	horseradish peroxidase
HSP	heat shock protein
ICH	international conference on harmonisation
IFN	interferon
IgG	immunoglobulin G
IgM	immunoglobulin M
IL	interleukin
ip	intraperitoneal
LCMS	liquid chromatography-mass spectrometry
LPS	lipopolysaccharide
mAb	monoclonal antibody
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MSA	mouse serum albumin
NMR	nuclear magnetic resonance
NMS	naive mouse serum
OD	optical density
OVA	ovalbumin
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PI	propidium iodide
pp	parts per
rFVIII	recombinant human factor VIII
RICS	raster image correlation spectroscopy

sc	subcutaneous
scFv	single chain antibody variable fragment
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEC	size exclusion chromatography
SEM	standard error of the mean
SEC-MALLS	size exclusion chromatography coupled with multi angle laser light scattering
T_{agg}	temperature of aggregation
TcR	T cell receptor
TEM	transmission electron microscopy
TGFβ	transforming growth factor β
Th	T helper
TLR	toll-like receptor
Treg	regulatory T cell
WHO	World Health Organisation

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CHAPTER 1:
INTRODUCTION

1 Introduction

1.1 Review Article

Immunogenicity of Therapeutic Proteins: Influence of Aggregation

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1.1.1 Abstract

The elicitation of anti-drug antibodies (ADA) against biotherapeutics can have detrimental effects on drug safety, efficacy, and pharmacokinetics. The immunogenicity of biotherapeutics is therefore an important issue. There is evidence that protein aggregation can result in enhanced immunogenicity; however, the precise immunological and biochemical mechanisms responsible are poorly defined. In the context of biotherapeutic drug development and safety assessment, understanding the mechanisms underlying aggregate immunogenicity is of considerable interest. This review provides an overview of the phenomenon of protein aggregation, the production of unwanted aggregates during bioprocessing, and how the immune response to aggregated protein differs from that provoked by non-aggregated protein. Of particular interest is the nature of the interaction of aggregates with the immune system and how subsequent ADA responses are induced. Pathways considered here include 'classical' activation of the immune system involving antigen presenting cells, and alternatively, the breakdown of B-cell tolerance. Additionally, methods available to screen for aggregation and immunogenicity will be described. With an increased understanding of aggregation-enhanced immune responses, it may be possible to develop improved manufacturing and screening processes to avoid, or at least reduce, the problems associated with ADA.

1.1.2 Introduction

Since the breakthrough of recombinant DNA technology in the 1970s, and the subsequent introduction of recombinant human insulin as a drug in the early 1980s (Johnson 1983), clinical use of protein therapeutics (also known as biotherapeutics) has increased dramatically. An availability of different classes of biotherapeutics, e.g., antibodies, hormones, and enzymes, provides useful tools in treatment of a wide range of diseases, including diabetes, rheumatoid arthritis, and hemophilia. Since their launch in the mid-1980s, therapeutic monoclonal antibodies (mAb) have evolved from mouse, to chimeric and humanized derivatives, to fully human molecules (Wang et al. 2009). Early, non-human biotherapeutics were expected to provoke an immune response, as they would be recognized as foreign. Recombinant human biotherapeutics, however, are not expected to evoke an immune response in humans given their similarity to endogenous proteins. Indeed, recombinant human proteins do display reduced immunogenicity compared with non-human sequences (Wadhwa and Thorpe 2007), yet formation of anti-drug antibodies (ADA) was noted after patient treatment with such therapeutics (Schernthaner 1993; Sauerborn et al. 2010).

ADA pose a challenge in the biotherapeutics industry and clinical medicine as they can cause adverse events (like neutralization of endogenous protein) or reduce efficacy of a biotherapeutic. Immunogenicity is therefore a key limitation for the clinical use of biotherapeutics. Protein aggregation can increase the immunogenicity of biotherapeutics, and can be a key factor in causing adverse events associated with immunogenicity in the clinic (Rosenberg, 2006). A reduction in yield due to protein aggregation can also have a significant impact on development and manufacturing costs. Thus, despite the success of biotherapeutics and their expansion in the market, a tendency of proteins to aggregate during production and post-production stages creates challenges for industry and in the clinic, and acts to bottleneck development (Wang et al. 2012).

1.1.3 Protein aggregation

Definition and classification of aggregates

Aggregation is a broad term, encompassing the interactions which result in the self-association of protein molecules into assemblies other than the native quaternary structure (Narhi et al. 2012). Protein aggregates include a diverse range of protein assemblies that can differ in their biochemical and biophysical characteristics. They can range considerably in size, from dimers up to subvisible and visible particles (see Figure 1.1), they can involve covalent or non-covalent linkages, be ordered or disordered in structure, soluble or insoluble, and their formation can be reversible or irreversible. To study protein aggregation and understand the pathways that result in aggregate formation, it is important to classify protein aggregates according to their characteristics; however, the size range and diversity of protein aggregates make such characterization difficult. Additionally, use of imprecise and overlapping terms in the description of some aggregates makes interpretation of reports in the literature difficult. For example, the terms ‘subvisible particles’ and ‘oligomers’ may both be used to describe the same aggregate species. Narhi et al. (2012) have attempted to remove these inconsistencies by suggesting standardized nomenclature to describe protein aggregates; this classification system accounts for aggregate size, reversibility of formation, conformation, chemical modification, and morphology. In this system, size is referred to using quantitative categories rather than imprecise terms, e.g., ‘1-100 μm’ is used rather than the term ‘subvisible particles’.

For the purpose of this review, ‘protein aggregation’ refers to the association of partially unfolded forms, which is relevant for the aggregation of therapeutic proteins during bioprocessing. It is important to note the distinction between these partially unfolded aggregate forms, and amyloid fibrils, which are insoluble fibrous protein structures composed of β-sheet aggregates. Aggregation promoted by ‘amyloidogenic’ regions of a protein can be more reliably predicted on the basis of

protein sequence. For example, regions with an increased capacity to form hydrogen bonds between peptide backbones, and regions with a high packing density confer a higher probability of amyloid fibril formation (Garbuzynskiy et al. 2010). Prediction of the aggregation of partially unfolded forms is more challenging, however.

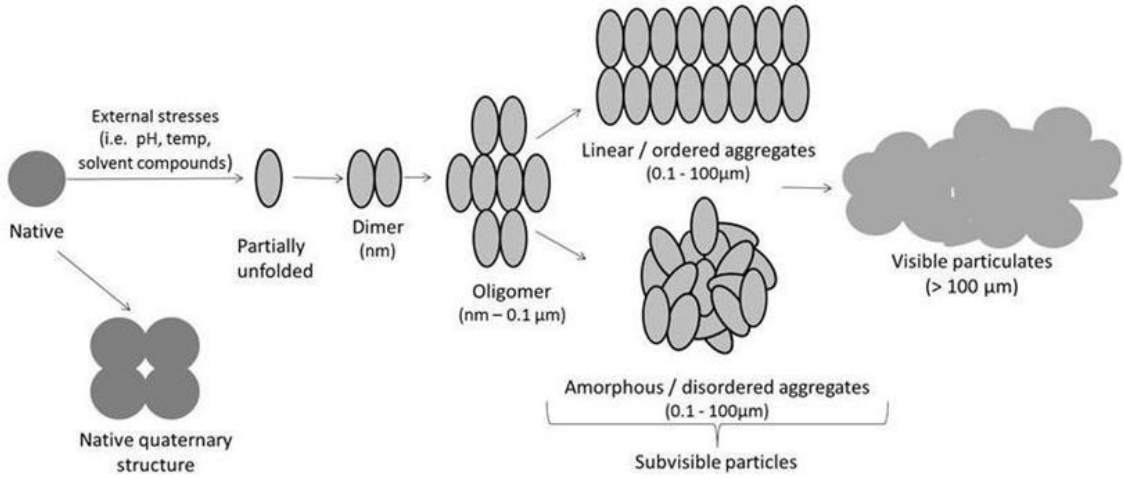

Figure 1.1 Schematic model of protein aggregation. Bioprocessing-associated stresses can trigger partial protein unfolding and initiate the aggregation process, beginning with the association of two or more protein molecules. Oligomers made from three or more monomers can form, leading to larger aggregates or 'subvisible particles'. Linear aggregates form where proteins associate in a uniform manner (e.g., amyloid-type), whereas amorphous aggregates form by the association of proteins in a disordered manner. Visible particulates can then form, as pre-existing aggregates act as nuclei for formation of larger aggregates. Aggregate components are not drawn to scale.

Aggregation and immunogenicity

Immunogenicity is defined as the ability of a substance (protein or chemical) to provoke an immune response. It is widely accepted in the scientific literature that protein aggregation can augment a protein specific immune response, and lead to formation of ADA in the case of protein therapeutics (Rosenberg 2006; Sauerborn et al. 2010). Given the ability of aggregation to enhance immune responses, aggregation

can be used as a predictor for immunogenicity of biotherapeutics, and minimizing the risk of aggregation may reduce the risk of immunogenicity in the clinic. However, the majority of protein formulations contain at least a low level of aggregates, although the extent and type of aggregation that could pose a risk is not known (Weiss et al. 2009). Understanding the immunological mechanisms through which aggregation can influence the immunogenicity of proteins is, therefore, of considerable importance. The manufacture of biotherapeutics involves methods that can cause aggregation; these must be taken into account in bioprocess design in order to reduce aggregation-associated immunogenicity.

Aggregate formation in bioprocessing

Non-native aggregation describes the process of non-native protein structures assembling from initially native proteins (Chi et al. 2003). The loss of native structure is a common response of proteins to external stresses such as temperature, pH, co-solutes and adsorption to air-liquid and solid-liquid interfaces. Structurally altered proteins have a strong tendency to aggregate and additives that maintain native protein structure have been shown to reduce protein aggregation (Manning et al. 2010). Conformational stability therefore appears to be an important factor in controlling aggregation rates. Non-native aggregation is frequently observed at many stages of bioprocessing, including protein expression, purification, and storage. This process is generally irreversible, so reducing aggregation during manufacture requires controlled conditions (Weiss et al., 2009). Aggregation can also occur under conditions where the native state is favored thermodynamically and in the absence of stresses (Krishnan et al. 2002). For example, large aggregates with native conformation can form through adsorption to micro-particles, or at high concentration through ‘salting out’. This phenomenon is caused by protein exceeding the solubility limit above that required to cause precipitation as the salt concentration increases

(Fink 1998). More commonly in the production of protein therapeutics, aggregation is preceded by the loss of native protein structure; the resulting protein is then more susceptible to aggregation (see Figure 1). The mechanisms of protein aggregation are still not fully understood, but current evidence supports the role of partially folded intermediates in aggregation (Kim and Yu 1996; Acosta-Sampson and King 2010), and it is thought that exposure of hydrophobic surfaces on non-native protein structures allows inter-molecular interactions, leading to aggregate formation.

Native protein stability can be compromised by physical and chemical stressors; those associated with aggregation can be encountered at various stages of product development (Frokjaer and Otzen 2005) from protein expression, to storage of the final product. Characterization of the protein is therefore required at each stage to ensure batch-to-batch uniformity and overall quality; this is particularly important for regulatory submissions. Proteins can aggregate early in product development at the protein expression stage. Therapeutic mAb are typically derived from mammalian cell culture and this process involves steps that can result in aggregation. For example, over-expression of recombinant proteins in cultures can lead to aggregation and intra-cellular inclusion body formation (Schrodel and de Marco 2005). Protein purification techniques can also expose a protein to conditions like high ionic strength, pH values far from neutrality, and high protein concentrations, all which contribute to aggregation (Cromwell et al. 2006).

Proteins are surface-active agents, and are attracted to hydrophobic interfaces where they are prone to unfolding and subsequent aggregation (Horbett 1988; Gidalevitz et al. 1999). Such interfaces are encountered at various points in the life of a biotherapeutic product (Dasnoy et al. 2011). Different biotherapeutic products have also been reported to aggregate at the air-liquid interface; here proteins can act as a detergent, migrating to the air-liquid interface, and this stress can cause partial unfolding. Susceptible proteins include insulin (Sluzky et al. 1991), factor VIII (Joshi et al. 2009), human growth hormone (hGH; Maa and Hsu 1997), and IgG variants

(Mahler et al. 2005; Kiese et al. 2008). Proteins are also subject to mechanical stress during the manufacturing process, i.e., as solutions of high protein concentration pass through piston pumps during fill-and-finish operations, molecules encounter high shear and mechanical stresses which can cause partial denaturation, and hence aggregation (DePalma 2006). Differences in product formulation can further impact on aggregation. Some solvent additives are used to stabilize protein preparations and prevent aggregation but, in contrast, some solvent compounds and contaminants promote aggregation (Arakawa et al. 1991). The stability of pH within formulations is important, as it is well known that acidic conditions can affect antibody structure, stability and folding. It has been recognized that ionic strength and pH can play a key role in aggregation of mAb (Hari et al. 2010). Additionally, high concentration liquid formulations are often required to achieve the desired therapeutic dose: this can lead to aggregation as high concentrations promote protein association (Treuheit et al. 2002).

In order to optimize capacity, it is common practice to conduct large scale freezing of bulk protein preparations for storage. During this process there will be freeze/thaw stress that contributes to aggregation. The molecular mechanism of this stress-induced aggregation is not fully understood but it is thought that proteins partially unfold at the ice-liquid interface and hydrophobic bonds weaken (Kreilgaard et al. 1998b). The formation of ice crystals can also be particularly damaging, as this can enhance protein unfolding (Hamada et al. 2009). The process of lyophilization involves dissolving a drug formulation in an aqueous matrix, which is then frozen, and the aqueous phase is removed by sublimation. This method is widely used in the biopharmaceutical industry to improve shelf life and increase stability (Tang and Pikal 2004). Lyophilization can result in protein unfolding and hence aggregation as the protein can be exposed to low temperature stresses (Townsend and Deluca 1990). Conversely, lyophilization can also be used to avoid the aggregation seen in liquid

formulations that is caused by partial unfolding due to agitation stress and exposure to liquid-solid interfaces within containers.

Some studies have suggested that the mechanism of aggregation is dependent on the nature of the stress applied (Krishnan and Raibekas 2009; Joubert et al. 2011). As a result, aggregates can be heterogeneous with qualities attributable to a particular type of stress. Protein characterization following exposure to different stress conditions may therefore help to identify the stresses that cause aggregation and influence the bioprocess pipeline. Small changes to manufacturing processes, such as increases in production scale or changes to the formulation, could alter the immunogenic potential of a protein. As manufacturing processes evolve, different factors are introduced that may contribute to immunogenicity. Regulatory guidelines that are in place to ensure the safety of biotherapeutics must therefore accommodate these frequent changes in the manufacturing process.

1.1.4 Methods to reduce aggregation

Excipients

There have been recent developments in the use of excipients such as surfactants, amino acids, and pH buffers to reduce protein aggregation in formulated products (DePalma, 2006). Certain excipients are used specifically to inhibit aggregation at the air-liquid interface; generally these work by competing with the therapeutic protein for adsorption to the interface, thereby protecting the protein from exposure (Katakam et al. 1995; Chou et al. 2005). For example, non-ionic surfactants polysorbate 20 and 80 are typically used to prevent adsorption at the interface and subsequent aggregation (Kerwin 2008). Silicone oil on the surface of syringes and stoppers can come into contact with biotherapeutics prior to delivery; this interaction has been implicated in aggregation. Silicone oil-induced aggregation can be reduced by polysorbate 20 (Thirumangalathu et al. 2009). Unfortunately, the use of polysorbate 80 in formulation can lead to formation of micelles that may cause

aggregation and enhance immunogenicity (Villalobos et al. 2005). This observation highlights the need for careful selection of excipients.

Other excipients used to prevent adsorption at the air-liquid interface include cyclodextrin derivatives (Serno et al. 2010), and amino acids such as arginine, lysine, and glutamic acid (Dasnoy et al. 2011). Carbohydrates such as sucrose, dextrose, and trehalose have also been used as stabilising excipients (Katakam and Banga 1995; Kreilgaard et al. 1998). These function by covering the surface of hydrophobic bonding sites to preserve the native structure (Andya et al. 2003). This is useful for the storage of freeze-dried proteins, as hydrophobic interactions play a key role in protein folding and support the native state; loss of these interactions under dehydration can destabilize the protein.

High throughput screening (HTS) technology may be used to assess the ability of excipients to protect against aggregation. Dasnoy et al (2011) developed a HTS stress test for studying aggregates at the air-liquid interface, allowing the evaluation of a large number of excipients for prevention of aggregation. Considerable variation (for example, in size and structure) is observed in aggregates formed during bioprocessing. Given the diversity of therapeutic proteins, the type of excipient used must be selected based on properties of each particular protein. Thus, optimal formulation for each protein of interest requires screening and optimization in order to achieve a formulation that provides minimal risk of aggregation. Research is required to understand whether formulation strategies used for typical aggregates can function effectively in the reduction of subvisible protein particles, which are believed to play an important role in aggregation of biotherapeutics (Carpenter et al. 2009).

Protein engineering

A protein engineering approach also can be used to minimize aggregation. For example, using a *Pichia pastoris* expression system, it has been shown that IgG molecules with lower levels of glycosylation were less prone to aggregation, and the presence of an N-terminal tetra-peptide extension increased the temperature at which aggregation was first induced (Schaefer and Pluckthun, 2012). Mutagenesis has also been used to prepare antibody variants with enhanced protein stability and reduced aggregation potential (Chennamsetty et al. 2009). In addition, *in silico* methods have been used to study protein aggregation in relation to bioprocessing; computational studies to examine the stabilizing effect of excipients look particularly promising (Cellmer et al. 2007). *In silico* methods for predicting protein aggregation and modelling optimized proteins are in their infancy, but they provide a potentially valuable tool in the design of biotherapeutics with reduced aggregation potential (Bratko et al. 2006).

1.1.5 Immunogenicity of biotherapeutics

Mechanisms of immunogenicity

In order for a protein therapeutic to be immunogenic, it must interact with immune cells. There are essentially two ways in which a protein therapeutic may induce immune responses in the patient. The first is if the therapeutic agent is sufficiently 'foreign' to be recognized as such and induce an adaptive immune response. Thus, the therapeutic agent is internalized, processed, and presented by antigen-presenting cells (APC) resulting in CD4+ T-cell responses and the elaboration of antibody (see Figure 1.2). If, however, the therapeutic agent shows high, or complete, homology with an endogenous protein to which the patient is immunologically tolerant then that B-cell tolerance will need to be broken for an antibody response to be induced. Antibody production is the primary effector mechanism in immune responses to biotherapeutics and while ADA can be without

clinical consequences, side effects can occur varying from a loss of drug efficacy to anaphylaxis.

Immune tolerance

T- and B-cells express T-cell receptors (TcR) and B-cell receptors (BcR), respectively, for antigen recognition. These are generated by random gene rearrangements (somatic mutation) to ensure a very wide repertoire and the ability to recognize a vast array of foreign antigens. By chance, some receptors are often able to recognize self-antigen. Central tolerance mechanisms exist to prevent the survival and proliferation of these self-reactive immune cells, thereby preventing autoimmunity. The process of negative selection results in the deletion of developing B- and T-cells that recognize self-antigens in the environment of the bone marrow and thymus, respectively. Any remaining self-reactive lymphocytes are controlled by anergy or peripheral tolerance. B-Cell peripheral tolerance is caused by exposure to circulating soluble antigen and low levels of BcR cross linkage (Andrews and Wilson, 2010). In CD4+ T-cells, peripheral tolerance is maintained by the following mechanisms; functional anergy rendering the T-cell unresponsive, deletion of the cell by apoptosis following cell activation, and suppression of T-cell activation by regulatory T (T_{reg}) cells (Abbas et al. 2004).

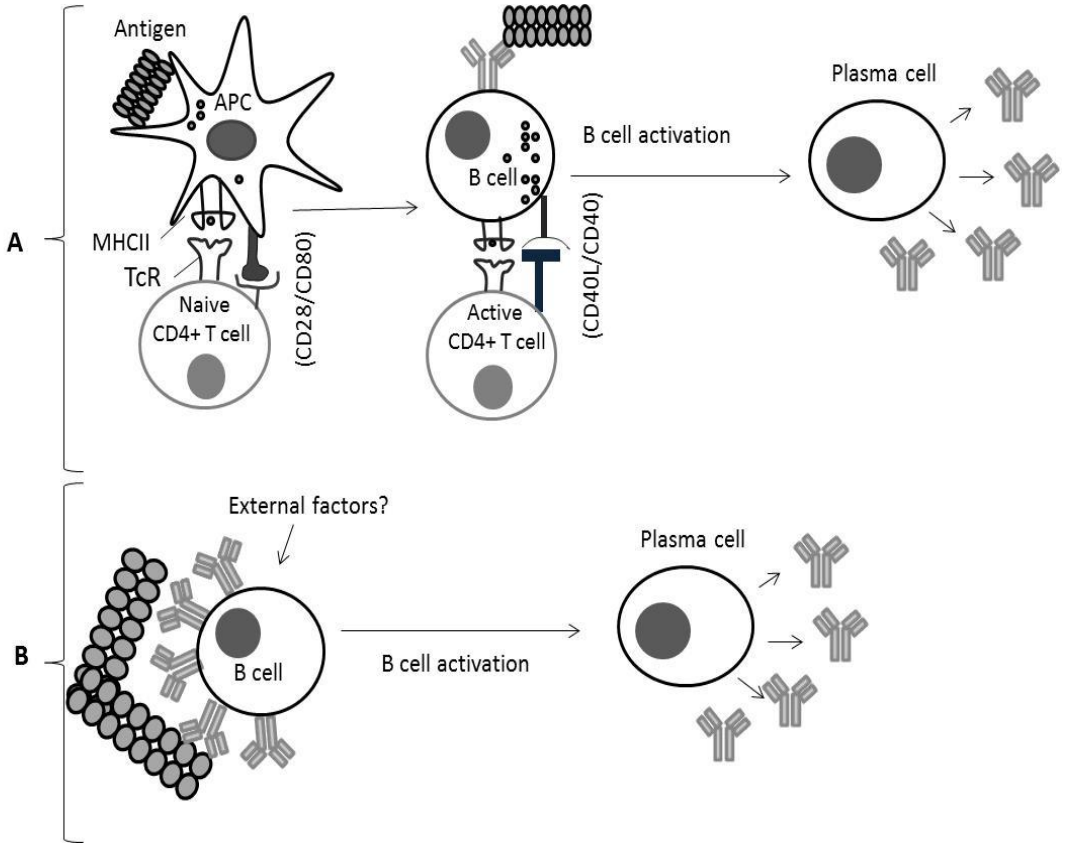

Figure 1.2. B-Cell activation mechanisms. (A) Classical response: Antigen is internalized by APC, and processed to peptide fragments that bind to major histocompatibility complex II (MHCII). Recognition by $CD4^+$ T-cells stimulates cytokine secretion and B-cell activation followed by differentiation to plasma cells. **(B)** Breakdown of B-cell tolerance: B-cells can be activated to plasma cells by antigens possessing repetitive epitopes which cross link antigen specific BcR, triggering activation signals. External factors/signals could play a role in this process (Sauerborn et al. 2010; Ragheb and Lisak 2011).

Classical immune response to foreign antigen

Foreign antigens trigger a 'classical' immune reaction that is dependent upon T-cell activation. This mechanism requires interaction of antigen with APC that, in turn, prime naïve T-cells. Primed T-cells may then interact with B-cells displaying the antigen within a major histocompatibility complex (MHC) molecule. Interaction with co-stimulatory molecules (such as CD28/CD80) further activates T-cells and

stimulates cytokine secretion, leading to the proliferation of B-cells and antibody production. Isotype switching from IgM to IgG is a hallmark of this T-cell mediated immune response (Avery et al. 2008). The presence of IgG ADA is often indicative of T-cell help, although some isotype switching can take place in the absence of T-cells (Sauerborn et al. 2010). This classical type of immune response is found in patients lacking immune tolerance to a human therapeutic protein, or in response to modified proteins containing non-self epitopes. Aggregates of recombinant human proteins may also induce this type of immune response. It is known that aggregates have the ability to activate APC, and are more easily phagocytozed (Scott and De Groot 2010). Furthermore, Joubert and colleagues (2012) recently demonstrated the ability of protein aggregates to induce an adaptive T-cell response *in vitro* using cultured human peripheral blood mononuclear cells stimulated with aggregated mAbs. Here, aggregates induced innate signals through cell surface receptors, including Toll like receptors that could develop into an adaptive T-cell response, characterized by $CD4^+$ T-cell proliferation and cytokine profiling. A cytokine signature, including interleukin (IL)-1β IL-6 and tumour necrosis factor-α, was identified as a potential biomarker for aggregate immunogenicity, and this may find application in the *in vitro* prediction of biotherapeutic immunogenicity. However, results recorded *in vitro* may not be truly representative of the *in vivo* situation; in particular, the relationship between this biomarker and the adverse effects associated with ADA formation is unknown.

Breakdown of B-cell tolerance

Unresponsive or anergic B-cells are unable under normal circumstances to respond to endogenous 'self' proteins that bear antigens for which they have specific receptors. However, these cells can be activated if they receive an appropriate signal from T-cells. The way in which antigen is displayed can also influence the responsiveness of tolerant B-cells, as organized structures have been found to be optimal for T-cell-independent immune responses to self-antigen in mice (Bachmann

et al. 1993; Ohashi and DeFranco 2002). Antigen organization in an ordered protein aggregate differs from the monomeric protein (see Figure 1). It has been hypothesized that repetitive epitope structures formed by aggregation may be capable of activating B-cells directly, as proposed by Bachmann and Zinkernagel (1997). Aggregates may crosslink BcR in a manner that stimulates a downstream signalling cascade followed by differentiation of B-cells into plasma cells and secretion of IgG ADA. The ability of highly ordered structures to elicit more potent immune responses compared with the monomeric protein has been demonstrated using viral coat protein from vesicular stomatitis virus (Bachmann and Zinkernagel 1997). Additionally, the highly ordered structure of papaya mosaic virus, used as a vaccine platform, has been shown to be critical for the generation of an efficient humoral response (Babin et al. 2013).

It is not unexpected that highly ordered oligomerized antigens can be more immunogenic, as they resemble the structure of foreign microorganisms such as viruses, and may be recognized as such by the immune system. Epitopes spaced 5-10 nm apart are characteristic of microbial antigens and it is hypothesized that the immune system has evolved to recognize and respond to this type of antigen (Hermeling et al. 2004; Kessler et al. 2006). Thus, a potential mechanism for the activation of the immune system by protein aggregates is the organization of protein structures into viral-like arrays and subsequent breaking of B-cell tolerance. The repetitive structure of pathogenic microbes is able to induce T-cell-independent responses (Szomolanyi-Tsuda and Welsh 1998). This may occur through BcR engagement, which has been shown to lead to the breakdown of tolerance (Kouskoff et al. 2000), possibly through receptor cross-linking and activation of downstream proliferative signals. The mechanism responsible may not be strictly T-cell-independent as T-helper (T_H) cells may also help B-cells to fully respond to antigen, possibly by the release of cytokines from activated T-cells or non-specific T-cell interaction (Hunziker et al. 2003; Soulas et al. 2005). Unlike the classical immune

response that leads to the development of immunological memory, direct activation of B-cells leads predominantly to the formation of low affinity IgM in the absence of memory. Although there will only be effective class switching if there is involvement of T_H cells, some isotype switching to IgG can occur via an unknown mechanism (Lange et al. 2008; Sauerborn et al. 2010).

Factors involved in immunogenicity of aggregates

Various intrinsic and extrinsic factors can affect the propensity of a protein to stimulate an immune response. Many extrinsic factors contribute towards determining the immunogenicity of biotherapeutic proteins; these can be treatment related factors such as the dosing regimen, patient related factors such as MHC variants, or product related factors such as product design (Singh 2011). These contributing factors (illustrated in Figure 1.3) can all influence the immunogenicity of a protein aggregate and make it difficult to attribute immunogenicity to one source. Intrinsic factors relate to the protein itself, these include the presence of epitopes that are recognized specifically by receptors of the immune system.

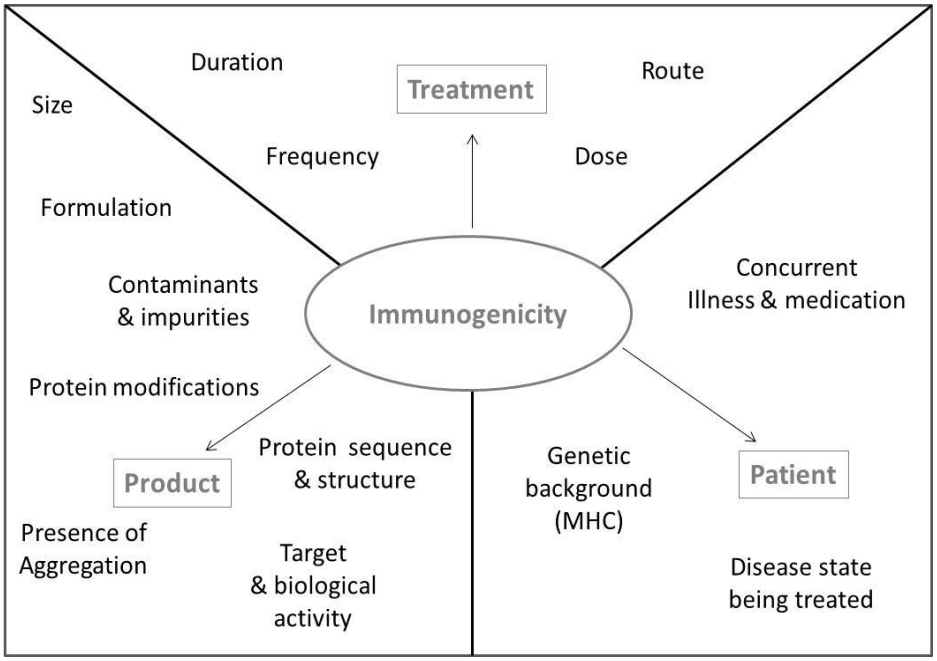

Figure 1.3. Factors that may influence biotherapeutic immunogenicity. Treatment, product, and patient related factors that can impact upon the immunogenic potential of a biotherapeutic.

1.1.6 Clinical examples of aggregation associated immunogenicity

There is clinical evidence, from early studies in 1960s through to more recent examples, that aggregation of therapeutic proteins can affect immunogenicity (Villalobos et al. 2005; Rosenberg 2006). ADA induced by the breakdown of B-cell tolerance generally first appear months after treatment and disappear after treatment is terminated; few proteins induce a classical vaccine-type immune response where antibodies may persist for years (Schellekens 2010). Biotherapeutic immunogenicity can result in no observable effect to a range of clinical manifestations; these include neutralization of therapeutic effectiveness, which may result in a worsening of the existing disease (Farrell et al. 2012), reactivity with host protein homologues (Casadevall et al. 2002), and adverse reactions such as haematotoxicity (Everds and Tarrant 2013). There is also the potential for biotherapeutics to induce anaphylaxis. Although chimeric/humanized antibodies result in much reduced immunogenicity compared with original mouse antibodies, even these antibodies can result in

anaphylaxis (Harding et al. 2010; Radstake et al. 2009). For example, anaphylactic shock has been recorded upon second exposure to chimeric anti IL-2 receptor (Baudouin et al. 2003). A patient treated with this therapy developed an IgE response that triggered anaphylactic shock on further exposure. It is possible that aggregation could exacerbate this effect. Examples of some specific biotherapeutics are discussed below to illustrate the clinical consequences of immunogenicity.

Eprex

A well-documented example of biotherapeutic immunogenicity is the antibody responses that developed in patients receiving treatment with human erythropoietin (epoetin-α) Eprex®. This was associated with the development of pure red cell aplasia (PRCA) among patients with chronic renal failure (Casadevall et al. 2002). Erythropoietin is a hormone that is required for red blood cell (RBC) development, and PRCA manifests as severe sudden-onset anemia that is characterized by the absence of red cell precursors in the bone marrow (Boven et al. 2005b). In Eprex®-treated individuals, PRCA was caused by the formation of neutralising antibodies against the administered protein, these antibodies also bound to the endogenous protein. PRCA incidence was rare in treated individuals; between 2001 and 2003, 50 patients out of 100 000 were affected. The mean time between initial exposure to Eprex® and diagnosis of PRCA was 9.1 months (Bennett et al. 2004). There is evidence to suggest that a number of factors such as micelle formation, route of exposure and contaminants from rubber stoppers may have contributed to the immunogenicity of Eprex®; these factors will be discussed in more detail.

PRCA was observed in patients treated with the epoetin-α formulation that contained polysorbate 80 (Villalobos et al. 2005). An earlier formulation of Eprex® contained human serum albumin; this was replaced with polysorbate 80 as a stabilizer (Haselbeck. 2003) and this new formulation was shown to encourage formation of

micelle associated epoetin. Multimeric epitopes are formed when epoetin-α molecules associate with micelles, and it has been suggested these multimeric epitopes formed by the protein in the micelles may be responsible for induction of ADA following BcR recognition and cross-linking (Hermeling et al. 2003). This is supported by evidence that protein multimerization can lead to more efficient immune responses compared with dimers and trimers of the same protein (Rosenberg, 2006). It is also relevant that in the case of Eprex®, the route of administration was an important contributory factor. Subcutaneous (SC) injection resulted in ADA formation, whereas this response was not reported following intra-venous (IV) administration. Hermeling et al (2003) proposed that micelle-associated epoetin may remain intact and interact with immune cells following SC administration, whereas following IV injection micelles are immediately dispersed in the bloodstream upon the dilution of polysorbate 80. Another factor thought to have contributed significantly to the immunogenicity of epoetin-α formulated with polysorbate 80 is the leaching of organic compounds from uncoated rubber stoppers in pre-filled syringes (Boven et al. 2005b); evidence indicates that these compounds could act as adjuvants (Boven et al. 2005a; Locatelli et al. 2007). Additionally, the presence of silicone oil in pre-filled syringes has been implicated in the induction of protein aggregation (Kossovsky et al. 1987; Thirumangalathu et al. 2009). Although it is clear that one or more aspects of the formulation was responsible for the immunogenicity of Eprex®, the proliferation of hypotheses that seek to explain the mechanism behind these adverse events suggests that the precise reason is still unknown (Thirumangalathu et al. 2009).

IFNβ

Interferon (IFN)-β is the main treatment for relapsing-remitting multiple sclerosis (MS). The induction of ADA poses a challenge for the treatment of MS with

recombinant human IFNβ. Antibodies produced in patients can be a mixture of neutralizing and non-neutralizing antibodies. Neutralizing antibody prevents IFNβ binding to its receptor; this can have serious effects in MS patients by reducing drug efficacy and accelerating disease progression, with an increase in relapse rates (Farrell et al. 2012). Approximately 25% of patients develop neutralizing antibody to IFNβ products; these generally appear 6-18 months after first exposure to IFNβ (Ross et al. 2000). In a clinical trial in which patients either received IFNβ1a by subcutaneous (Rebif®) or by intramuscular (Avonex®) injection, the subcutaneously administered protein was more antigenic with respect to both the incidence of neutralising antibody (25% and 2% for Rebif and Avonex, respectively) and the antibody titer. Similar numbers of patients remained relapse-free (75% and 63% respectively), indicating that the presence of antibodies may not always be associated with loss of clinical efficacy (Panitch et al. 2002). However, the authors do acknowledge that the relatively short time frame for this clinical trial (48 weeks) may have impacted on the lack of a relationship between the presence of neutralising antibody and efficacy (Panitch et al. 2002).

Two types of IFNβ are used therapeutically; one is IFNβ-1a produced in Chinese hamster ovary cells, with identical amino acid sequence and similar glycosylation patterns to the endogenous human protein. The second is IFNβ-1b produced in *Escherichia coli* that is therefore unglycosylated; this protein is less potent than IFNβ-1a, which is thought to be a consequence of the lack of glycosylation, as sugar residues can stabilize some proteins. Without the glycan chain, the protein can aggregate through disulphide-linked complexes, and it is this feature that is associated with increased immunogenicity and decreased biological activity (Farrell et al. 2012). IFNβ-1a and -1b therefore display differences in immunogenicity secondary to differences in glycosylation. The immunogenicity of IFNβ can also be enhanced by chemical modifications such as oxidation and deamidation, which can cause degradation (Hermeling et al. 2004). For example,

oxidation-mediated aggregation has been reported to increase immunogenicity of IFNβ-1a in immune-tolerant transgenic mice compared with aggregates formed in the absence of oxidative stress (van Beers et al. 2011).

Other examples

In other examples, immune reactions against aggregates of injected human gamma-globulin were reported in the 1960s, with the production of antibodies specific for antigen either formed or revealed during aggregation (Ellis and Henney 1969). Clinical studies of recombinant IFN-β2a for treatment of malignancies and viral diseases also reveal a strong correlation between immunogenicity and the presence of protein aggregation (Ryff 1997). Additionally, persistent antibodies have been observed in patients with growth hormone deficiency treated with heavily aggregated hGH, whereas transient antibodies were observed in patients treated with less-aggregated forms of hGH (Moore and Leppert 1980). Similarly, preparations of hGH with lower aggregate concentrations of smaller sizes resulted in reduced immunogenicity in mice (Fradkin et al. 2009).

Factor VIII is a clotting factor that is deficient in patients with haemophilia A. Recombinant human factor VIII (rFVIII) is used to treat this condition, however, 15-30% of treated hemophilia patients develop ADA. Studies have indicated that aggregation of rFVIII provides a distinct antigen, or neo-epitope, to which an immune response is mounted (Josic et al. 1999; Purohit et al. 2006). Importantly, in this case endogenous protein does not display the neoepitope, so such antibodies lack neutralising properties.

Omalizumab is a recombinant humanized anti-IgE mAb used to treat allergic asthma and rhinitis (Kim et al. 2010), approximately 0.1% of patients treated with this mAb suffer an anaphylactic reaction. There is no clear mechanism behind this reaction, although the poly-sorbate excipient used in product formulation has been associated with hypersensitivity reactions (Price et al. 2007). Links have been made

between adverse reactions in patients treated with omalizumab and those treated with Eprex®, which also contained a polysorbate excipient, as previously discussed.

Vaccines

In vaccine development, the induction of humoral immunity has been linked to protein aggregation (Wang et al. 2012). Thus, observations made in vaccine development may help to understand the role of aggregation in the unwanted immunogenicity of biotherapeutic products. In animal models oligomeric and multimeric forms of protein antigens have been shown to be more immunogenic than the monomeric forms (Denis et al. 2007; Rudra et al. 2010; Qian et al. 2012). For example, multimerization of papaya mosaic virus capsid protein as a carrier protein for hepatitis C virus is critical for immunogenicity of the vaccine in mice, which is absent with the monomeric form (Denis et al. 2007). This evidence supports the hypothesis that repetitive epitopes of protein aggregates can interact with immune cell receptors, leading to the breakdown of immune tolerance.

Increased immunogenicity of vaccine proteins is generally an advantage; however, the quality of immune response is also important. $CD4^+$ T_H cells polarize into distinct subsets: including T-helper 2 (T_H2) cells that activate naive B-cells to divide and secrete antibody, and T-helper-1 (T_H1) cells that activate macrophages and stimulate cellular immunity. T_H1 and T_H2 cells have reciprocal antagonistic effects. Vaccines inducing Type 1 immunity have been protective in animal models, whereas vaccines that stimulate Type 2 immunity can increase susceptibility to infection (Spellberg and Edwards 2001). Oculorespiratory syndrome (ORS), associated with respiratory symptoms and conjunctivitis (Skowronski et al. 2003b), was recorded as an adverse effect in patients treated with an inactivated influenza vaccine (Babiuk et al. 2004). The implicated vaccine had been manufactured using a different viral splitting agent and was found to contain large aggregates of around 500 unsplit virions, which were absent from competitor vaccines that did not provoke the

adverse effect. A trend towards Type 2 polarization was also reported among vaccinated patients with ORS (Skowronski et al. 2003a). Experimental (mouse) studies have also shown that the strength and type of cellular immune response to the vaccine varies with formulation and extent of aggregation (Babiuk et al. 2004). Further investigation would be required to understand how formulation can affect the polarization of an immune response.

1.1.7 Screening for aggregation and immunogenicity

Due to the costly loss of protein and immunogenicity associated with protein aggregation, screening for aggregation is used in the development of biopharmaceuticals. More recently attention has focused on subvisible particles below 10 µm due to concerns over immunogenicity (Carpenter et al. 2009; Zoells et al. 2012). Analysing protein aggregates can be challenging due to the unknown nature of the aggregates that may have formed in therapeutic protein formulations (den Engelsman et al. 2011). Protein aggregates are generally distinguished from protein monomers by mass balance or size; however, particles in the 1-100 µm size range are too small to be visible, but too large for size-exclusion chromatography (SEC) analysis, a standard technique for aggregate detection (Carpenter et al. 2009). Currently there is no single method available for detection of the whole size range of aggregates that may arise from bioprocessing (Kiese et al. 2008). Additionally, there is a lack of knowledge regarding the size and type of aggregates that can induce harmful ADA. Analytical methods differ with respect to measuring principle and the information that they provide, so a combination of techniques is necessary for characterization of aggregates. Some of the key techniques for analysis of protein particles and aggregates of different sizes are summarized in Table 1.1. Methods are discussed in greater detail in a previous review (den Engelsman et al. 2011).

Immunogenicity testing

Regulatory agencies have recognized the importance of screening for protein aggregation and there is consequently increased pressure on the biotherapeutics industry arising from concerns over safety and efficacy. In order to demonstrate clinical safety and efficacy, immunogenicity testing is now a key component of biotherapeutic drug development. Clinical studies are also required under International Conference on Harmonization (ICH) guidelines to measure various ADA and characterize the ADA response. The formation of neutralizing antibodies can affect safety and efficacy, but non-neutralizing antibodies can also be a concern due to effects on half-life and biodistribution (Shankar et al. 2006).

The induction of ADA in animals and patients is a key endpoint concerning the immunogenicity of biotherapeutics. To study the immunogenicity of protein therapeutics, methods to detect the presence of and to characterize antibodies are required. A range of techniques exist which are useful for investigating the presence of antigen-specific antibody; these include immunoassays that can identify antibodies capable of binding to antigen and bioassays that can distinguishing between neutralizing and non-neutralizing antibodies (Wadhwa and Thorpe 2007). A more detailed survey is beyond the scope of this article.

Animal models

Animal models are a potentially useful approach to measure antibody responses to bio-therapeutics. The 2011 ICH S6 Guideline (preclinical safety evaluation of biotechnology-derived pharmaceuticals) describes the need for detection and characterization of antibodies in repeat dose studies using animal models. A relevant species must be used for *in vivo* studies, i.e. one in which the target epitope is expressed. Although non-clinical immunogenicity studies are required, immune responses are species specific; therefore induction is not entirely predictive of antibody formation in humans (Shankar et al. 2006; Wang et al. 2008). Species specific immunogenicity is related to the lack of genetic diversity in animal models, as

genetic diversity is implicated in immunogenicity (Brinks et al. 2011). Rodent models for immunogenicity testing are therefore less useful than animals which show a higher degree of homology with humans and more genetic diversity than inbred mouse strains, such as non-human primates; however, these are not widely used due to ethical constraints. Conventional non-transgenic animal models can be useful for highly-conserved proteins, but a lack of immune tolerance to human proteins limits their use for immunogenicity testing. These animal models can be useful for comparing the immunogenicity of two similar products, i.e., the immunogenicity of an originator and biosimilar product; this may not reflect the human situation, but it may provide a warning against the advancement of a biosimilar if the immunogenicity profile observed differs to that of the originator.

Despite the limitations associated with the use of animals to predict immunogenicity, several transgenic animal models have been generated for this purpose. Transgenic mice are often the preferred *in vivo* model to predict immunogenicity as they are immune tolerant to the administered human protein (Hermeling et al. 2006; van Beers et al. 2010), and can be used to study the immunogenicity of biotherapeutic aggregates. For example, in a study by van Beers et al. (2010), the IFNβ-1a aggregate percentage and extent of denaturation were shown to influence the ability of aggregates to break tolerance in transgenic mice. In these experiments immune tolerant mice were immunized with IFNβ-1a formulations and antibody responses were measured. Only non-covalently bound aggregates that retained some native epitopes were able to break tolerance resulting in a transient immune response, removal of aggregates prevented this breakdown of tolerance (van Beers et al. 2010). Additionally, mice expressing human MHC molecules can be used to compare antibody and T cell responses to vaccines and protein therapeutics (de Groot et al. 2009). Use of animal models in immunogenicity testing is discussed more extensively in a recent review (Brinks et al. 2011).

Table 1.1. Methods for aggregate analysis in therapeutic protein development.

Table 1.1 abbreviations: SEC, size exclusion chromatography; AUC, analytical ultracentrifugation; Flow FFF, Flow field flow fractionation; DLS, dynamic light scattering; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; Native-PAGE, native-polyacrylamide gel electrophoresis.

Method	**Approximate size range**	**Measuring principle**	**Information obtained**
SEC	5 – 50 nm	Separation through porous matrix based on size	Hydrodynamic diameter
AUC	1 nm - 0.1 µm	Sedimentation rate in response to centrifugal force	Molecular weight and conformation
Flow FFF	1 nm - 1 µm	Separation by flow retention based on diffusion coefficient	Hydrodynamic diameter
DLS	0.5 nm – 10 µm	Fluctuations of scattered light signals	Hydrodynamic diameter
Mass spectrometry	Atomic resolution - MDa	Detection of mass/charge ratio of ionized molecules	Mass/charge ratio
SDS-PAGE	kDa- MDa	Separation of denatured protein in a gel in an electric field according to size	Molecular weight
Native-PAGE	kDa- MDa	Separation of native protein in a gel in an electric field according to size/charge	Charge and hydrodynamic size
Optical microscopy	1 µm - mm	Visualization of protein particles	Size and morphology
Electron microscopy	nm - mm	Visualization of protein particles and detection of chemical composition at high resolution	Size and morphology
Fluorescence spectroscopy	N/A	Protein fluorescence is induced and detected	Conformational changes and/or folding state

In vitro assays

A number of *in vitro* techniques can also be used to assess the immunogenic potential of therapeutic proteins. These could be used to predict the risk of immunogenicity in a preclinical setting. The expression of APC-surface molecules differs following activation; for example, the expression of MHC (Class I and II), costimulatory molecules and cytokine receptors is enhanced. Flow cytometry is an *in vitro* technique that can be used to determine differences in cell surface molecule expression indicative of APC maturation that may initiate T-cell responses (Gaitonde and Balu-Iyer 2011). [^{3}H]-Thymidine based T-cell proliferation assays are also useful tools to study the activation and proliferation of T-cells in the presence of antigen (Joubert et al. 2012). Additionally, the release of immunomodulatory cytokines can be characterized by enzyme-linked immunosorbent assay. This approach can be used to assess the quality of an induced immune response, as specific cytokines can be markers of T_H1 (IL-12 and IFNβ) or T_H2 immunity (IL-4 and IL-10). T cells that respond to a particular epitope *in vitro* can be labelled with MHC class II oligomers and sorted by flow cytometry, the phenotype of responsive T cells can then be determined using intracellular cytokine staining (de Groot et al. 2009; Tobery et al. 2006). In addition to the assays described above, *in silico* techniques have been developed for the prediction of antigenicity by identification of potential T-cell epitopes (Tovey et al. 2011). *In silico* methods have been shown to successfully identify MHC Class II restricted epitopes within biotherapeutics (Koren et al. 2007). Knowledge of aggregation-prone regions may also help in the design and selection of biotherapeutic candidates and reduce aggregation concerns (Wang et al. 2009). For example, aggregation motifs that lack charge have been found in the light chain regions of mAbs including Erbitux® and Raptiva®. This computational approach could therefore be useful to screen biotherapeutic candidates early in drug development.

1.1.8 Concluding remarks

The development of unwanted immunogenicity against biotherapeutics poses significant clinical, scientific, and manufacturing challenges. A breakdown in tolerance following the formation of aggregates with repetitive epitopes seems to be an important mechanism by which ADA are induced. However, the precise immunological mechanisms remain poorly defined. There are still many unanswered questions regarding the immunogenicity of protein aggregates, including whether ADA formation is caused primarily by a breakdown of B-cell tolerance through direct interaction with BcR, or a more classical mechanism involving APC and T-cell activation. It is also unknown what type or level of aggregation is required to induce an unwanted immune response. With these questions in mind, it is important to increase our understanding of mechanisms underlying protein aggregate immunogenicity; this may allow for more effective screening and improved manufacture to avoid aggregation associated adverse events.

1.2 Introduction

This section of the introduction is added to supplement and complement the review article comprising section 1.1 (Ratanji et al. 2014). Further studies on the influence of protein aggregation on immunogenicity have been published since the review, and will be discussed briefly in this section. Additionally, to introduce the background to experiments presented in this thesis, the use of *in vivo* and *ex vivo* assays to assess protein immunogenicity, and biophysical techniques to characterise protein aggregates will be discussed in more detail.

1.2.1 Immunogenicity testing

In vitro studies

Attempts have been made to evaluate the immunogenic potential of aggregated protein using *in vitro* methods. Human peripheral blood mononuclear cell (PBMC) derived dendritic cell (DC) cultures have been used to assess the ability of aggregated monoclonal antibody (mAb) preparations to stimulate DC activation, or adaptive primary T cell responses. Using assays of this type, highly aggregated mAbs have been shown to induce DC activation by upregulating DC maturation markers and T-cell co-stimulatory molecules that are necessary for $CD4^+$ T cell activation, whereas monomers do not (Rombach-Riegraf et al. 2014). A similar study using PBMC-derived DC (Telikepalli et al. 2015) demonstrated that treatment of DC with mAb particles that were 5-10 µm in size resulted in elevated cytokine secretion when compared with aggregates in other size ranges, both larger and smaller (2-5 µm, and 10-400 µm). *In vitro* cultures of human donor $CD4^+$ T cells with autologous antigen-pulsed DC have also been used to test the immunogenicity of licensed IFN-β1α formulations (Jaber and Baker 2007). T cell proliferation was measured by ^{3}H-thymidine incorporation, and cytokine release measured by ELISpot. This approach provided evidence for differential immunogenic potential between two Interferon-β1α (IFN- β1α)

formulations, thought to be due to the removal of human and animal-derived components with the new formulation, which resulted in reduced immunogenicity.

In vitro assays can therefore be useful to rank responses relative to other aggregates and select lead candidates in biotherapeutic development. Advantages of human PBMC-based assays include the ability, in theory at least, to reflect the HLA (human leukocyte antigen) diversity of the human population, and also to use human donors with known medical histories (Moussa et al. 2016). However, it is not known how well responses *in vitro* correlate with immunogenic responses *in vivo.*

In vivo studies

A number of *in vivo* studies have been published recently that use mouse models to compare monomer and aggregate protein immunogenicity. Bessa et al. (2015) found that aggregates of human IgG1 were not immunogenic in a human IgG tolerant transgenic mouse model unless aggregates were significantly covalently modified through artificial light stress, suggesting that the covalent modifications resulted in the formation of immunogenic neo-epitopes. In another human IgG tolerant transgenic mouse model (Bi et al. 2013) it was shown that mechanically aggregated human IgG_2 mAbs and native mAb-coated microspheres were able to enhance mAb-specific immune responses, whereas the monomer did not induce an immune response, indicating that covalent modifications are not the only way in which immune tolerance can be broken.

The role of native and non-native structures within protein aggregates has also been investigated *in vivo*, but with contradictory results. The immunogenicity of oligomer/nanosized (10-40 nm) native-like antibody aggregates, that maintained key structural features of the native protein, was shown to be increased when compared with the monomer, whereas the immunogenicity of non-native aggregates was similar to the monomer (Fathallah et al. 2015). This suggests that it is important for protein

molecules to retain a relatively 'native' state for aggregates to be immunogenic. In a separate study, large insoluble aggregates of a murine mAb (up to 100 000 particles/ml ≥1 μm, and up to 300 particles/ml ≥10 μm) with non-native structure were shown to be more immunogenic when compared with native aggregates in mice (Freitag et al. 2015). The basis for these apparently contradictory results is not known, although it is not surprising given that immunogenicity is dependent on so many different factors; differential antigen processing by DC, for example, or differences in T cell responses (even to the same epitope), are highly context dependent.

The induction of neutralising anti-drug antibodies (ADA) against human IFNβ poses a challenge in the treatment of multiple sclerosis, therefore a number of *in vivo* studies have been carried out using recombinant human IFN in order to understand how aggregation might affect its immunogenicity. The level of aggregation, as well as the size and structure of recombinant human IFNβ-1b aggregates, have been shown to be important in breaking immune tolerance in IFNβ transgenic mice by Abdolvahab et al (2016). In this study thermally stressed aggregates (1.82 μm; measured using fluid imaging microscopy) were more immunogenic compared with copper oxidised aggregates (5.36 μm). Of the mice treated with monomeric Avonex® drug, which has an IFNβ sequence similar to the human IFNβ expressed in the transgenic mice, 80% did not develop ADA. In this study different stress conditions resulted in aggregates of IFN with different biophysical properties and immunogenic potential. However, this is not always the case as a separate study demonstrated that mechanically-derived and metal-oxidised aggregates of human IFNα2b both induced equivalent levels of immunogenicity in transgenic mice (Human et al. 2015).

An investigation into the mechanisms underlying immunogenicity of recombinant human IFNβ in transgenic mice showed that ADA responses were $CD4^+$ T cell dependent, although responses failed to induce IFNβ specific T and B cell immunological memory (Kijanka et al. 2015). Furthermore, adjuvants were not

effective in enhancing the response, therefore whilst the response was $CD4^+$ T cell dependent in this model system, it was distinct from a classical T cell dependent response.

The section below provides greater detail on the *in vivo* experimental model which has been utilised in this thesis for the comparison of monomer and aggregate protein immunogenicity.

BALB/c intraperitoneal exposure model

Mouse models are commonly used for animal experiments as it is generally accepted that many aspects of immune regulation are shared with humans (Hausding et al. 2008, Flajnik 2002); the ability to use transgenic mice as described above is also a major advantage. BALB/c strain mice have been widely used to study the immunogenic or allergenic potential of proteins in the context of food protein and biopharmaceuticals (Chudzik-Kozlowska et al. 2013, Mao et al. 2012, Maas et al. 2007, Dearman and Kimber 2009), although other inbred mouse strains such as C57BL/6 and Swiss Webster (Fathallah et al. 2015, Bessa et al. 2015) can also be used. Intraperitoneal (ip) injection is the most commonly used route of exposure for investigating protein immunogenicity and allergenicity, and has proven to be suitable for studying food protein allergens (Dearman et al. 2001) and biotherapeutics (Abdolvahab et al. 2016). This route has been used in many experimental models to stimulate vigorous immune responses. Other routes of administration can also be used to investigate immunogenicity, such as subcutaneous injection (Human et al. 2015). In a study comparing routes of administration, the immunogenicity of human IFNβ was found to be similar following intramuscular, intraperitoneal or subcutaneous injections (Kijanka et al. 2013). Following intraperitoneal exposure to fluorescently labelled protein, fluorescence can be observed throughout the body of a mouse 3 h after injection (Kijanka et al. 2014). Therefore, labelled protein does not remain in the

peritoneum but is distributed systemically, enabling the injected protein to elicit an immune response. Since BALB/c mice are widely used for studying the immune response to proteins, and intraperitoneal injection is well-established as an effective route for immunisation, experiments described in this thesis will use this experimental model.

To provide more background for some of the methods that contribute to a bulk of the thesis, *in vivo* and *ex vivo* assays will be described below.

In vivo and ex vivo assays

In order to study protein immunogenicity, *in vivo* immunisations with the protein of interest are often used to study antibody production; protein specific antibodies in sera can then be measured *ex vivo*. This approach was used for investigations presented in this thesis. T-lymphocyte cultures can also be used to test T cell reactivity to epitopes in *ex vivo* assays. Experiments can be carried out with lymphocyte cultures from *in vivo* experiments, typically mouse studies, where lymphocytes taken from immunised animals can be re-stimulated *ex vivo* with the antigen (Zhao et al. 2011). Red blood cell depleted splenocytes are commonly used in this type of immunogenicity assay (Davtyan et al. 2013, Montagnoli et al. 2004) as a sufficient lymphocyte rich cell population can be achieved and with a higher yield than that derived from blood. A DC co-culture approach can be used with splenocytes to improve antigen presentation and T cell priming, as the number of splenic DC may be insufficient (Farkas et al. 2013); DC are antigen presenting cells that internalise, process and present antigens in the form of peptide-MHC (major histocompatibility complex) complexes to initiate and orchestrate T cell responses. The addition of these cells to an *ex vivo* T cell assay can improve T cell stimulation (Drutman and Trombetta 2010). It is important to note that background levels of proliferation are higher when naïve T cells are cultured with DC, as it is well known that DC have the ability to

stimulate general low level T cell proliferation in culture (Ge et al. 2002, Scheinecker et al. 1998). The DC-splenocyte co-culture approach has been used in the investigations described in this thesis in order to compare the immunogenic potential of monomeric and aggregated protein preparations.

The availability of different experimental approaches for characterising the immunogenic potential of aggregates has been discussed. *In vitro* tests allow a comparison of the immunogenic potential of different protein preparations. Animal models have more value in predicting the comparative immunogenicity of therapeutic proteins, and the potential clinical outcome of immunogenicity. Predicting the immune response in patients can be improved with the use of transgenic mice that express the endogenous human protein (Bessa et al. 2015, Brinks et al. 2011). Furthermore, *ex vivo* T cell activation assays are a useful pre-clinical tool alongside *in vivo* studies for immunogenicity assessment.

In assessing protein immunogenicity, the character of the immune response can be studied, in addition to the overall magnitude. This can provide an indication of the particular T cell subsets involved in the immune response. Assessing the quality of the immune response is important in the context of the response to biotherapeutics, as differential responses may result in different adverse effects, such as allergic reactions or complement fixing (Krishna and Nadler 2016). T helper (Th) cell differentiation, and the cytokines and antibodies that are markers for the activation of different Th cells are discussed briefly below.

T helper cell differentiation

Various cell types make up the immune system, with T and B lymphocytes comprising the major cellular components of the adaptive immune response. Cell mediated responses are controlled by T cells. Cytotoxic T cells or CD8 expressing T cells can

kill pathogen infected cells directly, and CD4 expressing T cells play a key role in coordinating the immune response, involving various cell types, and are important in facilitating B cell antibody production. $CD4^+$ T cells differentiate into distinct Th subsets in order to fulfil a variety of functions. The differentiated subpopulations of T lymphocytes provide a mechanism for the immune system to tailor the quality of immune responses such that specific antigenic challenges are dealt with effectively. These Th subsets are identified largely based on their cytokine profile and antibody isotypes that they promote (Zygmunt and Veldhoen 2011).

Th1 and Th2 cells were classified based on the selective production of IFNγ and IL-4 cytokines respectively. Th1 cells produce IFNγ and are capable of activating innate cells including macrophages and DC, and can enhance their ability to kill intracellular pathogens or to present antigen. They can also support $CD8^+$ T cells in their cytotoxic functions. Th2 cell mediated immunity is important to combat extracellular pathogens and to help regulate antibody class-switching in B cells. In terms of antibody distribution, different IgG subclasses act as surrogate markers for divergent T cell responses; in mice an elevation of IgG2a is indicative of Th1 immunity, and IgG1 is indicative of Th2 immunity. Since Th1 and Th2 subsets were first described, T regulatory (Treg) and Th17 cells have also been added to the $CD4^+$ T helper cell subset repertoire. Tregs express TGFβ and play an important role in regulating immune cell activation and dampening immune responses. Th17 cells express IL-17 and are important in inflammatory responses (Ouyang et al. 2008).

This thesis aimed to investigate whether protein aggregation influences the magnitude and/or character of induced immune responses. One of the challenges in this field of study is in understanding the biophysical properties of protein aggregates. The section below will discuss some of the methods available for protein aggregate characterisation, the information that they provide, and method limitations.

1.2.2 Biophysical methods for aggregate analysis

Due to the unknown nature of the aggregates that may have formed in therapeutic protein formulations, and limitations of the methods used to measure aggregates, analysing protein aggregates can be challenging (den Engelsman et al. 2011). Protein aggregates are heterogeneous in nature and can vary widely in size and with respect to physicochemical properties. There is also a lack of knowledge about the characteristics that confer on aggregates the ability to induce harmful ADA. Numerous techniques are available for the analysis of protein particles and aggregates of different sizes; some of the key biophysical methods that can be used to characterise protein aggregates are discussed below.

Size-exclusion chromatography

Size-exclusion chromatography (SEC), also known as gel filtration, gel permeation and gel chromatography, is used as a workhorse technique for the detection and quantification of protein aggregates in biopharmaceutical development (Yumioka et al. 2010). This method can also be used for determining molecular mass averages of protein molecules. The principle of SEC is quite simple; protein samples are passed through a column packed with porous beads; small soluble protein particles can penetrate the pores whereas molecules that are too large to fit through the porous matrix bypass the beads, resulting in different elution times. The hydrodynamic volume of a protein dictates its ability to enter the beads; so the elution position can be used to measure the hydrodynamic radius and approximate the molecular weight. The absorbance can also be used to quantify the extent of aggregation.

SEC can be combined with a multi angle laser light scattering (MALLS) technique, which is used to determine the molecular weight of soluble aggregates (Li et al. 2009). The SEC-MALLS method is used for a more reliable estimation of molecular mass in comparison with SEC alone. In this case the molecular mass is determined from the

angular dependence of scattered light intensity as a function of concentration (Beirne et al. 2011). Online coupling of SEC with liquid chromatography has also recently been developed to detect and quantify both proteins and excipients in biotherapeutic products; this can be used to ensure the integrity of a given formulation (He et al. 2012).

Although SEC remains the primary analytical tool for aggregate analysis of biotherapeutics, there are disadvantages associated with its use. The main disadvantage is that non-specific protein adsorption to the SEC column matrix can result in loss of protein and inaccurate aggregate detection, particularly with new columns. SEC also requires specific limited buffer conditions which often require high salt or solvent concentrations to prevent non-specific binding (Liu et al. 2006). Another disadvantage is that SEC has a limited dynamic range (range of molecular masses that can be separated) of up to 670 KDa (Hong et al. 2012), and attempts to increase this range weakens the resolution, therefore a balance must be made between the two (Arakawa et al. 2010). The upper limit of the size of detectable aggregate can be exceeded by aggregates (0.2 µm) as the column can act as a filter, and many columns have a pre-filter to remove larger particulates which would otherwise block flow through the column. If larger aggregates do reach the column resin, the column may then be overloaded and a build-up of protein can hinder column performance, increasing back-pressure, reducing resolution and protein yield (den Engelsman et al. 2011).

Due to the drawbacks associated with SEC, column-free techniques have become more frequently used in aggregation analysis. Alternative methods to SEC that do not require matrices which can influence the separation of protein species, and can be applied under a wider range of buffer conditions can be more attractive options (Liu et al. 2006).

Analytical ultracentrifugation

Analytical ultracentrifugation (AUC) is a versatile tool to detect and characterise aggregates in solution; this method relies on the hydrodynamic separation of protein species in a heterogeneous solution, and works by monitoring the sedimentation behaviour of macromolecules in the centrifugal field. A high speed centrifuge and optical systems are employed to measure the concentration gradients of proteins under centrifugal force. Studying the concentration distributions can then provide information on the structure and conformation of protein present in the sample. Advances in software and instrumentation for sedimentation velocity analysis have allowed AUC to become useful for monitoring the size distribution of aggregation in biotherapeutic formulations (Liu et al. 2006). AUC can be used to complement SEC as it can detect larger aggregates which may pass undetected in SEC due to filtering out or elution in the void volume, although, in common with other methods, AUC can be less accurate with complex mixtures of different sizes (Hughes et al. 2009).

Field flow fractionation

Field flow fractionation (FFF) is a family of elution techniques that are able to measure and separate macromolecules based on their diffusion coefficient (Giddings 1993). Fields that can be used for protein separation include flow, sedimentation, electrical and thermal. For the separation of protein aggregates the flow field is the best suited technique; here, separation occurs in a single-phase, removing the potential for protein adsorption to a matrix. A controlled flow profile acts as the separating mechanism rather than a matrix filled column (Gabrielson et al. 2007). Flow FFF uses a laminar flow of solvent and a cross field generated by cross flow, protein separation is then based on the diffusion coefficient of the solute (Messaud et al. 2009).

Dynamic light scattering

Dynamic light scattering (DLS), also known as quasi-elastic light scattering, is a useful tool to determine the size distribution of particles in solution within the size range of 0.3 nm to 10 µm diameter. This method measures time dependent fluctuations of scattered light signals. The scattered light intensity fluctuates due to particles undergoing Brownian motion, allowing an assessment of the hydrodynamic diameter of aggregates (Li et al. 2011). Unlike SEC, DLS is highly sensitive to large aggregates and is a relatively fast and straightforward method, although it has limited resolving power between aggregates of a similar size (Nobbmann et al. 2007).

Circular dichroism

Circular dichroism (CD) is a spectroscopic technique that can be used to study the secondary structure of macromolecules. This method measures the difference in the absorption of left-handed and right-handed circularly polarised light and occurs at wavelengths of light that can be absorbed by a chiral molecule. The majority of amino acids are chiral and so a key use of CD is to study protein folding and structure. The CD spectrum of a protein is influenced by its 3D structure, and therefore changes in the structure of chiral macromolecules can be detected. CD spectra can be recorded within minutes so it is useful for following changes in protein structure with altered conditions. For example, a protein can be compared before and after temperature or pH treatment to monitor changes in folding and structure. Unlike other methods discussed, CD does not provide information about size distributions. As CD is used to detect changes in secondary structure, which can be correlated to protein aggregation, CD has been used to monitor conformational changes with aggregation. It has been shown that CD is sensitive enough to detect conformational changes in protein structure of a monoclonal antibody caused by thermal stress (Joshi et al. 2014).

Atomic force microscopy

Atomic force microscopy (AFM) is a type of scanning probe-microscopy that measures surface roughness. The technique is based on the interaction between a cantilever tip and the sample surface, allowing the production of 3D surface maps (Vahabi et al. 2013). Most modern AFMs are optical systems that use a laser-beam detection mechanism. AFM tips made from silica or silicon nitride come into contact with the sample surface, resulting in forces that cause bending of the cantilever and laser deflection. AFM can be useful for imaging protein aggregates and providing additional information about the 3D structure of aggregates. For example, the stages of amyloid aggregation of β-lactoglobulin have been analysed using AFM, revealing that fibrils have a multi-stranded, twisted ribbon-like structure (Adamcik et al. 2010). This method has also recently been used to characterise the morphology of subvisible aggregates of an IgG1 mAb, caused by mechanical agitation (Ghazvini et al. 2016). If AFM is carried out in dry non-physiological conditions, one disadvantage is that any salt present in buffer can form salt crystals and disturb the images.

Raster image correlation spectroscopy

Raster image correlation spectroscopy (RICS) is a relatively novel technique for measuring molecular dynamics using commercial laser scanning microscopes, where images are generated by measuring fluorescence intensity across an area (Brown et al. 2008). A laser beam illuminates a region of the sample and the intensity of the emitted fluorescence is measured, providing a value for each region termed a 'pixel' (Rossow et al. 2010). Protein dynamics and aggregate states, including size, can be determined by image analysis and spatial-temporal fluorescence image correlation methods. The method also provides a way of measuring diffusion coefficients and binding. RICS has been used to detect protein complexes in live cells by obtaining laser scanning confocal image sequences (Digman et al. 2013); a map of protein complexes and their spatial distribution within the cell can be constructed from these sequences. (Kim et al. 2010). A weakness of RICS is the limited spatial and temporal

resolution; spatial resolution limited to the sub-region in which spatial correlation is calculated and temporal resolution limited by the scan rate (Brown et al. 2008). Additionally, photobleaching of the protein can occur during sample collection.

Transmission electron microscopy

Electron microscopy (EM) is a technique that allows the visualisation of protein aggregates, providing information on aggregate size and structure. Transmission electron microscopy (TEM) is a type of EM in which a beam of electrons is transmitted through the specimen and unscattered electrons are detected, providing a 'shadow' image. This method has been used to study antibody aggregates; it is capable of providing detailed information on the microstructure of aggregates and visualising the macromolecular structure, from nm sized molecules to larger assemblies of several microns (Sung et al. 2015). One disadvantage of TEM is that it relies on imaging aggregates under non-physiological conditions. For example, negative staining requires drying of the sample which can alter the appearance of protein particles, or even lead to the collapse of protein assemblies (De Carlo and Harris 2011).

Mass spectrometry

Mass spectrometry is an analytical technique that measures the mass/charge ratio of ions to identify and quantify molecules. One particular application of mass spectrometry is in conjunction with hydrogen/deuterium exchange (HDX) for the analysis of protein aggregates (Bronsoms and Trejo 2015). The combination of HDX with electrospray ionization mass spectrometry can be used to determine peptides involved in protein aggregation. Protons involved in H-bonded secondary structures exchange protons more slowly with deuterium than solvent-exposed and non-H bonded regions. Therefore, by assessing the susceptibility for proton exchange to deuterium, regions involved in secondary structure, and the conformational dynamics of proteins can be studied (Kheterpal et al. 2006). This technique has been used to

study the structure of amyloid aggregates (Nettleton and Robinson 1999) and to provide information of higher order structure of monoclonal antibody complexes (Zhang et al. 2014).

Nuclear magnetic resonance

Nuclear magnetic resonance spectroscopy (NMR) relies upon the magnetic properties of atomic nuclei, and can be used to determine the physical and chemical properties of molecules within a sample. Diffusion measurements performed with NMR techniques can show changes in the hydrodynamic radius of a protein resulting from denaturation (Wilkins et al. 1999). NMR is not widely applied to studying protein aggregates, but it can be used to compare native and non-native states of a protein and to characterise folding transitions through measurement of the hydrodynamic radii (Jones et al. 1997, Indrawati et al. 2007). Measuring the dimensions of the protein structure can provide information on the conformational properties of a protein species.

To summarise, there have been advances in recent years in the available analytical methods for the characterisation of protein aggregates. While each method has its relative merits, there are limitations with regard to the type of information that they provide, sample processing requirements or resolving power. A selection of techniques has been discussed here and in the review (Ratanji et al. 2014), although these are not exhaustive of all the available methods, more of these techniques are described in a number of reviews (Amin et al. 2014, den Engelsman et al. 2011). The appropriate characterisation technique(s) to use can be dependent upon the individual aggregate properties, and often multiple techniques are required to obtain information on the conformational state, as well as the secondary and tertiary protein structure.

1.2.3 Thesis aims

The overall aim of the work described in this thesis was to begin to characterise the relationship between biophysical characteristics of protein aggregates and the intensity and character of induced immune responses in BALB/c mice.

These investigations were focussed on investigating whether protein aggregation influences the intensity and/or character of induced immune responses, and not in predicting the immunogenicity of a given protein. Therefore, the deliberate strategy was to choose foreign reference proteins that would cause an immunogenic response, so that the impact of aggregation on that response could be studied. Firstly, a humanised scFv was chosen as a reference protein; a scFv consists of variable heavy and light chains of an antibody, connected with a linker peptide. This particular scFv was an anti-c-met scFv raised in a rabbit, that had been previously redesigned by grafting its CDR (Complimentarity Determining Region) with human frameworks for variable heavy and light chains (Edwardraja et al. 2010). The scFv format has relevance for the biopharmaceutical industry with the emergence of novel formats and antibody fragments on the market (Holliger and Hudson 2005, Goel and Stephens 2010). Ovalbumin (OVA) was included as an additional reference protein as it is a well characterised antigen with a different size and structure to the scFv. Mouse serum albumin was then used to see if the lessons learnt from these foreign proteins could also be applied to a homologous protein.

For the purpose of creating aggregates for immunogenicity studies, various stress methods were tested on the proteins described above. Work focussed initially on the humanised single chain variable fragment (scFv) which was expressed recombinantly and purified from *E.coli*. Stress conditions required to aggregate the scFv were determined. Additional proteins (OVA and mouse albumin) were also stressed and characterised using similar techniques. Efforts were made to ensure that stressors used were relevant to those encountered during bioprocessing, and to achieve aggregates within the subvisible size range. Subvisible aggregates (up to 10 µm)

were sought due to concerns over their immunogenicity (Ahmadi et al. 2015, Carpenter et al. 2009). Elevated temperature and stirring stress were the chosen methods for generating aggregates, and stressed protein preparations were evaluated using a number of analytical methods. For example, dynamic light scattering was used for size analysis, and circular dichroism and microscopy techniques were used to analyse secondary and 3D structure. The stability of aggregates was also tested as they were required to withstand freeze-thaw stress for storage purposes.

The second objective was to compare the immunogenicity of monomeric and aggregated forms of the same protein. In these investigations immune responses induced by monomeric and aggregated preparations of scFv and OVA were compared in BALB/c strain mice following intraperitoneal (ip) immunisation protocols. In some experiments subcutaneous administration was compared with ip immunisation using the scFv protein as the immunogen. Protein-specific antibody responses [IgG, IgG1, IgG2a and IgM antibodies] in individual serum samples were measured using ELISA systems.

Immunogenicity was also studied at the cellular level by measuring lymphocyte proliferation and cytokine secretion in response to *ex vivo* treatment with antigen. Splenocytes from immunised mice were cultured alone and with bone marrow derived DC, the latter in an effort to improve assay sensitivity. Cell proliferation was measured by ^{3}H-thymidine incorporation with β scintillation counting, and cytokine production by analysis of supernatants by ELISA. Collectively, the use of antibody ELISAs and cell based assays allowed both the magnitude and character of induced immune responses to be studied.

The final objective was to investigate the impact of host cell impurities (HCP) on the immunogenic potential of protein aggregates. Host cell proteins (HCP) can remain in

biotherapeutic preparations as a process related impurity. Heat shock proteins, otherwise known as chaperones, are one type of HCP that can potentially influence the immunogenicity of biotherapeutics (de Zafra et al. 2015). Under normal conditions chaperones constitute a major portion of total cell protein and play a role in protein folding, binding to partially unfolded proteins to fulfil this role (Bukau and Horwich 1998). The impact of chaperones on protein immunogenicity is of particular interest in these investigations, which are focussed on protein aggregation, as aggregates consist of partially unfolded proteins and chaperones by nature will bind to exposed hydrophobic regions. Chaperone proteins have been implicated in the stimulation of the immune system (Srivastava 2002), and can act as adjuvants towards a co-administered antigen. Therefore, the effect of aggregation and HCP impurities (chaperones in particular) on protein immunogenicity is of interest.

In experiments described in this thesis *E.coli* HCP were identified in purified oligomeric preparations of the scFv. Of the identified HCP, *E.coli* chaperone protein DnaK (Calloni et al. 2012) was chosen as a candidate protein for further investigation. Recombinant *E.coli* DnaK was spiked into scFv preparations at varying concentrations, with 1pp1000 being the highest level of DnaK impurity. The effect of DnaK on the immunogenic activity of scFv was determined for both monomeric and heat aggregated scFv preparations to characterise the effects of aggregation and DnaK separately and together. In addition, mouse albumin was used to determine whether the DnaK impurity and/or aggregation of a murine protein could break immune tolerance *in vivo*. Immunogenicity was assessed using antibody ELISA assays to study protein specific total IgG, IgG1 and IgG2a subclasses, and IgM in individual serum samples.

1.3 Alternative format

The thesis is being presented in the alternative format in accordance with the rules and regulations of the University of Manchester. The three results chapters and part of the introduction presented herein are in manuscript form, and are presented in the style of the publishing journal or intended journal of submission. However, elements have been reformatted to ensure these chapters form a cohesive body of work. In addition, a chapter containing supplemental data has also been added. Below are the details of each manuscript, its publishing journal or intended journal of submission, and contribution of each author to work presented.

Chapter 1 **(Introduction: review article) Immunogenicity of therapeutic proteins: Influence of aggregation**

Authors: Kirsty D. Ratanji, Jeremy P. Derrick, Rebecca J. Dearman, and Ian Kimber

Publishing Journal: Journal of Immunotoxicology

Contribution of authors: As first author on this paper, I was fully responsible for writing the text of the manuscript. The manuscript was reviewed and commented on by all

co-authors. These comments were then synthesised by myself to produce the final manuscript.

Chapter 2: **Producing and characterising subvisible protein aggregates for immunogenicity studies**

Authors: Kirsty D. Ratanji, Rebecca J. Dearman, Ian Kimber, Maryam Hussain and Jeremy P. Derrick

Intended Journal: Journal of Pharmaceutical Sciences

Contribution of authors: This manuscript is representative of experiments of which I contributed a large portion. Dr James Austerberry assisted with the circular dichroism analysis. Maryam Hussain carried out the raster image correlation spectroscopy RICS analysis. Atomic force microscopy was carried out with Dr Steven Marsden from the Bioimaging core facility at the University of Manchester. Electron microscopy experiments were carried out by Dr Richard Collins within the Electron microscopy facility at the University of Manchester. My supervisors Prof. Jeremy Derrick, Prof. Ian Kimber and Dr. Rebecca Dearman provided advice and guidance on all experimental work. As first author on this paper, I was fully responsible for writing the text of the manuscript. The manuscript was reviewed and commented on by all co-authors. These comments were then synthesised by myself to produce the final manuscript.

Chapter 3: **Subvisible aggregates of immunogenic proteins promote a Th1-type response**

Authors: Kirsty D. Ratanji, Rebecca J. Dearman, Ian Kimber, Robin Thorpe, Meenu Wadhwa and Jeremy P. Derrick

Publishing journal: Toxicological Sciences

Contribution of authors: This manuscript is representative of experiments for which I am solely responsible. My supervisors Prof. Jeremy Derrick, Prof. Ian Kimber and Dr. Rebecca Dearman, Dr Meenu Wadhwa and Dr Robin Thorpe provided advice and guidance on all experimental work. Lorna Beresford carried out the immunisations. As first author on this paper, I was fully responsible for writing the text of the manuscript. The manuscript was reviewed and commented on by all co-authors. These comments were then synthesised by myself to produce the final manuscript.

Chapter 4**:** ***E.coli*** **chaperone protein DnaK modifies and enhances protein aggregate immunogenicity**

Authors: Kirsty D. Ratanji, Jeremy P. Derrick, Ian Kimber, Robin Thorpe, Meenu Wadhwa and Rebecca J. Dearman.

Journal of submission: Immunology

Contribution of authors: This manuscript is representative of experiments for which I am solely responsible. My supervisors Prof. Jeremy Derrick, Prof. Ian Kimber and Dr. Rebecca Dearman, Dr Meenu Wadhwa and Dr Robin Thorpe provided advice and guidance on all experimental work. Lorna Beresford carried out the immunisations. As first author on this paper, I was fully responsible for writing the text of the manuscript. The manuscript was reviewed and commented on by all co-authors. These comments were then synthesised by myself to produce the final manuscript.

CHAPTER 2:

PRODUCING AND CHARACTERISING SUBVISIBLE PROTEIN AGGREGATES FOR IMMUNOGENICITY STUDIES

2 Paper 1: Producing and characterising subvisible protein aggregates for immunogenicity studies

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Planned submission: Journal of Pharmaceutical Sciences

2.1 Abbreviations

BCM barycentric mean
CD circular dichroism
DEAE diethylaminoethanol
DLS dynamic light scattering
E.coli *Escherichia coli*
FTIR fourier transform infrared spectroscopy

mAb monoclonal antibody

MSA mouse serum albumin

OD optical density

OVA ovalbumin

RICS raster image correlation spectroscopy

scFv single chain variable antibody fragment

SEC size exclusion chromatography

SEC-MALLS size exclusion chromatography - multi angle laser light scattering

TEM transmission electron microscopy

2.2 Abstract

The formation of protein aggregates in biotherapeutic preparations is a major concern for the biopharmaceutical industry. It is well known that aggregation has the potential to enhance protein immunogenicity, therefore efforts are being made to detect and characterise aggregates in biotherapeutic preparations. A wide range of biophysical methods are available that can provide information on protein conformational stability, particle size and structure. However, since limitations are associated with individual methods, and each method provides different information, there is no standard set of techniques that is used for biophysical analysis. Here, a selection of techniques was compared for their ability to analyse protein preparations that have been subjected to thermal and mechanical stresses. For these experiments, and future immunogenicity studies, a single chain variable antibody fragment, ovalbumin and mouse serum albumin were selected as test proteins. Dynamic light scattering (DLS) and raster image correlation spectroscopy (RICS) were employed to analyse particle size distributions, circular dichroism (CD) was used to compare the secondary structure of monomeric and stressed preparations, and transmission electron microscopy and atomic force microscopy were tested as methods to examine the macromolecular structures and dimensions of aggregates. It was shown that subvisible sized aggregate preparations could be achieved for each test protein, although results from DLS and RICS differed slightly. Additionally, differing mechanical and thermal stress conditions were required to produce aggregates of each of the three test proteins. Furthermore, changes in secondary structure content after stresses could be detected by CD. However, limitations associated with the microscopy techniques prevented images of aggregates from being acquired. It is concluded that a number of techniques in combination provide a more comprehensive biophysical analysis of aggregated preparations.

2.3 Introduction

Protein aggregation can limit protein yield during purification and also has the potential to enhance immunogenicity resulting in the production of anti-drug antibodies, which can impact upon drug safety and efficacy in the clinic (Ratanji et al., 2014). The aggregation of biotherapeutics is a major concern within the biopharmaceutical industry, therefore efforts are being made to detect, characterise and reduce protein aggregates present during the development of biotherapeutics (den Engelsman et al. 2011).

External stresses that are encountered during bioprocessing can compromise native protein stability and increase the risk of protein unfolding. Protein unfolding results in the exposure of hydrophobic regions on the protein surface, which may interact with apolar regions on another protein molecule, resulting in the self-association or aggregation of the partially unfolded molecules. Bioprocessing-related stresses that can lead to protein unfolding include: acidic or alkaline pH, shear stress, elevated temperature and high protein concentration. The term ‘aggregates’ summarises all multimeric protein species that are formed by either covalent or non-covalent interactions (Mahler et al. 2009). Aggregate formation is propagated by the formation of a nucleus, i.e. an unfolded monomer or multimer that is polymerised. While protein particles themselves can act as nuclei for aggregation, foreign micro or nanoparticles can serve a similar function. For example, silicone particles which are shed from peristaltic pumps that are used in fill and finish operations can end up in a final biotherapeutic product (Saller et al. 2016). Due to this polymerisation mechanism, protein aggregates by nature often form heterogeneous populations that cover a wide size range. A major challenge in aggregate analysis and characterisation is that there is no single analytical method with the ability to detect the full size range or type of aggregate that may be present in a given preparation.

The relationship between protein aggregation and immunogenicity is well established in the literature, although, it is currently not known which biophysical characteristics

of protein aggregates (i.e. size or morphology) affect the immune response. Studies on the influence of protein aggregation on immunogenicity have been published: in these investigations a number of stress methods were employed to produce protein aggregates. The stresses applied were generally relevant to biopharmaceutical industry processes. For example, stirring stress, syringe stress, thermal stress and freeze/thaw stress have all been used to produce monoclonal antibody (mAb) aggregates for immunogenicity studies (Joubert et al. 2012; Tellikepalli et al. 2015; Rombach-Riegraf et al. 2014).

A number of orthogonal techniques are available for characterising subvisible protein aggregates (Manning et al. 2014). Subvisible particles are usually defined as particles that are not visible by eye, with a size of 0.1-100 µm. The quantification of subvisible particles that are greater than 10 µm and 25 µm in size is required by the United States Pharmacopeia <788> (Murphy 2015); this size range is typically characterised using imaging or light obscuration methods. Oligomers and multimers below 0.1 µm in size can be characterised using size exclusion chromatography, although recently there has been an increased focus on characterising subvisible particles from 0.1 to 10 µm in size due to concerns over immunogenicity (Carpenter et al. 2009), and there are few well-established techniques for size-quantification within this range. There is no standard set of methods used for the biophysical characterisation of protein aggregates, a point reflected in the wide range of different techniques that have been used in published studies where the immunogenicity of protein aggregates has been investigated. These techniques include nanoparticle tracking analysis, Fourier transform infrared spectroscopy (FTIR), UV-spectroscopy, size-exclusion chromatography and circular dichroism (Joubert et al. 2012; Fathallah et al. 2015; Human et al. 2015). It is important to note that not all of these methods used are for size quantitation; some give information on secondary and tertiary structure. Circular dichroism, for example, is used to measure protein secondary structure (Greenfield

2006). For the purpose of making aggregates for immunogenicity studies, understanding conformation is important as it indicates the potential for formation of neo-epitopes, which can have implications for protein immunogenicity.

The aim of the investigations described herein was to use a number of biophysical characterisation techniques to study aggregates derived from different protein preparations after the application of thermal and mechanical stresses. Reproducibility of the aggregates formed here was important as aggregate preparations needed to fall consistently into particular size ranges. The work was initially focussed on a single chain variable antibody fragment (scFv), as this was the main protein to be used in future immunogenicity experiments. In addition, ovalbumin (OVA) and mouse serum albumin (MSA) were also tested in a similar fashion, again as a prelude to immunogenicity studies.

2.4 Methods

Single chain variable antibody fragment purification

A humanised scFv (amino acid sequence is detailed in the appendix) (Edwardraja et al. 2010), was cloned into a pET-22b vector in Shuffle T7 express *Escherichia coli* (*E.coli)* cells (New England Biolabs*,* Beverly, MA*,* USA). Transformants were cultured at 30°C to an optical density (OD) of 0.8 at 600 nm, induced with isopropyl β-D-1-thiogalactopyranoside and incubated overnight at 16°C. Cell pellets were resuspended, sonicated and centrifuged at 28672 g for 30 min. scFv was purified from supernatants using DEAE (diethylaminoethanol) Sepharose anion exchange chromatography. A column packed with DEAE resin (DEAE resin from Sigma-Aldrich, St Louis, Missouri) was washed with 2 column volumes (CVs) of 25 mM TrisHCl 1M NaCl pH 8.5 followed by 5 CVs 50 mM TrisHCl 25 mM NaCl pH 8.5. The column was loaded with sample followed by 1CV DEAE buffer. Flow through was collected for protein A purification. Protein A affinity chromatography was then carried out on the DEAE flow through using a column packed with rProtein A Sepharose resin or pre-packed rProtein A Sepharose Fast flow columns (GE Healthcare, Uppsala, Sweden), according to manufacturers' instructions. Bound protein was eluted with 0.1M Na/citrate buffer (pH 3.5). Eluted sample was neutralised to pH 7 immediately with 1.5M Tris (The purification protocol was optimised, see supplementary chapter Figure 5.1 for further information).

SEC and SEC-MALLS

SEC was carried out using an AKTA FPLC system (GE Healthcare). SEC was carried out using a SD75 24 or 120ml column (GE Healthcare) in Dulbeccos phosphate buffered saline (PBS) without Ca^{+2} or Mg^{+2} (Sigma-Aldrich) at a flow rate of 0.5 or 1 ml/min and collected in 1 ml fractions. Monomeric, and higher molecular weight peaks were pooled separately. Protein concentrations were determined by measuring the absorbance at 280 nm using an extinction coefficient of 58580 M^{-1} cm^{-1}. The samples

were then diluted using PBS to a final protein concentration of 1 mg/ml snap frozen in liquid nitrogen and stored at -80°C.

For SEC-MALLS (SEC coupled with multi angle laser light scattering) analysis the method was the same as described above except that the column outlet was connected to a Dawn Helios MALLS photometer (Wyatt, Santa Barbara, CA, USA) followed by OptiLab T-rEX differential refractometer. Data were processed using Wyatt-QELS software. Samples were processed by staff at the Biomolecular analysis facility at the University of Manchester.

Protein stress conditions

scFv: Thermal stress; Monomeric scFv (1 mg/ml; concentration determined by absorbance at 280 nm) in PBS was heated at 40°C for 25 min. Mechanical stress; To induce stir-stressed aggregates 1 ml of 1 mg/ml scFv was stirred with an 8x2 mm Teflon stirrer bar in a 5 ml glass tube for 6 h.

Ovalbumin (OVA)*:* Thermal stress; Lyophilised OVA (Sigma-Aldrich) was reconstituted in PBS to a concentration of 10 mg/ml and heated at 80°C for 1.5 h. Mechanical stress; OVA was diluted to 1 mg/ml in PBS and stirred with an 8x2 mm Teflon stirrer bar in a volume of 1ml in a 5 ml glass tube for 24-28 h. Dynamic light scattering (DLS) was used to monitor aggregation status and stirring stopped once the desired subvisible size range achieved.

Mouse serum albumin (MSA)*:* Thermal stress; Lyophilised MSA (MP Biomedicals, Santa Ana, CA, USA) was reconstituted in PBS to 5 mg/ml and heated at 60°C for 24 h, then diluted to 1 mg/ml in PBS. Mechanical stress; 1 ml of 1 mg/ml MSA was stirred with an 8x2 mm Teflon stirrer bar in a 5 ml glass tube for 1 week.

Static light scattering

Monomeric scFv at 1 mg/ml was subjected to a temperature ramp experiment between 30-85°C and analysed by static light scattering (SLS) using the Avacta Optim® 1000 with 266 nm and 473nm lasers (Pall corporation, Port Washington, NY). Static light scattering combined with thermal ramping was used to determine the temperature of aggregation onset (T_{agg}).

Dynamic light scattering

Measurements of DLS were performed with a Malvern Zetasizer Nano ZS ZEN3600 (Malvern, Herrenberg, Germany) equipped with a 633 nm laser. Each sample (70 µl) was measured in a Suprasil® quartz cuvette (Hellma GmbH, Muellheim, Germany) with a path length of 3 mm and 200-2500 nm spectral range. Monomeric and stressed samples at 1 mg/ml were measured at 25°C to determine the volume-based average protein particle diameter in solution.

Raster image correlation spectroscopy

Analysis of particulates with confocal microscopy (RICS)

SYPRO® Red (Thermo Scientific, Leicestershire, UK), used to label protein aggregates, was added 15 minutes prior to visualisation with confocal microscopy. A Zeiss 510 Confocor 2 (Zeiss, Jena, Germany) confocal microscope equipped with a c-Apochromat 40×/1.2NA water-immersion objective was used for image acquisition. Samples were labelled with SYPRO® Red prior to imaging protein aggregates but following stress. Imaging was carried out by exciting the dye with a Helium-Neon laser at 543 nm and the emitted fluorescence collected above 585 nm. Confocal image time series of 1,024 × 1,024 pixel resolution were captured over 100 frames with a corresponding pixel dwell time of 6.4 microseconds. In-house RICS software (ManICS) was applied to analyse images acquired using confocal microscopy. A full description of the RICS algorithm has been described elsewhere (Hamrang et al. 2012; Digman et al. 2005; Hamrang et al. 2015). The aforementioned image time

series were sub-divided into 32 x 32 pixels sub-regions and the diffusion coefficients (D) of each region of interest (ROI) was generated. All fits possessing a R^2 below 0.7 were discarded from the fit data prior to generation of particle size distributions.

RICS-derived diffusion coefficients were subsequently converted to particle diameter using the Stoke-Einstein equation (following determination of solvent viscosity):

$$D = {}^{kT}/_{3\pi\eta a}$$

Where *D* refers to the diffusion coefficient, *k* refers to the Boltzmann constant, *T* the temperature at which the measurements were performed, *η* solvent viscosity and *a* the hydrodynamic diameter.

RICS analysis was carried out by Maryam Hussain at the University of Manchester.

Circular dichroism

Far-UV circular dichroism (CD) was used to study the secondary structure of the protein before and after stress treatments. The measurements were performed with a Jasco J-815 CD spectrometer in combination with a Jasco PTC-423S temperature controller (Jasco International, Tokyo, Japan) at 25°C. The samples were measured in quartz cuvettes (Hellma GmbH) with a path length of 1 mm. CD spectra were collected in a continuous scanning mode from 190 to 260 nm. The measurements were performed at a scanning speed of 50 nm/min, a response time of 2 s, a bandwidth of 1 nm, a sensitivity of 100m, steps of 0.5 nm, and an accumulation of 6 scans. Data were analysed using the Spectra Analysis Software Dichroweb and reference data set SMP180 (Abdul-Gader et al. 2011). Data were transformed to mean residue ellipticity according to previously described equations using a mean amino acid residue weight of 113.

Transmission electron microscopy

Protein aggregate samples at 1 mg/ml were dialysed into 20mM Tris 150mM NaCl pH 7 prior to analysis. TEM analysis was carried out by the Electron Microscopy Core

Facility at the University of Manchester. Samples were assessed using a Tecnai 10 transmission electron microscope (TEM) operated at 100 keV using a reported protocol (Smith et al., 2008). To prepare samples, carbon-coated copper grids (No. 400) were glow discharged for 5 s and placed shiny side down onto a 10 µl droplet of aggregate for <5 s. Loaded grids were immediately placed on a 10 µl droplet of dH_2O for 60 s, and then stained with a 10 µl droplet of 4% (w/v) uranyl acetate for 60 s and blotted. Data were recorded onto Kodak SO-163 films and images were subsequently scanned using a UMAX2000 transmission scanner providing a specimen level increment of 3.66 Å/pixel. A crystallographic analysis suite (CRISP (Hovmoller)) was used to process and select a variety of scanned images with aggregates of different lengths/widths. Selected aggregates were CTF (contrast transfer function) corrected and fast Fourier transform (FFT) analysis was performed on cropped areas of 1024 × 1024 pixel boxes.

Atomic force microscopy

Atomic force microscopy (AFM) was used to image aggregates. AFM analysis was carried out by the Bioimaging Core Facility at the University of Manchester. Samples were dialysed after aggregation into Dulbeccos PBS diluted 1 in 30 in 0.2 µm filtered water. 5 µl of the sample solution was deposited onto freshly cleaved V1 mica and allowed to dry at room temperature. The sample was imaged using a 2nd generation Multimode-Picoforce AFM (Bruker, Billerica, MA, USA) by tapping mode, in air, with silicon RTESP AFM probes (Bruker) oscillated at 300KHz and with a scan speed of 0.5Hz. The AFM software used was Nanoscope v7.3.

2.5 Results

In order to determine the mass of scFv from elution peaks observed on SEC, separate protein peaks from SEC were subjected to SEC-MALLS analysis (SEC coupled to multi angle laser light scattering). The higher Mw peaks, referred to as Fraction A, were pooled. The monomeric peak, referred to as Fraction M, was analysed separately.

Three separate populations were identified by SEC-MALLS analysis of fraction A, the mean Mw for each peak was as follows: (1) 110 KDa (2) 81 kDa (3) 53 kDa (Fig 1A). Thus, fraction A contained peaks of material which eluted at positions consistent with being a dimer, trimer and tetramer of 26.7 kDa scFv; hence it is a mixture of these species. Fraction M from SEC (Fig 2.1B) eluted in a single peak on SEC-MALLS and is consistent with it being exclusively monomeric. For further experiments, as a starting point for the formation of aggregates, monomeric scFv was used exclusively.

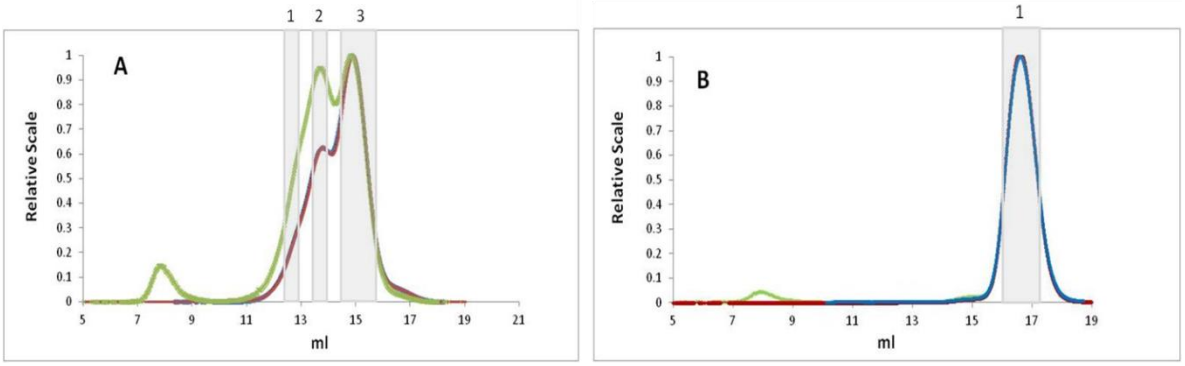

Figure 2.1. scFv SEC-MALLS Higher molecular weight (fraction A) and monomeric (fraction M) elution scFv fractions obtained from SEC were pooled and run on SEC-MALLS. On each graph, light scattering (LS; green), UV absorbance at 280 nm (UV; red) and refractive index (RI; blue) are plotted. Approximate fractions where molecular masses within peaks have been identified are highlighted in grey boxes; **A)** Fraction A (1, 2 and 3) **B)** Fraction M (1).

Static light scattering (SLS) was used to analyse the aggregation behaviour of monomeric scFv with increasing temperature. The AvactaOptim® 1000 system measures SLS and trytophan (Trp) fluorescence as a function of temperature. This can detect aggregation as SLS measures mean solute mass and Trp fluorescence provides an indication of conformational state. A temperature ramp was applied to scFv to monitor conformational stability with increasing temperature from 30 to 85°C. Analysis of the SLS data revealed a change in colloidal stability, i.e. the ability of particles in solution to resist aggregation, at approximately 39°C, and a loss of conformational stability between 40-45°C (Fig 2.2).

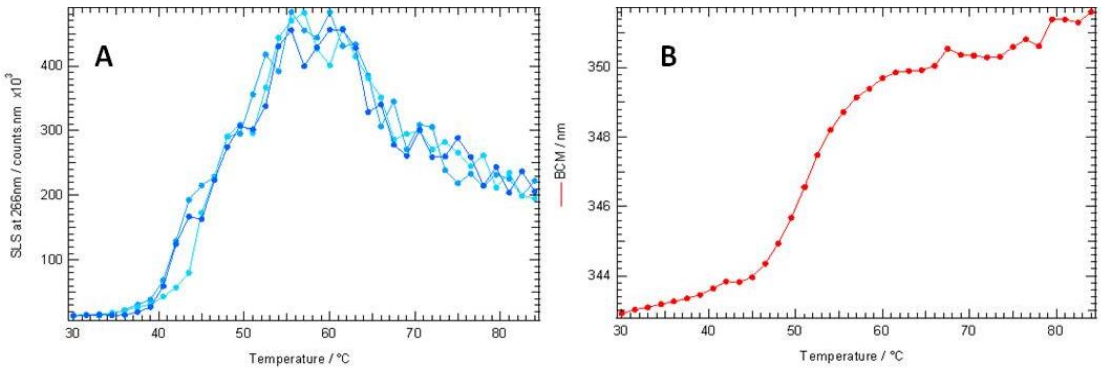

Figure 2.2. SLS temperature ramp analysis of scFv. Monomeric scFv at 1 mg/ml in PBS pH 7 was subjected to temperature ramp analysis with static light scattering (SLS) from 30 to 85°C. **A)** Graph of colloidal stability and aggregation. SLS at 266nm is plotted against temperature. The three lines represent 3 independent repeat samples. **B)** Graph of intrinsic fluorescence conformational stability. Barycentric mean (BCM), a value of protein fluorescence, is plotted against temperature for a single representative run.

The change in light scattering with temperature was mirrored by a change in intrinsic fluorescence. Since the T_{agg} for scFv was shown by SLS to be between 39 and 40°C, in order to aggregate the scFv with gentle heat stress, a 1mg/ml preparation was incubated at 40°C for 25 min. To aggregate the scFv by mechanical stress, a 1 mg/ml preparation was stirred for 6 h using a magnetic flea. In addition to the scFv, OVA

and mouse albumin were subjected to various thermal and mechanical stresses in order to optimise conditions for reproducible aggregation for each of the materials. Dynamic light scattering (DLS) was used to analyse the mean protein particle diameter before and after stress treatments. The lowest temperature and stirring time to produce reproducible aggregates within the subvisible size range was investigated and associated DLS data are presented in Figure 2.3.

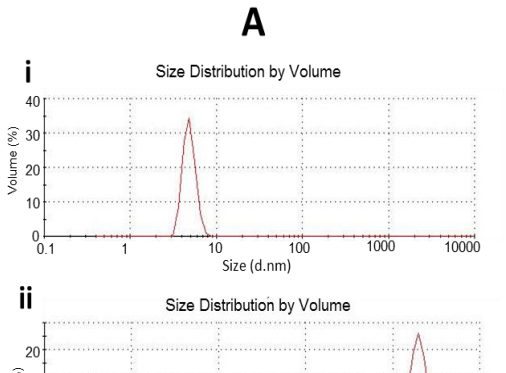

Figure 2.3. Mean protein particle diameter analysis by DLS. scFv OVA and mouse albumin were subjected to thermal and mechanical stresses. Unstressed and stressed samples were analysed by DLS. Mean protein particle diameter by volume percentage is presented for each sample. Single representative DLS plots are presented. **A) scFv** i) Monomer ii) scFv at 1 mg/ml incubated at 40°C for 25 min iii) scFv at 1 mg/ml stirred in a volume of 1 ml for 6 h. **B) OVA** i) Monomer ii) OVA at 10 mg/ml heated at 80°C for 1.5 h then diluted to 1 mg/ml iii) OVA subjected to stir stress at 1mg/ml in a volume of 1ml for

26h. **C) MSA** i) Monomer ii) MSA at 5 mg/ml heated at 60°C for 24 h, then diluted to 1 mg/ml in PBS. iii) MSA subjected to stir stress at 1mg/ml in a volume of 1ml for 1 week.

Heating of the scFv at 40°C resulted in the production of a homogenous aggregate population of ~2 µm. scFv samples were stirred in a volume of 1 or 2 ml with micro-stirrer bars for 6 h at room temperature. Stirring in a 2 ml volume in a 7ml Bijou resulted in a much lesser extent of aggregation, with the majority remaining monomeric (data not shown). Stirring in a volume of 1 ml in a 7 ml Bijou, however, resulted in a reproducible increase in size from 7 nm to ~2 µm, similar to the heat stress condition (Fig. 2.3A).

In order to aggregate OVA, a higher temperature and longer stirring time were required when compared with the scFv. Heating at 80°C for 1.5 h, and stirring for over 24 h resulted in reproducible aggregate profiles. Stir aggregates of OVA were less homogenous than those observed with the scFv preparation. The mean diameters as a percentage of volume were: 80% ~0.8 µm, 10% ~0.1 µm and 10% ~5 µm (Fig. 2.3B). MSA was very difficult to aggregate with thermal and mechanical stress. Stirring for up to a week still resulted in the majority of protein remaining monomeric. Heat stress of a 5 mg/ml solution at 60°C for 24 h did result in aggregation. However, these aggregates were much smaller than scFv and OVA aggregates at ~50 nm in size (Fig. 2.3C). DLS analysis showed identical size distribution profiles before and after two freeze-thaw cycles of all of the monomeric and aggregated protein preparations (data not shown).

Aggregated preparations of scFv, OVA and MSA (heat and stir stressed scFv, stir stressed OVA and heat stressed MSA) were subjected to analyses with Raster Image Correlation spectroscopy (RICS) for particle size-quantitation to complement the analysis carried out with DLS in Figure 3. Although DLS indicated that scFv heat and stir stressed aggregates were homogenous in size, RICS analysis showed the

majority of the protein to be within the 0.1-2 µm size range (Fig. 2.4), but with smaller and larger aggregates also present in lower amounts. Analysis of OVA stir aggregates was roughly in keeping with the DLS data, which showed a heterogeneous population with the majority at ~800 nm. Similarly, the measurements made with MSA mostly agreed with DLS data, with some larger aggregates also detected.

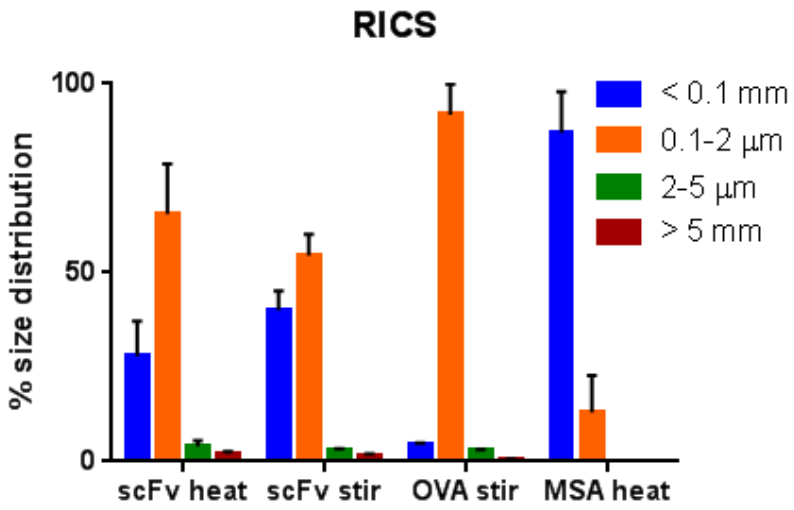

Figure 2.4. Size analysis of scFv, OVA and mouse albumin aggregates by RICS. Stressed protein samples were labelled with SYPRO red and size distribution obtained from RICS measurements of 1 mg/ml aggregate samples. Particles per femtolitre within each size distribution were measured. Size distributions are represented as a percentage of the total particles detected for each sample. Mean values for 3 separate RICS readings ±SEM are presented for each sample. scFv heat: 1 mg/ml scFv at 40°C for 25 min. scFv stir: 1 mg/ml scFv stirred for 6 hours at room temperature. OVA stir: 1 mg/ml OVA stirred for 26 h at room temperature. MSA heat: 1 mg/ml MSA heated at 5 mg/ml at 60°C for 24 h, then diluted to 1 mg/ml.

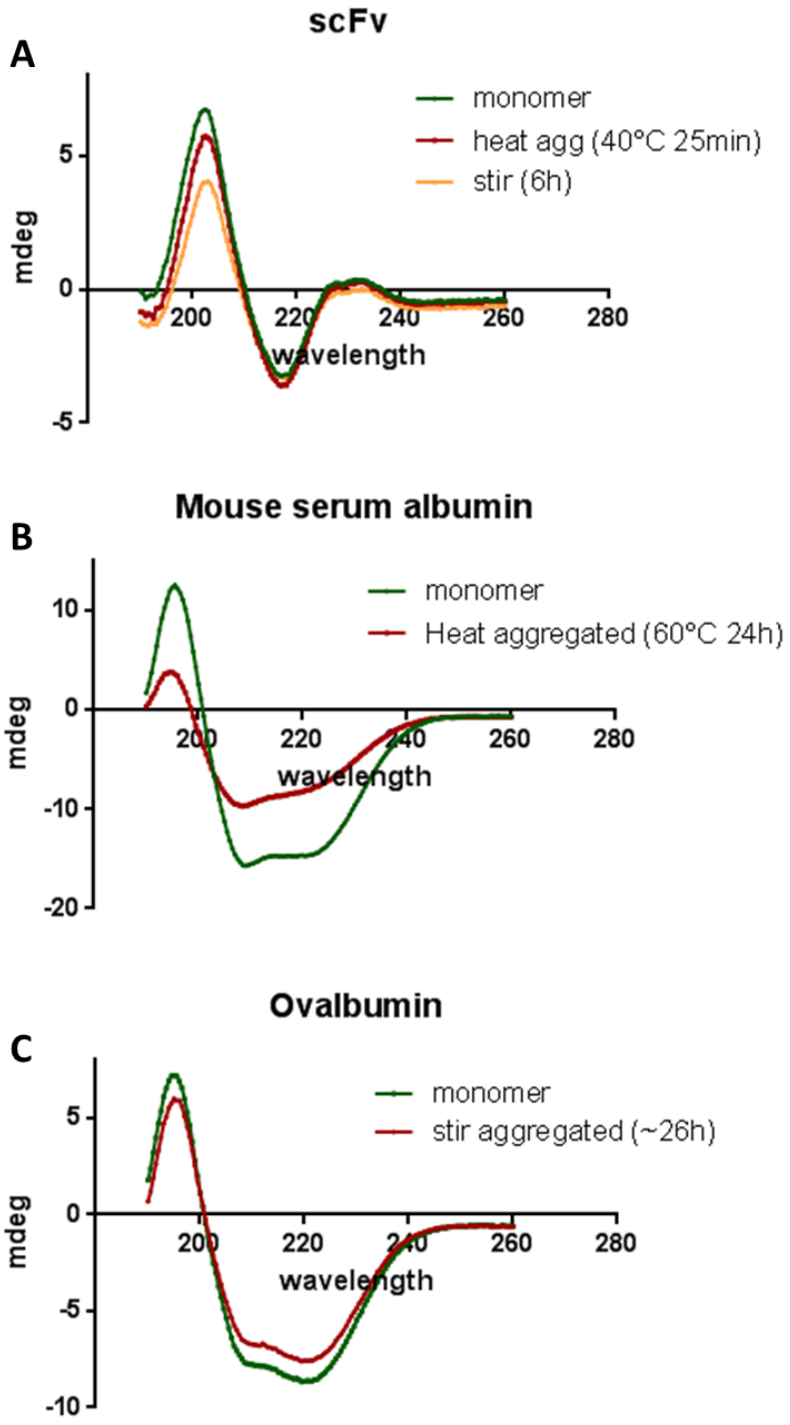

Figure 2.5.

Secondary structure analysis by CD. Monomeric and aggregated preparations of scFv, OVA and MSA were analysed separately by CD. Far-UV CD spectra at a wavelength of 190-260 nm are presented for each protein. A) scFv monomer (green); 1 mg/ml scFv heated at 40°C for 25 min (red) 1 mg/ml scFv stirred for 6 h (yellow) B) MSA monomer (green); MSA heated at 5 mg/ml at 60°C for 24 h, then diluted to 1 mg/ml (red) C) OVA monomer (green); OVA stirred for 26 h at room temperature (red).

Thermal and mechanical stresses can result in partial unfolding, allowing self-association of protein molecules. Circular dichroism (CD) was therefore utilised to analyse the secondary structure of aggregates compared to monomers and to look more closely at the extent of protein unfolding in each case. The CD spectra obtained for each protein are presented in separate graphs for a comparison of monomeric and aggregated preparations (Fig. 2.5).

Circular dichroism analysis of the scFv indicated a high β sheet content, which was slightly reduced with both thermal and stir stresses (β-sheet structure is indicated on Fig 2.5A by the peaks at a wavelength of ~200). The monomer contained 73% β sheet, whereas heat and stir stressed aggregates contained 71% and 65% respectively. Stir aggregation of OVA resulted in a slight reduction in α-helix content (α-helix structure is indicated on Fig 2.5B/C by the peaks between wavelengths 200-240), from 48% to 44%. MSA was subjected to heat stress (60°C) that was considerably harsher than the conditions used for aggregating the scFv and OVA preparations; this resulted in a more marked protein unfolding whereby the alpha helix content of the monomer was reduced from 72% to 45% after heat stress.

Techniques discussed so far have addressed protein particle size and secondary structure. In order to visualise the macromolecular structure of aggregates, transmission electron microscopy (TEM) was employed.

TEM analysis was carried out on both scFv aggregates (heat and stir stressed) and OVA stir stressed aggregates. Samples were negatively stained with uranyl acetate for imaging by TEM. Unfortunately, aggregate images could not be acquired for the scFv samples. Images from the OVA stir aggregate sample were acquired and are presented in Figure 2.6.

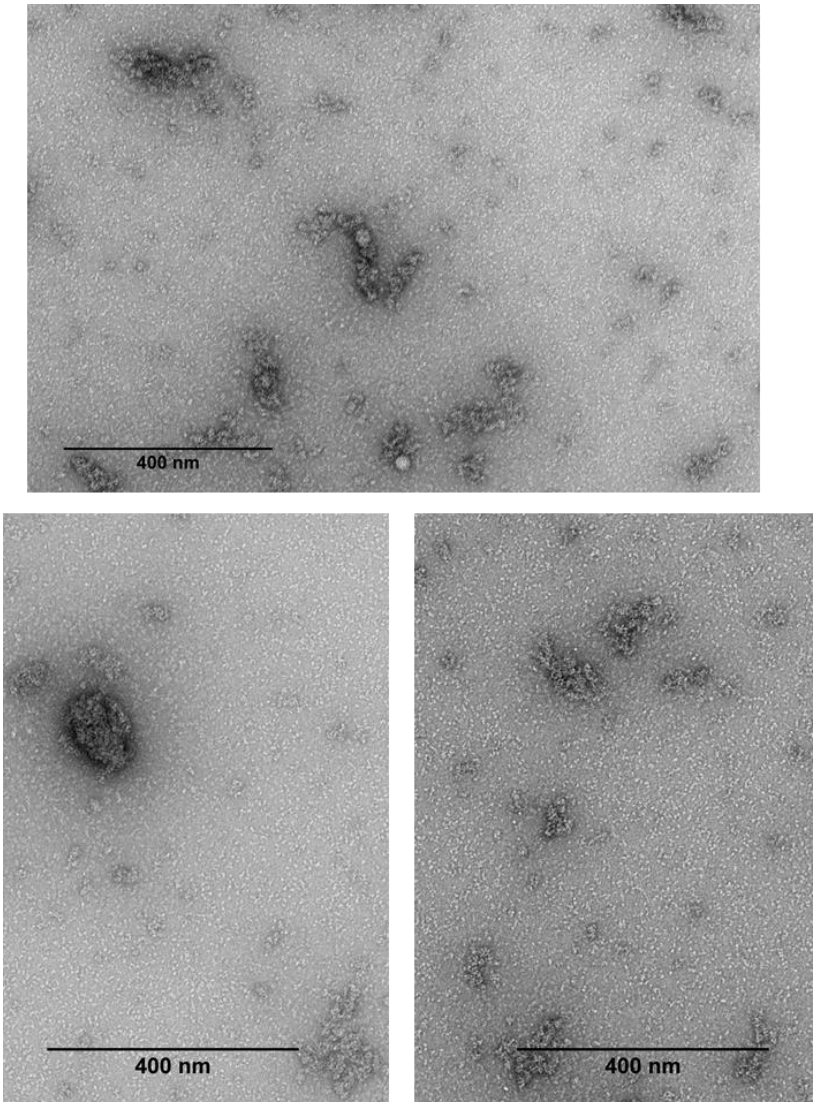

Figure 2.6. TEM analysis of OVA stir aggregates. OVA was stirred for 26 h at room temperature then dialysed into 20mM Tris 150mM NaCl. Three images of OVA stir aggregates are presented after negative staining with 4% uranyl acetate and TEM analysis. Scale bar = 400 nm.

The results revealed aggregates that were approximately 200 nm in size or smaller. Larger OVA aggregates, that were detected using DLS and RICS, were not visualised using this method. Atomic force microscopy (AFM) was also utilised in another attempt to visualise scFv, OVA and MSA aggregates. Aggregates were dialysed into a lower salt concentration (PBS diluted 1 in 30) in an effort to minimise salt peaks when samples were dried onto the mica surface. When aggregates were dialysed into pure water with no salt, the aggregate profiles by DLS were altered, so this was felt

to be inappropriate for imaging the aggregates. Unfortunately, even the lower salt concentration resulted in salt crystals that interfered with imaging of the protein aggregates, thus it was not possible to visualise the proteins. A representative image of a salt crystal, taken from the OVA stir aggregate sample, is shown in Figure 2.7.

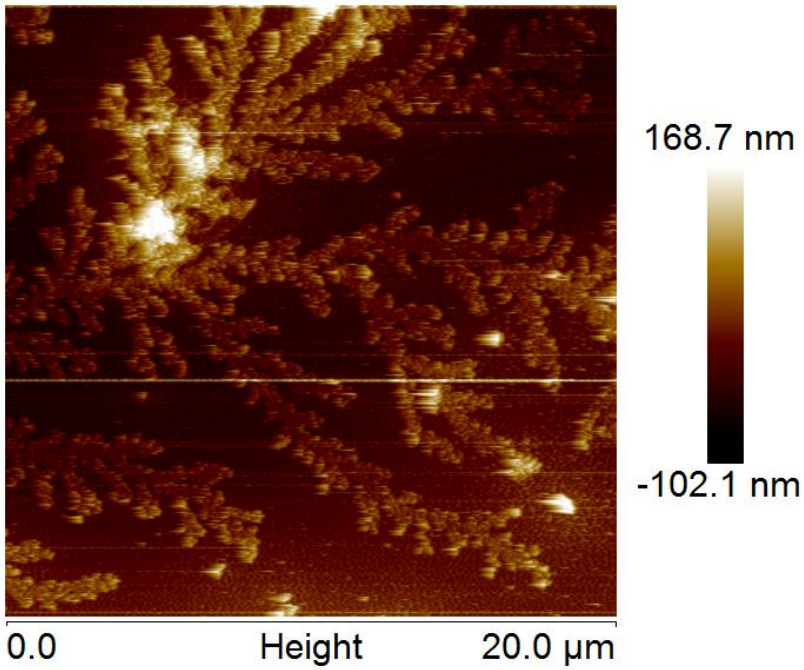

Figure 2.7. Aggregate imaging by AFM. An OVA stir aggregated sample was dialysed into PBS diluted in filtered water 1 in 30. A 5 µl sample was deposited onto a freshly cleaved mica surface and air dried at room temperature. The sample was imaged by tapping mode in air using a silicone RTESP probe, oscillated at 300 KHz.

2.6 Discussion

Due to the potential impact of aggregation on protein immunogenicity, there has been an increased focus on detecting and characterising protein aggregates present in biopharmaceutical preparations. There are a wide range of technologies available for the biophysical analysis of proteins, and these can provide information about the conformational stability or structure of protein molecules (Amin et al. 2014). There is no standard method or set of methods that is used routinely for the characterisation of biotherapeutic aggregates, as the information that they provide can differ, and there are limitations associated with many of them. The orthogonal nature of analytical techniques also means that comparisons between data sets from different methods can be difficult.

In these experiments one aim was to determine stress conditions required to produce subvisible aggregate preparations to be used in future immunogenicity experiments. Another aim was to test a selection of biophysical techniques to examine the conformational stability, protein particle size and structure of stressed protein preparations. Different stress types are known to give rise to distinct aggregation mechanisms and aggregates of different sizes and morphologies (Zhang et al. 2012), therefore both thermal and mechanical stresses were employed in order to provide a range of aggregates for assessment.

A scFv antibody fragment was selected for initial experiments, as this format has relevance for the biopharmaceutical industry with the emergence on the market of novel formats and antibody fragments. Purification of scFv using anion exchange and Protein A chromatography resulted in two separate populations when run on SEC, these were designated fraction A and fraction M. Fraction A and M were identified as oligomeric and monomeric forms of the scFv, respectively, using the SEC-MALLS technique. While the SEC-MALLS technique was only used after purification and not following thermal or mechanical stress, it identified protein association at the early

stages of aggregation, with oligomerisation of scFv molecules. This oligomerisation may be explained by partial unfolding caused by low pH stress (pH 3.5) during the elution step of protein A chromatography (Shukla et al. 2007).

A temperature ramp experiment with SLS was used to determine the temperature of aggregation (T_{agg}) of the scFv, as it is well known that structural changes due to thermal stress can cause protein aggregation (Chi et al., 2003). SLS data showed the T_{agg} to be between 39 and 40°C. Based on these results, scFv was heated for 25 min at 40°C and DLS was used to analyse the size distribution of protein particles. A reproducible aggregate population with a mean diameter of ~2 µm was formed; this size profile was satisfactory for studies examining the effect of subvisible particle size on immunogenicity, especially since aggregates form a heterogeneous population that can be comprised of protein particles that cover a wide size range (Das 2012).

Mechanical stress was also tested, as this may be more relevant to some stresses encountered during bioprocessing, thus scFv preparations were subjected to stirring stress. Stirring in a 2 ml volume resulted in a small amount of aggregation, with the majority remaining monomeric, however, when the volume was reduced to 1 ml, all protein was aggregated when analysed by DLS. This difference in protein particle size may be explained by different levels of stress encountered; in a volume of 1 ml the stirrer was close to the solution meniscus, creating surface tension with stirring, whereas this stress was not present in a volume of 2 ml as the stirrer was farther away from the meniscus when using the same vessel. It is known that protein stability and aggregation can be affected by changes in surface shear rheological properties (Hirano et al. 2007; Dickinson. 1999). Thus in all subsequent experiments this parameter was kept constant.

OVA and MSA were selected as additional reference proteins for immunogenicity studies, and subjected to thermal and mechanical stresses alongside the scFv. DLS

was used to examine the size distribution of protein particles in solution. OVA reproducibly formed subvisible aggregates, (majority ~800 nm in diameter) when heated at 80°C at a concentration of 10 mg/ml. The higher temperature and protein concentration required for aggregation illustrated the increased protein stability of OVA compared to the scFv. (see Supplementary Chapter, Fig. 5.8 for antibody binding data against OVA heat aggregate vs monomer) Stirring stress for a longer period of time was also required to aggregate OVA; 26 h stirring resulted in a heterogeneous subvisible aggregate profile. MSA aggregated with 60°C heat treatment at a 5 mg/ml concentration. However, the aggregates formed were considerably smaller than scFv and OVA aggregates, with a homogenous peak at ~ 50 nm when analysed by DLS. Additionally, MSA remained monomeric (97% monomer) after stir stress for up to 1 week using conditions whereby the scFv would form aggregates within 6 h. Similar to OVA, these results indicated increased protein stability of mouse albumin in comparison with the scFv. This is not surprising as albumin proteins share a globular structure (Singh 2007).

Freeze thaw stress did not alter the aggregate or monomeric populations of scFv, OVA and MSA, as DLS analysis showed identical profiles before and after two freeze-thaw cycles. This is important, as freeze thaw stress can induce aggregation, and may have altered the aggregate profile, or induced aggregation of the monomer (Kreilgaard et al. 1998).

Raster image correlation spectroscopy (RICS) capitalises on the natural scanning pattern of a confocal microscope, where pixels are separated on a µsec time scale. Correlations of fluorescence intensity fluctuations can yield concentration and size information with the RICS technique. The use of RICS for measuring size distribution has been previously investigated, and shown to be capable of characterising the size of protein aggregates over a broad size range (10 nm to ~100 µm) (Hamrang et al. 2015). However, the capability of RICS to accurately size particles in overcrowded

and polydisperse samples is not yet known. In these experiments RICS analysis of scFv, OVA and MSA aggregates was used to complement the size analysis carried out with DLS. The concentration in particles per femtolitre was measured within specific size ranges from < 0.1 µm to > 5 µm. Results from RICS were generally in agreement with the DLS data, although aggregate profiles that appeared homogenous by DLS (forming a single peak), were shown to be more heterogeneous with the RICS analysis. For example, scFv heat and stir aggregates both appeared as homogenous peaks on DLS at ~2 µm in size, but by RICS analysis these preparations contained up to 40% of particles sized <0.1 µm and some aggregates (<5%) larger than 2 µm. Since RICS and DLS are orthogonal techniques, slight differences in results were expected. For each protein, the size-range with the largest protein concentration detected by RICS was in accordance with the aggregate size determined by DLS.

Secondary structure cannot be directly studied using the protein particle-sizing methods. Circular dichroism (CD) is a technique that allows protein folding and secondary structure to be estimated (Greenfield 2006), so monomeric and aggregated preparations were compared using this method. Modest differences in secondary structure were identified between monomeric and aggregated scFv, indicating that heat and stir aggregates still retained secondary structure, with only slight unfolding. A difference in CD spectra between monomeric and aggregated scFv preparations suggested a decrease in the β-sheet conformation following both heat and stir stress. OVA and MSA are both globular proteins with α-helical structures. When OVA monomer and stir stressed samples were compared, a modest change in CD spectra was observed with a 4% decrease in α-helix structure following stir stress. MSA, however, exhibited a pronounced change in CD spectra following heat stress; here a decrease from 72% α-helix structure with the monomer was reduced to 45%

after heat stress. This change is not surprising given the harsh stress treatment that it was necessary to subject MSA to in order to achieve aggregation.

Advanced microscopy techniques can be applied for the imaging of protein aggregates. In these experiments transmission electron microscopy (TEM) and atomic force microscopy (AFM) were both tested for this purpose. TEM was used to image scFv heat and stir aggregates, and OVA stir aggregates. The scFv aggregate preparations could not be imaged using TEM, this may be due to the fact that during sample processing the protein preparations were subjected to negative staining; Negative staining requires drying of the sample which can alter the appearance of protein particles, or even lead to the collapse of protein assemblies (De Carlo and Harris 2011). The stir stressed OVA preparation could be imaged, although the aggregates observed were ~200 nm in size and below. Analysis by DLS showed 10% of the OVA aggregates to be 100-200 nm in size, which is in keeping with the aggregates imaged by TEM. However larger aggregates detected by DLS were not observed with TEM. It is possible that sample preparation with negative staining also affected the larger aggregate structures of OVA, causing them to dissociate or collapse. Additionally, uranyl acetate used for negative staining is acidic, which can cause artefacts. Cryo-EM offers the possibility to examine aggregates without such distortions, and could be tested as an alternative method in future experiments (Thompson et al. 2016).

Aggregate preparations of the scFv, OVA and MSA were then imaged using AFM. In order to perform AFM, samples were dialysed into a lower salt concentration (PBS diluted 1 in 30). Samples were then air-dried onto a mica surface for imaging by tapping mode in air. In these experiments AFM was carried out in dry non-physiological conditions, one disadvantage of this is that any salt present in solution can form salt crystals and disturb the aggregate images. Unfortunately, the lower salt buffer that samples were dialysed into still resulted in salt peaks when samples were

air-dried, such that images of protein aggregates could not be attained. Brighter areas on the AFM image in Figure 7 show more raised areas on the salt crystal. It is possible that during the drying process salt peaks formed around protein aggregates, which acted as a nucleation point for salt crystallisation. Therefore, it is possible that the bright spots could indicate the presence of protein aggregates, with salt crystals over the top. It is possible to carry out AFM with the sample in solution, in 'wet mode' for a more physiologically relevant condition (Gaczynska and Osmulski 2008). For further experiments it would be interesting to use this method to see if subvisible protein aggregates could be visualised without salt crystal interference.

Biophysical analyses have been carried out on aggregate preparations that have been used in published immunogenicity studies. These can be compared to the subvisible aggregate preparations described herein. For example, Joubert *et al* (2012) stressed human IgG mAbs using thermal, stir and syringe stresses; a temperature of 65°C for 1 h was used for thermal stress, and stirring for 20 h – 3 days for stir stress. Aggregate particle size/number were measured using light obscuration methods; the stresses each resulted in heterogeneous aggregate populations with particles ranging in size from <2 µm to > 10 µm. Bessa et al (2015) reported partial aggregation of a humanised mAb following light, pH or process-related stresses; here oligomers to higher molecular weight aggregates were detected using light obscuration and resonant mass measurements, but only a low residual content of subvisible particles was detected. Furthermore, thermal (37°C incubation for 1 week) and oxidative stresses resulted in submicron and micron sized aggregates in IFN-1β preparations, these were analysed using DLS, fluid imaging microscopy and resonant mass measurements (Abdolvahab et al. 2016). Published studies highlight the range of stresses that can aggregate proteins, and the need to monitor a wide size range in biopharmaceutical preparations.

In conclusion, aggregated protein preparations for use in immunogenicity studies have been characterised in these investigations using a selection of biophysical techniques. Different mechanical and thermal stress conditions were required to produce subvisible aggregates of each of the three test proteins, illustrating that individual proteins respond differently to external stresses. Furthermore, mechanical and thermal stresses resulted in different aggregate profiles for the albumin proteins, but not for the scFv. The limitations associated with some protein aggregate characterisation techniques are highlighted by the data presented herein. For example, the size analysis by the orthogonal methods DLS and RICS yielded slightly different results with regard to particle size distributions. Additionally, microscopy techniques for imaging the aggregates required further optimisation in order to yield data for some preparations. Given the limitations of individual methods, the use of multiple techniques in conjunction can provide a more comprehensive analysis of aggregate biophysical properties.

2.7 Acknowledgements

We thank Dr James Austerberry for assisting with the CD analysis, Maryam Hussain for carrying out the RICS analysis, the Bioimaging Core Facility at the University of Manchester for assisting with the AFM measurements, and the Electron microscopy facility at the University of Manchester for carrying out the TEM experiments.

2.8 Appendix

The amino acid sequence for the humanised scFv is detailed below:

QEQLVESGGGLVQPGGSLRLSCAASGFDFSSHWIYWVRQAPGKGLEWVSTIYTG
SDSTYYATWAKGRFTISKDNSKNTVYLQMNSLRAEDTAVYYCARDLGGSSSTSYI
SDLWGQGTLVTVSSGGGGSGGGGSGGGGSELVLTQSPATLSLSPGERATLSCTL
SSAHKTYSIAWYQQKPGQAPRYLIQLKSDGSYTKGTGVPARFSGSSSGADRTLTIS
SLEPEDFAVYYCSTDYATGYYVFGQGTKVEIKR

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CHAPTER 3:

SUBVISIBLE AGGREGATES OF IMMUNOGENIC PROTEINS PROMOTE A TH1-TYPE RESPONSE

3 Paper 2: Subvisible aggregates of immunogenic proteins promote a Th1-type response

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Running title: Immunogenicity of subvisible aggregates

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3.1 Abstract

Protein aggregation is associated with enhanced immunogenicity of biotherapeutics. As a result, regulatory guidelines recommend screening for aggregation during bioprocessing. However, the mechanisms underlying the enhanced immunogenicity of aggregates are poorly understood. In the investigations described herein, the immunogenicity in mice of a humanized single chain variable antibody fragment (scFv) purified after expression in *Escherichia coli* has been examined. Reproducible scFv aggregates were obtained within the subvisible particle size range (mean diameter 2 μm) using thermal and mechanical stresses. Intraperitoneal immunization of BALB/c strain mice with 1 mg/ml of aggregated or monomeric scFv induced similar IgG and IgG1 antibody responses. In contrast, aggregate preparations stimulated significantly higher levels of anti-scFv IgG2a antibody than did the monomer. In comparative studies, aggregates of ovalbumin (OVA) within the subvisible particle size range were prepared by stir stress, and their immunogenicity compared with that of monomeric OVA in mice. Aggregated and monomeric OVA induced similar anti-OVA IgG and IgG1 antibody responses, whereas IgG2a antibody levels were significantly higher in aggregate-immunized mice. Furthermore, cytokine profiles in supernatants taken from splenocyte-dendritic cell co-cultures were consistent with aggregated preparations inducing a T helper (Th) 1-type response. Aggregated proteins within the subvisible range were therefore shown to induce a preferential Th1 type response, whereas monomeric proteins elicited a selective Th2 response. These data indicate that protein aggregation can impact on both the magnitude and character of immune responses.

Keywords: immunogenicity, biotherapeutic, protein aggregation, scFv, OVA.

3.2 Introduction

Unwanted immunogenicity of recombinant biotherapeutics has the potential to impact upon drug efficacy and patient safety. Even biotherapeutics consisting of completely human-derived sequences have the potential to elicit an immunogenic response in patients. For example, immunogenicity of Interferon-β (Ross et al. 2000) and Adalimumab (Bartelds et al. 2011) have resulted in compromised drug efficacy in the clinic. Eprex® (epoetin α) and thrombopoietin are examples of biotherapeutics that have resulted in adverse events in some patients, due to the development of neutralizing antibodies (Casadevall et al. 2002; Li et al. 2001). Eprex® is a well-documented example of immunogenicity and, while the causative factor has not been identified, various factors including micelle formation, and change of formulation, have been implicated (Schellekens and Jiskoot 2006).

Various treatment, patient and product-related factors may contribute to biotherapeutic immunogenicity (Singh 2011). Protein aggregation is a product-related factor that has the potential to enhance immunogenicity (Ratanji et al. 2014), and regulatory authorities therefore recommend screening for and minimising the presence of aggregates in parenteral biotherapeutic products (European Directorate for the Quality of Medicine [EDQM]. 2010; Food and Drug Administration [FDA], 2014).

Proteins are subject to many stresses at different stages in bioprocessing and manufacture, from protein expression in the host cell system to storage and handling. Physical and chemical stressors such as mechanical stress, pH, elevated temperature, high protein concentrations, and repeated freeze-thaw can all result in partial unfolding and aggregation (Frokjaer and Otzen 2005). Since the immunogenicity of proteins is confounded by a number of potential variables, including route of administration, formulation, product container and host cell protein

impurities, it can be challenging to determine the influence of aggregation *per se* on protein immunogenicity. Furthermore, protein aggregates can be diverse in their biophysical and biochemical characteristics. Aggregate size is one variable that could influence immunogenicity. Aggregates are known to range in size from oligomers of nanometer dimensions, to subvisible and visible particulates (Narhi et al. 2012), but it is not clear how such variations in size affect the immune response.

In recent years research into protein aggregate immunogenicity has increased. *In vitro* approaches using human peripheral blood mononuclear cells (Joubert et al. 2012; Telikepalli et al. 2015) or dendritic cells (DC) (Rombach-Riegraf et al. 2014) have had some success when used to screen aggregates for their potential to influence cytokine expression profiles and surface marker expression. However, although *in vitro* assays may be useful in screening for immunogenicity to select lead candidates, they do not provide a holistic appreciation of the immunogenicity of a product, nor do they necessarily facilitate characterisation of the relevant immunological mechanisms. In addition, *in vivo* approaches have been adopted to compare and contrast immune responses elicited by aggregated proteins and their monomeric counterparts. A common approach is to measure antibody responses provoked in mice by immunizations with the monomeric or aggregated protein (Freitag et al. 2015; Shomali et al. 2014). Furthermore, transgenic therapeutic-tolerant mouse models have been used to assess the ability of aggregates to break immunological tolerance (Bessa et al. 2015; Braun et al. 1997; Kijanka et al. 2015).

However, to our knowledge, previous investigations have not sought to evaluate the character as well as the intensity of induced immune responses *in vivo,* using both antibody isotyping and *ex vivo* cellular assays. Functional subpopulations of T helper (Th) cells provide a mechanism for the development of different qualities of immune responses, depending on the nature of the immune challenge. Th1 cells are responsible for cell-mediated immunity, whereas Th2 cells produce cytokines that

promote humoral responses (Abbas et al. 1996). A more comprehensive analysis of the character of induced immune responses (i.e. Th1 or Th2) could provide a better understanding of the fundamental mechanisms underpinning aggregate immunogenicity.

The aim of the investigations described here was to characterize the relationships between aspects of protein aggregation, including aggregate size, with the magnitude and character of induced immune responses in mice. The aim was not to develop a method for predicting the potential of a protein to provoke an immune response in humans, but rather to investigate the influence of aggregation on immune responses. For this purpose a humanized single chain variable antibody fragment (scFv) and ovalbumin (OVA) were selected as test proteins. In using proteins that are foreign to mice, a baseline level of immunogenicity was expected to be achieved with monomers, and changes to this baseline with aggregation were studied. For both proteins, preparations of protein aggregates within distinct size ranges were produced using thermal and mechanical stress and the immunogenicity profile assessed in BALB/c strain mice in terms of antibody production and cytokine expression, in comparison with their monomeric counterparts.

3.3 Materials and Methods

Single chain variable antibody fragment purification

A humanized single chain variable antibody fragment (scFv), as reported by (Edwardraja et al. 2010), was cloned into a pET-22b vector in Shuffle T7 express *Escherichia coli* (*E.coli)* cells (New England Biolabs*,* Beverly, MA*,* USA*)*. Transformants were cultured at 30°C to an optical density (OD) of 0.8 at 600 nm, induced with isopropyl β-D-1-thiogalactopyranoside and incubated overnight at 16°C. Cell pellets were resuspended, sonicated and centrifuged at 28,672 g for 30 min. scFv was purified from supernatants using DEAE (diethylaminoethanol) Sepharose anion exchange chromatography, followed by Protein A affinity and size exclusion chromatography.

Generation of aggregates

scFv: Purified monomeric scFv was diluted to 1 mg/ml in Dulbeccos phosphate buffered saline (PBS) without Ca^{+2} or Mg^{+2} (Sigma-Aldrich, St Louis, Missouri) and stressed by heating at 40°C for 25 min. To induce stir-stressed aggregates 1 ml of 1 mg/ml purified scFv was stirred with an 8x2 mm Teflon stirrer bar in a 5 ml glass tube for 6 h. *OVA:* Lyophilized OVA (Sigma-Aldrich) was diluted to 1 mg/ml in Dulbeccos PBS and stirred in a volume of 1ml in a 5 ml glass tube for 24-28 h. Dynamic light scattering (DLS) was used to monitor aggregation status and stirring stopped once the desired subvisible size range achieved.

Monomeric preparations were passed through a 0.2 µm filter (Millipore, Billerica, MA, USA) and centrifuged prior to use in immunisations or assays. These protein preparations were confirmed to be monomeric using DLS.

Endotoxin measurement

The endotoxin content of protein preparations was measured chromatographically by limulus amebocyte (LAL) assay according to the manufacturer's instructions (Cambrex BioSciences, Wokingham, UK). Endotoxin levels were <100EU/mg of protein; levels which have been shown previously to be without impact on *in vivo* antibody responses (Dearman and Kimber 2007).

Analysis of aggregates

Measurements of DLS were performed with a Malvern Zetasizer Nano ZS ZEN3600 (Malvern, Herrenberg, Germany) equipped with a 633 nm laser. Each sample (70 µl) was measured in a Suprasil® quartz cuvette (Hellma GmbH, Muellheim, Germany) with a path length of 3 mm and 200-2500 nm spectral range. Monomeric and stressed samples at 1 mg/ml were measured at 25°C to determine the volume-based average protein particle diameter in solution.

Animal experiments

Female BALB/c strain mice were used for these experiments (Envigo, Bicester, UK). All procedures were carried out in accordance with the Animals (Scientific Procedures) Act 1986, and approved by Home Office licence. Mice were immunized by intraperitoneal (ip) injection (or subcutaneous [sc] injection) with 250 µl of 1 or 0.1 mg/ml protein (monomeric or aggregate) in PBS on days 0 and 7 and exsanguinated on day 14. In some experiments mice received an additional immunization on day 14 and were terminated on day 21. Spleens and serum were isolated for evaluation.

Generation and culture of murine bone marrow derived DC

Murine bone marrow (BM) derived DC (BMDC) were generated using a previously described method (Lutz *et al.*, 1999). Briefly, BM was extracted by flushing the femurs and tibias with PBS. The cell suspension was centrifuged at 112 g for 5 min. The pellet was then resuspended in warmed culture medium (RPMI 1640, GIBCO, Paisley, UK), supplemented with 10% fetal calf serum (FCS) (PAA laboratories,

GmbH, Austria) containing 400 µg/ml penicillin/streptomycin, 292 µg/ml L-glutamine, 0.05 mM 2-mercaptoethanol and 20 ng/ml granulocyte macrophage-colony stimulating factor (GM-CSF) (Miltenyi Biotech, Bisley, UK). Viable cell counts were performed by trypan blue exclusion (0.5%, Sigma-Aldrich). Cells were cultured at approximately $2x10^6$ cells per 100 X 15mm petri dish at 37°C in a humidified atmosphere of 5% CO_2 in air. Medium was refreshed every 3 days, and cells harvested by gentle agitation. BMDC were used in co-culture with splenocytes on day 7.

Splenocyte-BMDC co-culture

Single cell suspensions of splenocytes were prepared by mechanical disaggregation. Red blood cells were lysed by incubation in 0.85% ammonium chloride for 3 min. Splenocytes were washed and resuspended in culture medium, supplemented with 10% FCS containing 400 µg/ml penicillin/streptomycin, 292 µg/ml L-glutamine and cultured at 3×10^5 cells per well in 96 well round bottomed tissue culture plates, alone or in co-culture with 3×10^4 BMDC per well. Triplicate wells per individual mouse were cultured with monomeric or aggregated protein at 100 µg/ml, or with the T cell mitogen Concanavalin A (conA; Sigma-Aldrich) as a positive control at 2 µg/ml, or with an equal volume of medium alone for negative control wells and cultured at 37^{o}C in a humidified atmosphere of 5% CO_2 for 72 to 144h. Supernatants were harvested by centrifugation.

^{3}H-Thymidine incorporation assay: In parallel, aliquots of cells were pulsed with 0.2 MBq ^{3}H- thymidine (^{3}HTdR) (PerkinElmer, Waltham, MA, USA) per well 24 h before harvesting. Cells were harvested onto glass fibre filter mats with a multichannel semi-automated harvesting device (Titertek, Skatron AS, Lierbyen, Norway). Incorporation of ^{3}HTdR was measured as disintegrations per minute (dpm) in a liquid scintillation cocktail (Fisher Scientific, Loughborough, UK). Standard error of mean (SEM) was calculated from averages of three replicate wells.

Cytokine enzyme-linked immunosorbent assay (ELISA): Interferon-γ, Interleukin-13 and Interleukin-4

Splenocyte culture supernatants were tested for Interferon-γ (IFNγ), Interleukin-13 (IL-13) and Interleukin-4 (IL-4) protein using specific ELISA Duosets from R&D Systems. (R&D Systems, Minneapolis, US). The lower limits of accurate detection were: 31.25 pg/ml for IFNγ, 62.5 pg/ml for IL-13 and 15.6 pg/ml for IL-4. ELISAs were performed following the manufacturer's instructions.

ELISA for protein-specific antibody classes and subclasses

Plastic Maxisorb® plates (Nunc, Copenhagen, Denmark) were coated with 10 µg/ml of protein in PBS overnight at 4°C. Plates were blocked with 2% bovine serum albumin (BSA)/PBS (Sigma Aldrich) at 37°C for 30 min. Doubling dilutions of serum samples were added (starting dilution 1 in 32 or 1in 64 for anti-IgG; 1 in 128 or 1in 64 for anti IgM antibody analyses) in 1% BSA/ PBS (as a negative control naïve mouse serum [NMS] samples were added to plates) and incubated for 3 h at 4°C. Plates were incubated for 2 h at 4°C with horseradish peroxidise (HRP) labelled sheep anti-mouse IgG diluted 1 in 4000 (Cat no: AAC10P), goat anti-mouse IgG1 diluted 1: 2000 (Cat no: STAR132P), goat anti-mouse IgG2a diluted 1: 1000 (Cat no: STAR133P) (all Serotec, Oxfordshire) or goat anti-mouse IgM diluted 1: 6000 (Cat no: 62-6820; Invitrogen, Paisley, UK). Plates were washed between incubations with 0.05% Tween 20 in PBS. Plates were incubated with substrate *o*-phenylenediamine (OPD) and urea hydrogen peroxide for 15 min and reactions were stopped with 0.5M citric acid. Absorbance was read at 450 nm using an automated reader (Multiscan, Flow Laboratories, Irvine, Ayrshire, UK). Data are displayed as OD450 nm values and mean antibody titers. Titer was calculated as the maximum dilution of serum at which an OD450 reading of 0.3 or above is recorded (3 times reagent blank [all reagents except for serum] reading of 0.1). If an OD 450 nm reading of 3 times the reagent

blank at the highest serum concentration was not achieved, a nominal titer value of 16 was assigned.

Statistical analyses

Statistical analyses were performed using the software Graphpad Prism 6. Analysis of variance (ANOVA) was used to determine statistical significance of differences between groups. Experiments were analyzed by non-parametric one way or two way ANOVA followed by the Tukey post hoc test (*$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$).

3.4 Results

Monomeric and heat stressed aggregates of scFv induce differential antibody responses

To produce aggregates by heat stress, 1 mg/ml scFv in PBS was incubated at 40°C for 25 min and DLS was employed to observe changes in mean particle diameter. A reproducible increase in size from 7 nm to 1000-3000 nm was observed (Fig. 1A). Aggregate and monomer preparations were also shown to be stable by DLS following two freeze/thaw cycles. Initial experiments with the scFv monomer demonstrated that a concentration of 0.1 mg/ml resulted in a weak or undetectable IgG antibody response in some animals. A robust, detectable response was required in these experiments for comparison to the aggregated protein (data not shown). A 1 mg/ml dose was found to provide a consistent antibody response, so for all further experiments this concentration was used.

Preparations of monomer and aggregate protein (1 mg/ml) were administered via ip injection to mice on days 0 and 7 (two independent experiments; n=5 and n=3 per group) and sera isolated on day 14. To compare immune responses induced by monomer and aggregate, the presence of anti-scFv IgG, IgG1, IgG2a and IgM antibody in serum samples was analyzed by ELISA (Fig 3.1B).

Analysis of the serum dilution curves and antibody titers revealed that there were no false positive IgG, IgG1 or IgG2a antibody readouts for scFv in the negative control naïve mouse serum samples, regardless of whether aggregated or monomeric protein was used as the substrate (Fig 3.1B). Although comparatively low levels of IgM antibody were apparently present in naïve sera, this was due to non-specific binding that was also observed with BSA and OVA substrates (data not shown). Relatively high level expression levels of anti-scFv IgG, IgG1 and IgM antibodies were found in sera isolated from monomer or aggregate immunized mice, with virtually

identical titration curves irrespective of whether immunization was with the monomer or the aggregated form, or whether the substrate was monomeric or aggregated. In contrast, only immunization with the aggregated form of scFv resulted in a high level of IgG2a antibody production. Further investigations revealed that this was a robust and reproducible finding, with this same pattern observed in each of the two independent experiments. Thus, in each experiment, equivalent anti-scFv IgG, IgG1 and IgM antibody titers were recorded following immunization with monomer or aggregated protein, and identical titers were observed regardless of which material was used as substrate in the ELISA. However, a significantly higher titer IgG2a antibody response was recorded in sera from aggregate compared with monomer immunized mice (*$p<0.05$).

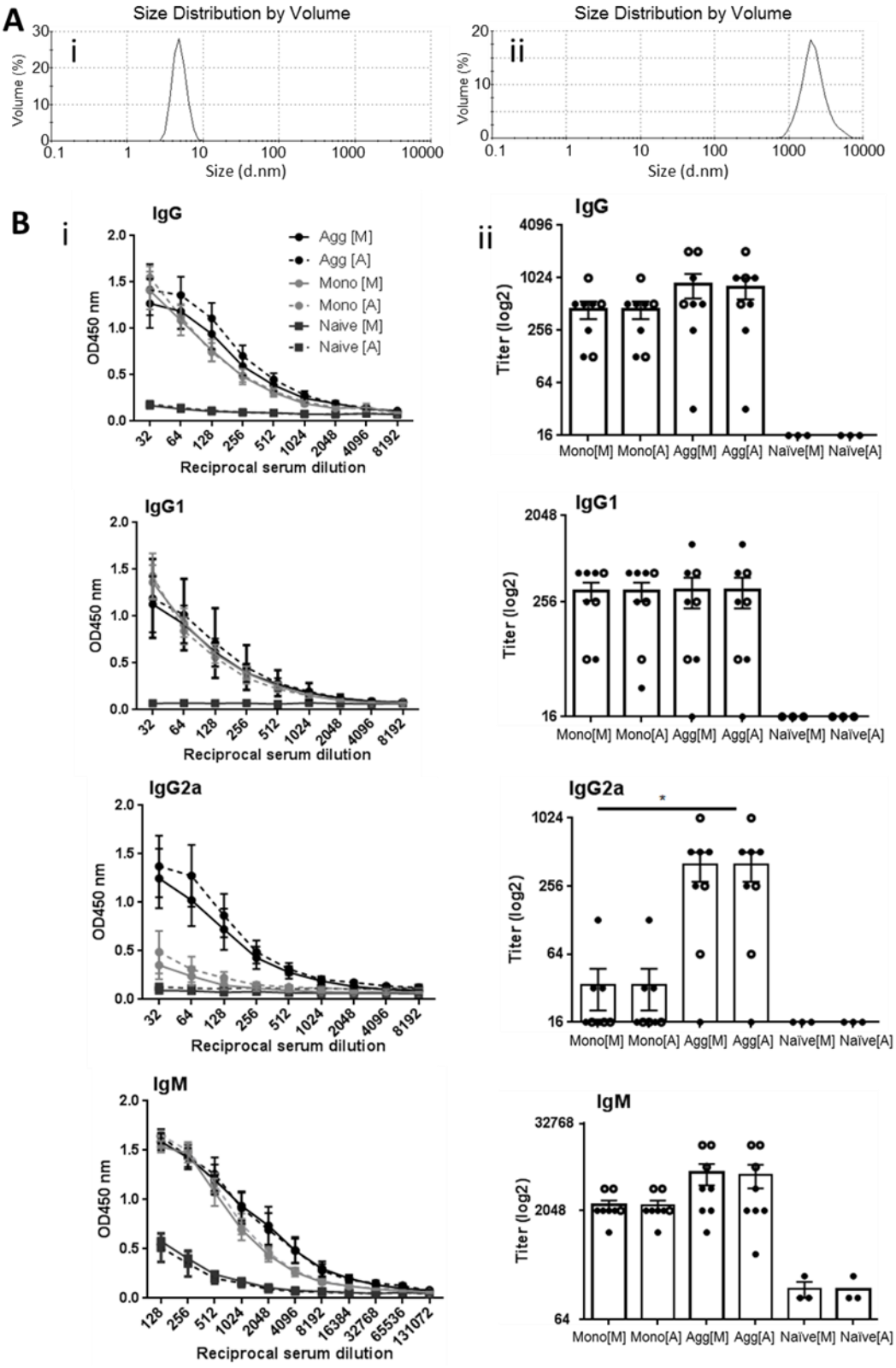


Figure 3.1. Characterisation of immune responses to scFv: comparisons of monomer and heat stressed aggregates. scFv at 1 mg/ml in PBS pH 7 was subjected to heat treatment for 25 min at 40°C. A) The mean particle diameter was measured by DLS before (i) and after

(ii) the 40 °C incubation. B) Mice were immunized by ip injection with 250 µl of 1 mg/ml monomer or heat aggregated scFv on days 0 and 7 and serum isolated on day 14 (two independent experiments; n=5 and n=3 per group). Doubling dilutions of serum samples from scFv monomer (Mono) and aggregate (Agg) immunized animals and negative control naïve serum samples were analyzed against both scFv substrate proteins (versus monomer [M] and vs aggregated protein [A]) by ELISA for IgG, IgG1, IgG2a and IgM anti-scFv antibody content. (i) Data are displayed as OD450 nm (±SEM) for each reciprocal serum dilution (32 to 8192 for IgG antibodies; 128 to 131072 for IgM antibodies) (ii) Data are displayed with respect to antibody titer (log2) calculated as the lowest serum dilution at which 3x the ELISA substrate blank OD450nm reading was reached. Individual titers are displayed with overall mean and SEM. Statistical significance of differences in antibody detection between all sera groups against each substrate was calculated using a one way ANOVA (*p<0.05).

Aggregation and IgG2a skewing is independent of either dose or method of aggregation

In subsequent experiments, the ability of aggregated scFv to induce IgG2a antibody skewing was confirmed. Mice (n=3-5) were immunized with monomer or heat stressed aggregate at 1 or 0.1 mg/ml using a more vigorous dosing regimen with an additional immunization (day 14), and termination on day 21 (Fig. 3.2).

For ELISA analyses of serum samples, the substrate used was the same as the immunizing material, as it had already been confirmed that identical responses were recorded with monomeric and aggregated scFv substrates (Fig. 3.1B). Sera isolated from mice immunized 3 times with monomer or aggregate displayed comparable responses to those observed in mice immunized twice (Fig. 3.1). Thus, comparable IgG and IgG1 antibody responses were recorded for both forms of protein, but the aggregate protein provoked significantly (*p<0.05) higher titer IgG2a antibody responses. Although IgM antibody titers were not significantly different, the maximal OD values recorded in the ELISA were significantly (**p<0.01) different between

monomer and aggregate immunized mice. Furthermore, mice immunized with a considerably lower dose (0.1 mg/ml) of protein displayed a similar trend, although overall titers were lower compared with the higher dose. In addition, a comparison of ip and sc routes of administration was conducted. Results from studies using these different routes of administration were comparable (Supplementary Data Fig. 3.1): only the aggregated material stimulated robust IgG2a antibody responses, regardless of the route of exposure.

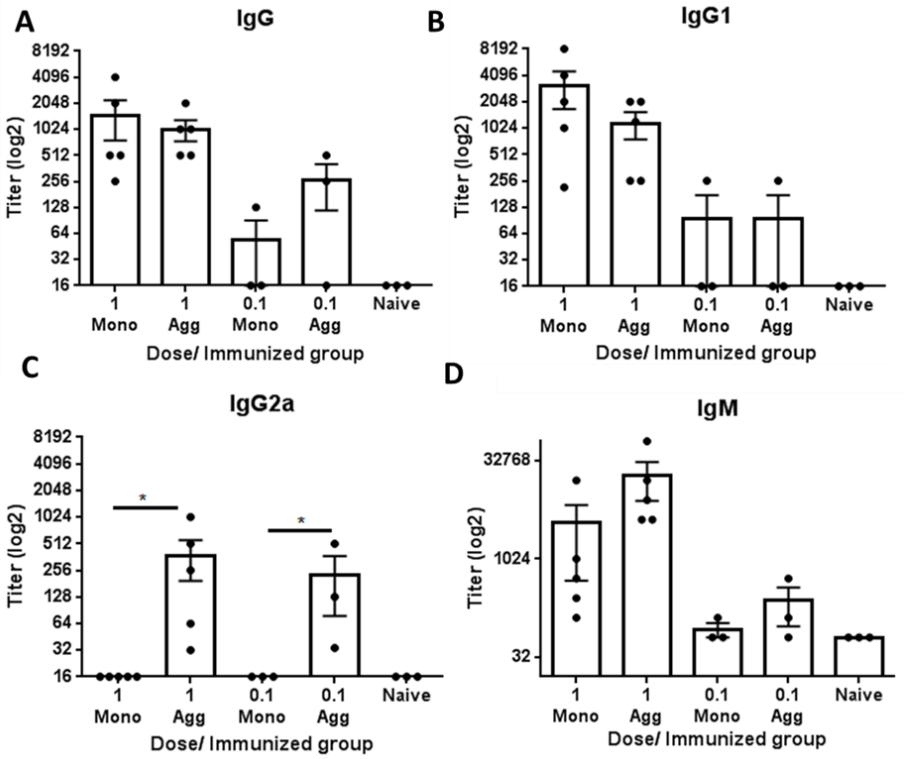


Figure 3.2. Antibody response to scFv: influence of dose and aggregation status. Mice were immunized by ip injection with 250 µl of 1 (n=5) or 0.1 mg/ml (n=3) monomer or heat aggregated scFv on days 0, 7 and 14 and serum isolated on day 21. Doubling dilutions of serum samples (starting dilution 1 in 32) from scFv monomer (Mono) and aggregate (Agg) immunized animals and negative control naïve mouse serum samples were analyzed against scFv substrate proteins (versus immunizing protein only results are shown) by ELISA for IgG (A), IgG1 (B), IgG2a (C) and IgM (D) anti-scFv antibody content. Data are displayed with respect to antibody titer (log2), calculated as the lowest serum dilution at which 3x the ELISA substrate blank OD450nm reading was reached. Individual titers are displayed with overall

mean and SEM. Statistical significance of differences in antibody binding between all treatment groups against substrate were calculated using a one way ANOVA (*$p<0.05$).

The aggregates studied thus far were formed using thermal stress, and were found to consistently induce higher titer IgG2a antibody responses than did the monomeric protein. Stir stress was employed to determine whether scFv aggregates within a similar size range could be formed using a mechanical stress method. The mean protein particle diameter of aggregates was analyzed by DLS (Fig. 3.3A), from which it was apparent that aggregates produced using stir stress were similar to heat stressed aggregates. Stir stressed aggregates also had a mean particle diameter of 1000-3000 nm and were stable following 2 freeze/thaw cycles. Anti-scFv antibody production patterns were identical, irrespective of whether stir aggregate or monomer was used as a substrate in the ELISA (data not shown). Monomer and aggregate protein preparations formed using heat or stir stress were administered via ip injection to mice on day 0, 7 and 14 (two independent experiments; n=3 in each) and sera isolated on day 21.

Sera isolated from monomer, heat and stir stressed aggregate immunized mice induce comparable IgG and IgG1 antibody titers (Fig. 3.3). In contrast, IgG2a titers were significantly (*$p<0.05$) higher in animals exposed to heat or stir stressed protein compared with monomer immunized mice. IgM antibody titers were somewhat higher in aggregate compared with monomer immunized mice and, although titers were not significantly different, maximal OD values recorded at the top serum dilution were significantly different (***$p<0.001$) between monomer and both groups of aggregate immunized mice.

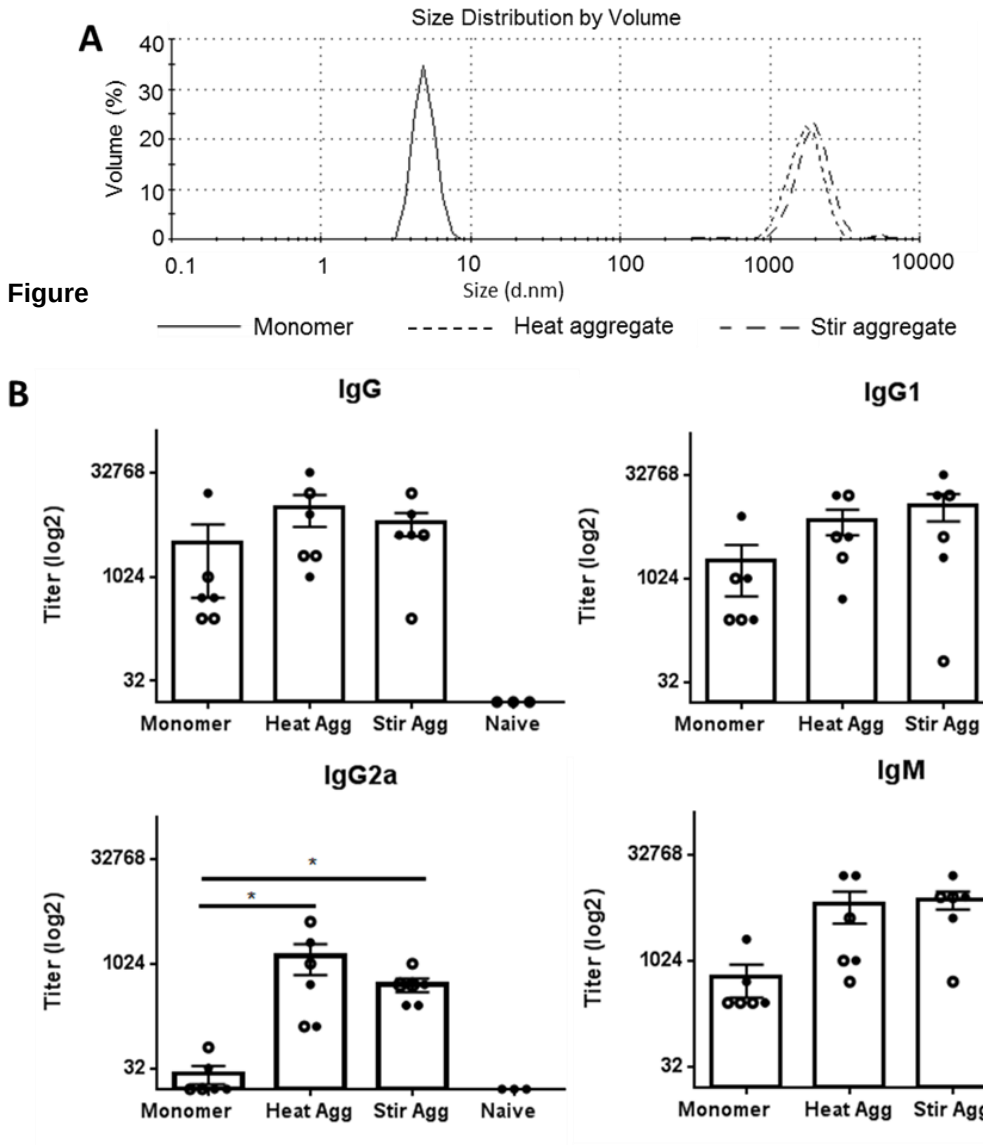


Figure 3.3. **Antibody responses to scFv monomer, heat stressed and stir stressed aggregates.** ScFv at 1 mg/ml in PBS pH 7 was subjected to heat treatment for 25 min at 40°C for thermal stress. To induce stir stress samples were stirred for 6 h at room temperature. A) The mean particle diameter was measured by DLS using the Malvern zetasizer before and after the 40 °C incubation or stir stress. B) Mice (n=6) were immunized by ip injection with 250 µl of 1 mg/ml monomer or heat (Heat Agg) or stir aggregated (Stir Agg) scFv on days 0, 7 and 14 and serum isolated on day 21. Doubling dilutions of serum samples (starting dilution 1 in 32 for IgG and 1 in 128 for IgM) from immunized animals and negative control naïve mouse serum samples were analyzed against scFv substrate proteins (versus immunizing protein only results are shown) by ELISA for IgG, IgG1, IgG2a and IgM anti-scFv antibody content. Data are displayed with respect to antibody titer (log2), calculated as the lowest serum dilution at

which 3x the ELISA substrate blank OD450 nm reading was reached. Individual titers (open and closed symbols) are displayed for two independent experiments (n=3 per group), with overall mean and SEM. Statistical significance of differences in antibody detection between all treatment groups against substrate were calculated using a one way ANOVA (*p<0.05).

Aggregation of the scFv induced differential cellular responses: proliferation and cytokine production

As antibody production profiles suggested that aggregated protein was inducing Th1 type skewing (associated with selective IgG2a antibody responses) we sought to examine this at the cellular level. To this end, antigen-driven proliferation and cytokine production by cultured lymphocytes prepared from the spleens of immunized mice were measured. Splenocytes from immunized and naïve mice were cultured either alone or in the presence of BMDC, the latter included to enhance assay sensitivity. Cultures were primed with monomeric or aggregated scFv and proliferation measured using ^{3}H-thymidine incorporation at a 72 h time point (Fig. 3.4).

Splenocyte only cultures derived from naïve and monomer or heat aggregate immunized mice did not proliferate significantly more in response to stimulation with scFv in any of the formats compared with medium alone. Only stir aggregate immunized mouse splenocytes displayed any activity by responding more effectively to stimulation with all of the scFv preparations (Fig. 3.4). Interestingly, the nature of the scFv used for antigenic stimulation in culture did not impact on proliferative activity. It should be noted that the baseline level of proliferation for naïve splenocytes was somewhat higher than those observed for cells from the immunized mice; due to logistics the naive cell cultures were not conducted concurrently. The addition of BMDC in co-culture with the splenocytes resulted in improved assay sensitivity, such that both heat and stir aggregate immunized mouse cell cultures responded significantly to scFv compared with medium alone. Again, the nature of the scFv used did not impact on proliferative activity. Naïve and monomer immunized mouse

splenocyte cultures did not respond to scFv in culture, regardless of the presence of BMDC.

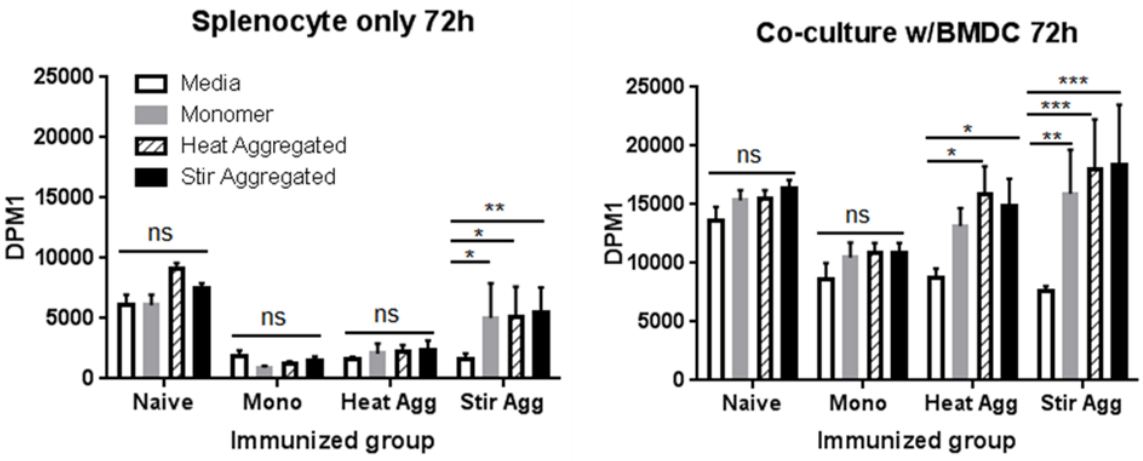


Figure 3.4. Splenocyte culture ^{3}H-thymidine incorporation following *ex vivo* scFv challenge. Splenocytes from monomer (Mono), heat (Heat Agg) or stir aggregated (Stir Agg) scFv immunized (as described in Fig. 3 legend), or naïve* mice (n=3 per group) were cultured alone and in co-culture with BMDC and challenged with 100 µg/ml monomeric scFv, heat or stir aggregated scFv or with media alone. Cells were pulsed with ^{3}H-thymidine 24h before harvesting at 72 h and β scintillation counting. Each culture condition was performed in triplicate for splenocytes derived from each individual animal and a mean calculated. Group mean proliferation measured as disintegrations per minute (DPM) is shown ±SEM. Statistical significance of differences were calculated using a two way ANOVA (*p<0.05, **p<0.01, ***p<0.001). *Experiments with naïve mice were conducted independently but utilised the same batches of mice and protein.

To characterise further the Th1/Th2 response, culture supernatants in parallel experiments were analyzed for the presence of IFN-γ (a Th1 signature cytokine), IL-4 and IL-13 (Th2 cytokines) (Fig. 3.5). Secretion of IFN-γ was generally highest under splenocyte-BMDC co-culture conditions. Immunization with heat and stir aggregated scFv resulted in an increased level of IFNγ secretion from splenocyte-BMDC co-

cultures in response to stimulation with scFv compared with medium alone. Monomer immunized mouse cultures did not respond to the scFv, by proliferation or by cytokine production, therefore aggregation of the immunizing material resulted in primed splenocytes that were capable of being re-stimulated by antigen in culture. Again, baseline levels of naïve cells were somewhat higher than those of immunized mice, but there was no evidence of antigen specific stimulation of these cells. Again, the nature of the scFv used for antigen challenge in culture failed to influence cytokine production levels. A similar pattern was observed for IL-13 secretion, in so far as BMDC co-culture was required for optimal production, and splenocytes derived from aggregate immunized mice displayed higher levels of cytokine production than did the monomer immunized counterparts. However, significantly higher IL-13 secretion compared with the medium control was only observed in the stir aggregate immunized co-culture group. No change in IFNγ or IL-13 production was observed with naïve splenocyte cultures. IL-4 secretion was measured in addition to IL-13 and IFN-γ, however, levels were below the limit of detection (15.6 pg/ml; data not shown.)

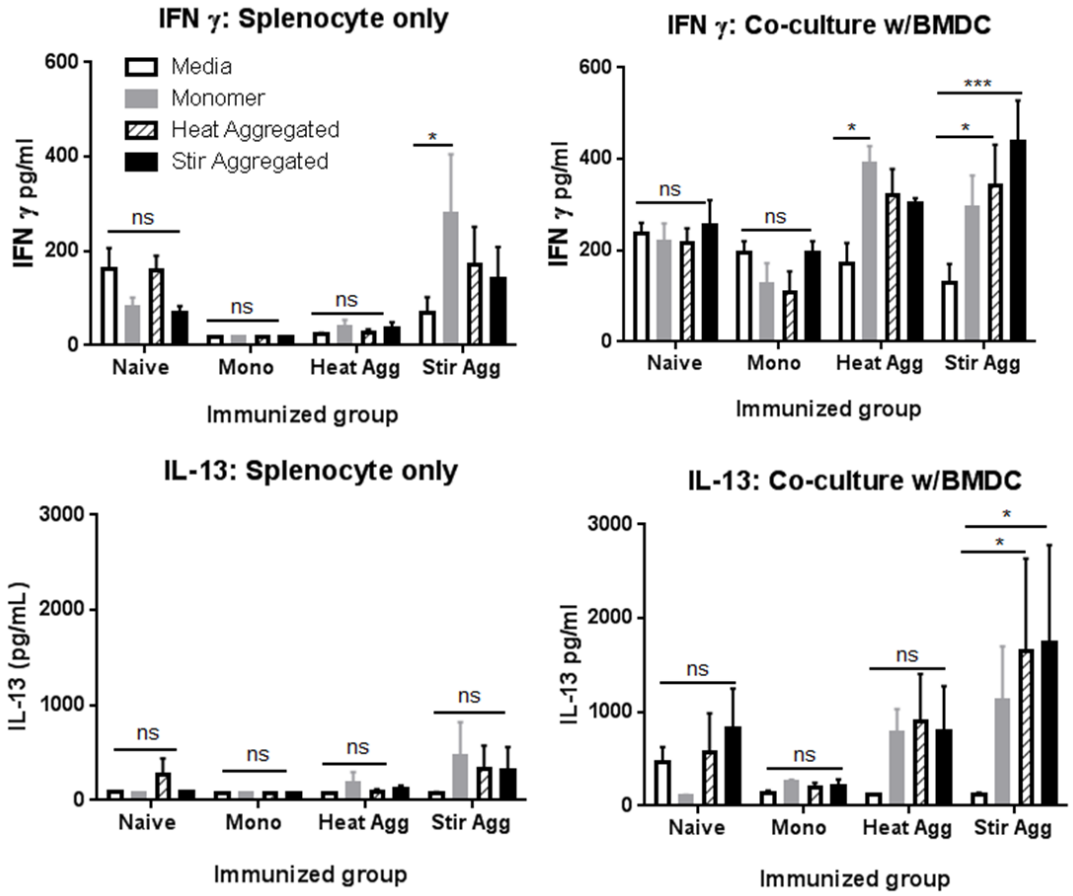


Figure 3.5. Splenocyte culture IFN-γ and IL-13 cytokine secretion following *ex vivo* scFv challenge. Splenocytes from monomer (Mono), heat (Heat Agg) or stir aggregated (Stir Agg) scFv immunized (as described in Fig. 3 legend) or naïve* mice (n=3 per group) were cultured alone (splenocyte only) and in co-culture with BMDC (w/BMDC) and challenged with 100 µg/ml monomeric scFv, heat or stir aggregated scFv or media alone. Each culture condition was performed for a single aliquot of splenocytes derived from each individual animal. Supernatants were harvested at 144 h and analyzed for the presence of IFNγ and IL-13 by cytokine specific ELISA. Data are shown as group mean ±SEM. Statistical significance of differences were calculated using a two way ANOVA (*p<0.05, **p<0.01, ***p<0.001). *Experiments with naïve mice were conducted independently but utilised the same batches of mice and protein.

Aggregation of the unrelated protein OVA results in similar Th1 skewing

To determine whether aggregation and Th1 skewing was a feature solely of scFv protein or a more general property of protein aggregation *per se*, OVA was used as an alternative immunogenic protein. Stir stress was employed to aggregate OVA within the subvisible size range and DLS used for analysis. The mean diameters of OVA aggregates as a percentage of volume were: 80% ~0.8 µm, 10% ~0.1 µm and 10% ~5 µm (Fig. 3.6A). Although these aggregates were less homogenous than observed with the scFv preparation, the mean diameter of the main population was similar to that of the scFv aggregates (1 µm). Monomer and aggregate protein preparations were administered via ip injection to mice on days 0, 7 and 14. On day 21, spleens and sera were isolated.

Analysis of the serum dilution curves and antibody titers revealed that there was no binding above the background level to either monomer or aggregate OVA substrates with naïve sera (Fig. 3.6B). Sera isolated from both OVA monomer or aggregate immunized mice displayed detectable IgG and IgG1 antibody, with very similar antibody titers. A significantly higher IgG2a antibody response (*$p<0.05$) was recorded in sera from aggregate immunized compared with monomer immunized mice. There was no significant difference between IgG2a responses in naive and monomer immunized sera. Antibody titers were comparable when either aggregated or monomeric protein was used as a substrate in the analysis. In addition, anti-scFv IgM OD values and titers were higher in aggregate immunized mouse sera compared with monomer immunized mice, but not significantly different.

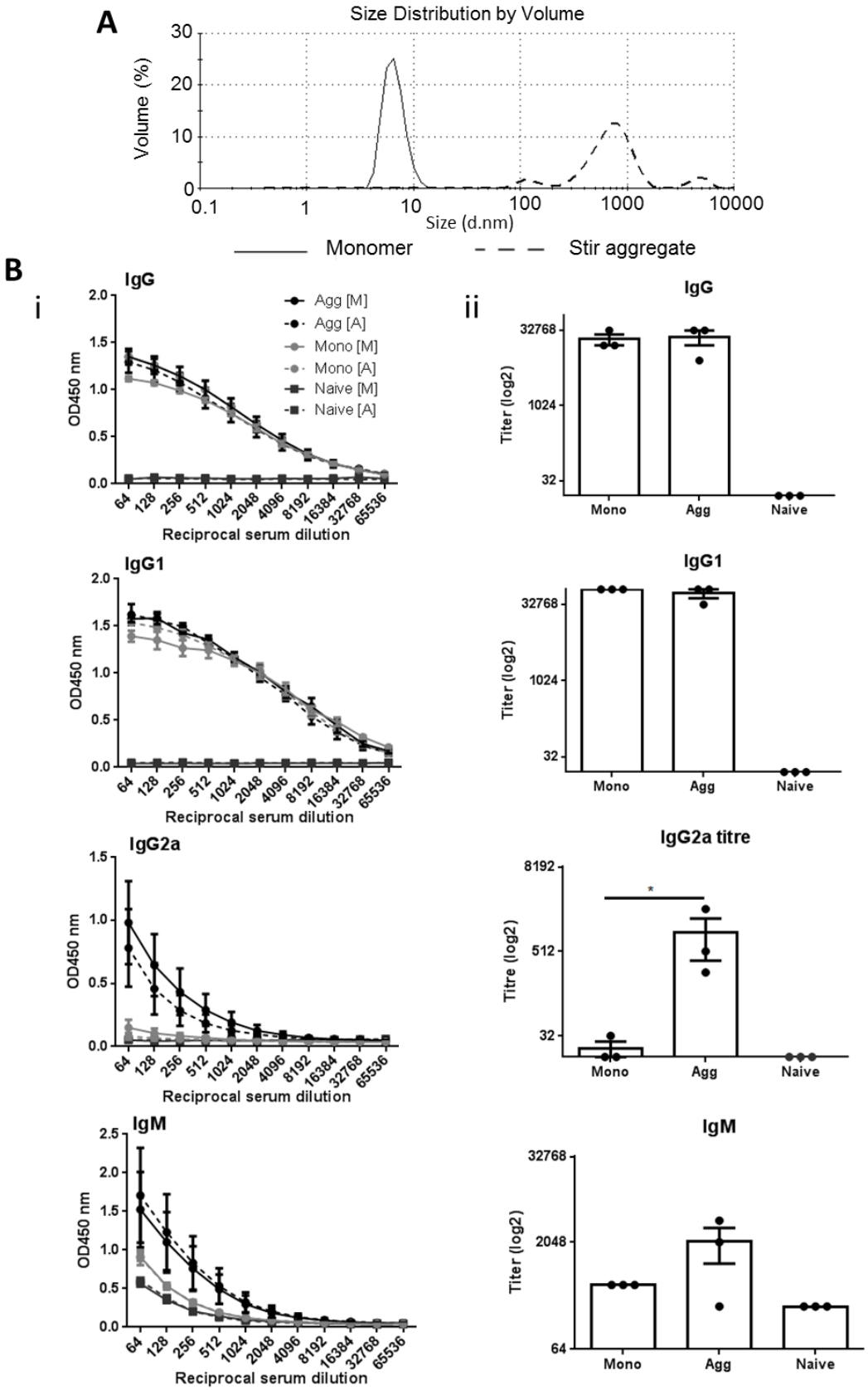


Figure 3.6. Characterisation of immune responses to OVA: comparisons of monomer and stir stressed aggregates. 1 mg/ml OVA in PBS pH 7 was subjected to stir stress for 24-28 h at room temperature. A) The mean particle diameter was measured by DLS using the

Malvern zetasizer before and after stir stress. B) Mice (n=3 per group) were immunized by ip injection with 250 µl of 1 mg/ml (n=3) monomer or stir aggregated OVA on days 0, 7 and 14 and serum isolated on day 21. Doubling dilutions of serum samples (starting dilution 1 in 64) from OVA monomer (Mono) and aggregate (Agg) immunized animals and negative control naïve mouse serum samples were analyzed against OVA substrate proteins (versus monomer [M] and vs aggregated protein [A]) by ELISA for IgG, IgG1, IgG2a and IgM anti-OVA antibody content. (i) Data are displayed as OD450 nm ±SEM for each reciprocal serum dilution (ranging from 64 to 66532).) (ii) Data are displayed with respect to antibody titer (log2) calculated as the lowest serum dilution at which 3x the ELISA substrate blank OD450 nm reading was reached. Individual titers are displayed with overall mean and SEM (versus immunizing protein only results are shown). Statistical significance of differences in antibody detection between all treatment groups against substrate were calculated using a one way ANOVA (*p<0.05).

Aggregation of OVA induced differential cellular responses: proliferation and cytokine production

Cellular assays with splenocyte cultures were conducted to measure antigen driven lymphocytes proliferative responses and cytokine expression. Naïve splenocyte cultures did not proliferate in response to OVA (Fig. 3.7A). Splenocytes from monomer and aggregate immunized mice each responded similarly in the proliferation assay (Fig. 3.7B and C); splenocyte-DC co-cultures proliferated significantly more in response to monomer and aggregate compared with medium controls (****p<0.0001). Additionally, cultures proliferated more vigorously in response to aggregated protein than to monomer (**p<0.01).

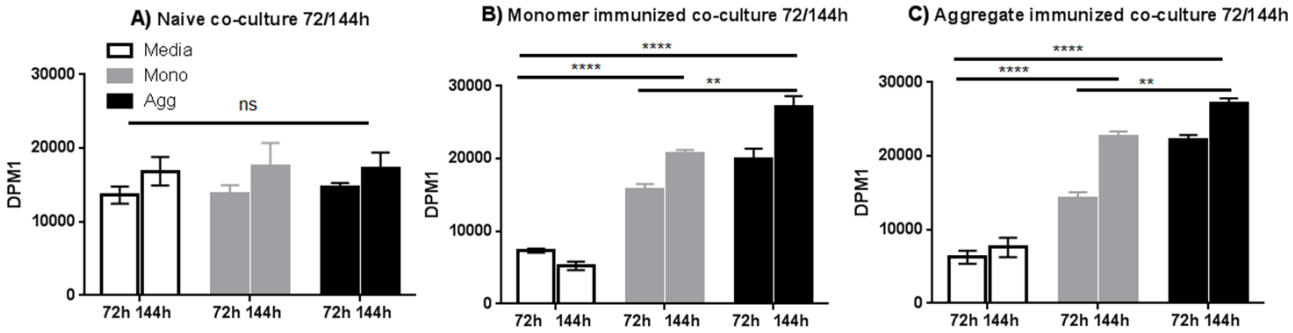


Figure 3.7. Splenocyte culture ^{3}H-Thymidine incorporation following *ex vivo* OVA challenge. Splenocytes from naïve* (A) and monomer (B) or aggregate (B) immunized mice (as described in Fig. 6 legend; n=3 per group) were co-cultured with BMDC and challenged with 100 µg/ml monomeric OVA (Mono), stir aggregated OVA (Agg) or media alone. Cells were pulsed with ^{3}H-thymidine 24 h before harvesting at 72 and 144 h and β scintillation counting. Each culture condition was performed in triplicate for splenocytes derived from each individual animal and a mean calculated. Proliferation measured as disintegration per min (DPM) is shown ±SEM (2 bars represent 72 and 144 h time points). Statistical significance of differences in proliferation between all groups were calculated using a two way ANOVA (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). * Experiments with naïve mice were conducted independently but utilised the same batches of mice and protein.

Antigen-induced cytokine production by cultured splenocytes was also measured (Fig. 3.8). Splenocytes secreted significantly more IFN-γ when stimulated with aggregate compared with monomer or medium alone (Fig. 3.8A). In the presence of BMDC there was an increased level of IFNγ secretion in response to monomeric protein. IL-4 and IL-13 secretion was also increased by provision of BMDC (Fig. 3.8B and C): here, monomer and aggregate treated wells secreted significantly more IL-4 and IL-13 compared with medium alone controls. Stimulation with monomer challenge induced significantly higher levels of IL-13 production (**p<0.01) compared with aggregate challenge in co-cultures from aggregate immunized mice. Naïve

splenocytes did not secrete significantly more cytokine in response to OVA treatment in culture when compared with the medium alone control. These data are indicative of a Th1 skewed response against the aggregated OVA preparation, and a Th2 skewed response against the monomeric preparation.

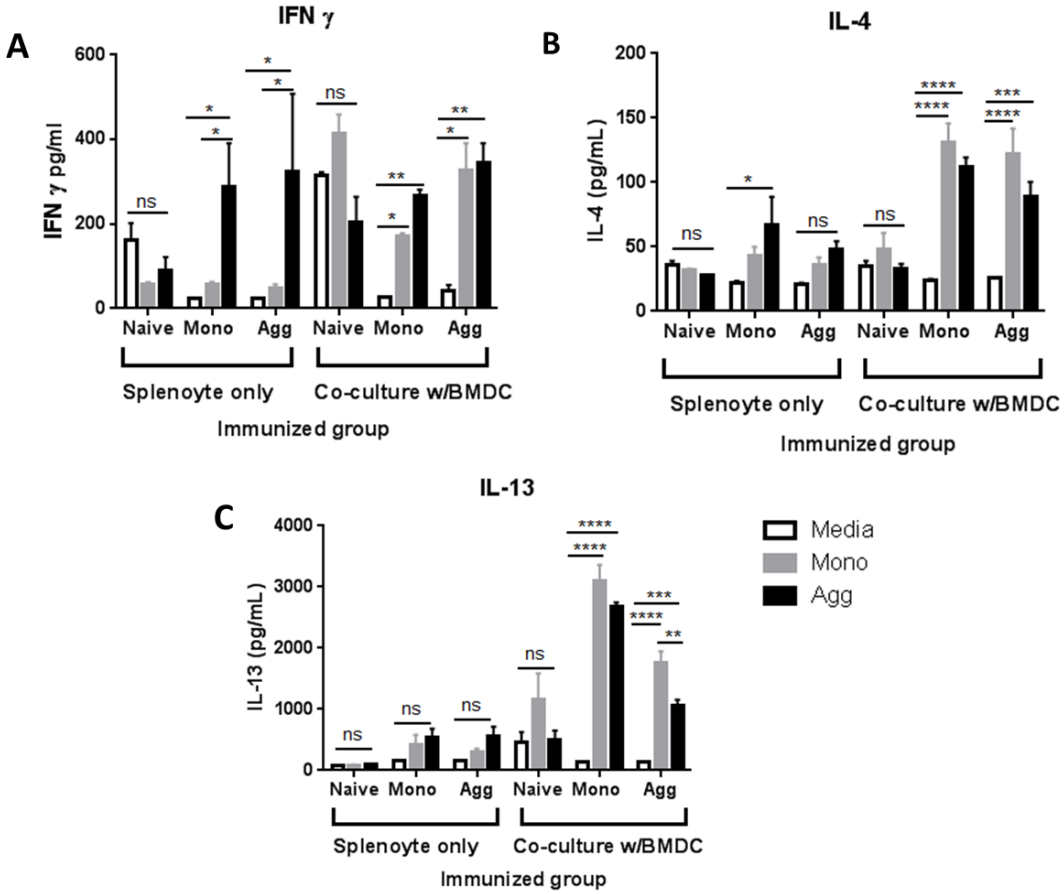


Figure 3.8. Splenocyte culture IFN-γ, IL-4 and IL-13 cytokine secretion following *ex vivo* OVA challenge. Splenocytes from naïve* and monomer (Mono) or aggregate (Agg) immunized mice (as described in Fig. 6 legend; n=3 per group) were cultured alone (splenocyte only) or in co-culture with BMDC (w/BMDC) and challenged with 100 µg/ml monomer (Mono), aggregate (Agg) or media alone. Each culture condition was performed for a single aliquot of splenocytes derived from each individual animal. Supernatants were harvested at 144 h and analyzed for the presence of A) IFNγ B) IL-4 and C) IL-13 by cytokine specific ELISA. Data are shown as group mean ±SEM. Statistical significance of differences were calculated using a two way ANOVA (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). *Experiments with naïve mice were conducted independently but utilised the same batches of mice and protein.

3.5 Discussion

Although protein aggregation is widely acknowledged to have an important influence on the immunogenicity of biotherapeutics, the cellular and molecular mechanisms through which such effects are induced are unclear. Concerns about aggregate immunogenicity may be exacerbated in the future by the introduction of novel formats and/or glycoengineered molecules. For example, antigen-binding fragments (Fab), such as Lucentis® (ranibizumab) and Cimzia® (certolizumab pegol) have been approved (Goel and Stephens 2010; Ferrara et al. 2006), and more antibody fragments, including scFv, are in the biotherapeutics pipeline (Holliger and Hudson 2005; Nelson 2010). In these experiments the protein chosen to study was a humanized scFv (Edwardraja et al. 2010); this was expected to provide a baseline response level of immunogenicity in mice, which could be compared with aggregate preparations. OVA was selected as a second, unrelated, antigen.

Protein aggregation can be difficult to control, and the relationship between size and immunogenicity of protein aggregates is not well understood at present. Attention is currently focused on subvisible particles, with sizes below 10 µm, due to concerns about their immunogenicity (Joubert et al. 2012; Zoells et al. 2012). Against this background, we aimed to generate protein aggregates that fell within the subvisible particle size range, and with a high degree of homogeneity (i.e. covering a distinct size range), since aggregates often form heterogeneous populations that cover a wide size range (Das 2012). Mild heat treatment (40°C) or stir-stress of the scFv generated homogenous populations of aggregates in the µm range. Subvisible OVA aggregates formed by stir stress were not completely homogenous, but deemed sufficiently close in size range and homogeneity to be comparable to the scFv aggregates.

As expected, an immune response was mounted against the scFv in mice. Identical antibody binding patterns were obtained regardless of whether aggregate or monomer was used as a substrate in the ELISA, indicating that the antibody epitopes were not corrupted during heat treatment. Analysis of antibody isotype distribution revealed a Th1 skewing of the humoral immune response associated with aggregation. The data also indicated that mice immunized with aggregate had somewhat higher (but statistically insignificant) IgM titers compared with monomer. Upon antigen recognition one of the first immunoglobulins expressed by naïve B cells is IgM; further B cell activation results in class switching where the immunoglobulin constant heavy chain changes but antigen specificity remains. In these experiments relatively high total IgG and IgG2a responses demonstrated class switching of the antibody response (Kracker and Durandy 2011); the observed levels of IgM in sera therefore were unexpected. An additional immunization was added to the protocol to determine whether IgM could be reduced with extended *in vivo* exposure. However, three immunizations induced strong anti-scFv IgM levels that were higher in the aggregate compared to monomer immunized mice. Higher IgM levels following aggregate administration compared with monomer immunization may suggest that the aggregate can stimulate a strong immature B cell response, in addition to B cell activation and class switching, potentially due to its structure or diversity of antigen epitopes. A lower scFv dose of 0.1 mg/ml was expected to induce a response with reduced vigor. Indeed IgG, IgG1 and IgM antibody levels in all immunized mice were reduced at the lower dose. The IgG2a response however, was still induced in mice immunized with 0.1 mg/ml aggregated scFv, and with a similar vigor of response compared with the 1 mg/ml dose. Therefore the skewing of the immune response appears more pronounced at the lower scFv dose.

Protein aggregation is known to occur under different stress conditions which can result in aggregates with different biophysical properties (Abdolvahab et al. 2016). A

stirring method was employed to produce aggregates mechanically, in part to imitate industrial processes where stir stresses are common. Antibody binding against the stir-aggregated substrate was similar to the monomer, indicating that antibody epitopes were not disrupted substantially with stirring. Stir and heat-stressed aggregates of the scFv were almost identical in size and antibody responses were very similar against both scFv aggregates, with a significant increase in IgG2a titers compared with monomer immunized sera. This indicates that the size range of these aggregates may be important in inducing a differential immune response or that heat and stir stresses resulted in a common misfolding of protein structure, providing aggregates with similar morphologies and therefore immunological responses. While a similar trend was observed with both ip and sc injections, it is possible that the impact of aggregation on immunogenicity could vary according to the route of exposure, potentially due to the micro-environment or immune milieu at different sites.

Studies using cultured splenocytes from immunized mice provided further evidence of differential responses of aggregate compared with monomeric scFv. Splenocytes were also co-cultured with BMDC in an effort to improve antigen presentation and assay sensitivity for proliferation and cytokine analysis (Sun et al. 2013). Indeed, we found that BMDC co-culture did improve assay sensitivity. In co-cultures from aggregate, but not monomer, immunized mice, scFv challenge resulted in increased proliferation. However, an immune response had been induced by the monomer *in vivo,* as evidenced by total IgG antibody titers which were comparable with aggregate immunized groups. This trend was also observed with results from cytokine analysis. Splenocyte cultures from aggregate immunized mice displayed enhanced secretion of both IL-13 and IFNγ with scFv stimulation; however, this effect was not observed in naïve or monomer immunized mouse cell cultures. Therefore, whilst aggregation resulted in Th1 skewing at the level of antibody production *in vivo*, an overall increase in immunogenicity was seen with respect to cytokine expression. It is worth noting

that serum antibody titer represents a cumulative measure of immune activation whereas the cytokine data are a snapshot at a single time point.

OVA was chosen as an alternative candidate protein antigen to investigate; OVA differs both in size and structure to a scFv, and is a well-studied protein (Huntington and Stein, 2001), known to induce a Th2 type immune response in BALB/c mice (Carvalho Gouveia et al. 2013). It was therefore of interest to determine if OVA, when aggregated in the subvisible size range, would induce a Th1 skewed immune response. Antibody analysis of immunized mouse sera did demonstrate a Th1 skewing with aggregation. Anti-OVA IgG and IgG1 levels were identical in both sets of mice; however, IgG2a antibody was significantly higher in aggregate compared with monomer immunized mouse sera. IgM binding appeared slightly higher in aggregate immunized sera, as observed with the scFv. In OVA immunized splenocyte co-cultures, aggregate culture treatment stimulated significantly higher proliferation compared with the monomer. One possible explanation for this might be that the aggregates were more easily recognised because of their size or distribution of epitopes. Furthermore, cytokine assays with splenocyte culture supernatants were consistent with Th1 skewing by aggregated OVA. Monomer and aggregate immunized splenocyte cultures secreted significantly more IFNγ in aggregate compared with monomer treated wells; however, monomer challenge induced more IFNγ secretion with the BMDC co-culture. This is likely due to improved antigen presentation in the presence of DC compared with splenocytes alone. This would also explain increased IL-4 and IL-13 secretion in co-culture compared with splenocytes alone. Increased IFNγ secretion in aggregate treated wells and IL-4 and IL-13 in monomer treated wells is in keeping with the antibody data, illustrating a Th1 skewed phenotype with aggregate treatment, and a Th2 phenotype with monomer treatment.

In the studies described here, antibody subclass distribution is a surrogate marker of divergent T cell responses. Th1 responses are typically mounted against intracellular

bacteria and viruses whereas Th2 responses are associated primarily with responses in atopy and multicellular parasite infections (Berger 2000). Viruses generally induce a Th1 type immune response in BALB/c mice (Huber and Pfaeffle, 1994). Data presented are consistent with the hypothesis that aggregates can mimic the multiple antigen copy distribution characteristics of pathogenic microbes and viruses (Kastenmueller et al. 2011; Rosenberg 2006). Vaccine studies have demonstrated Th1 skewing of immune responses by larger virus particles in BALB/c mice (Hovden et al. 2005); in these experiments it was shown that a whole virus vaccine was more immunogenic and induced a more dominant Th1 antibody response compared to a split virus vaccine. In addition, BALB/c strain mice are reported to be biased towards a Th2 immune response (Fukushima et al. 2006; Schulte et al. 2008), so the use of these mice in this study strengthens the finding that subvisible aggregates can enhance a Th1 type immune response. It is suggested that the Th1 skewing observed with aggregation in these experiments is a direct effect of differential antigen processing and presentation.

This work shows that subvisible scFv and OVA aggregates can be achieved by heat and/or stir stress. Monomeric proteins induced a Th2 dominant immune response but when proteins were aggregated, the response gained a Th1 phenotype. This indicates that subvisible aggregates are recognised differently by immune cells, resulting in differential responses. Further work is needed to elucidate whether smaller or larger aggregates also replicate this effect, or whether other variables (i.e. hydrophobicity) also play a role. Although extrapolation of our observations to humans is more difficult, our results do at least indicate that aggregation can stimulate immunological responses which differ in kind, and that the composition of aggregates could be important. The speculation is that if patients were exposed to aggregates of a similar size, the immune response may be influenced similarly, however, further work is required to understand the potential clinical implications of these findings.

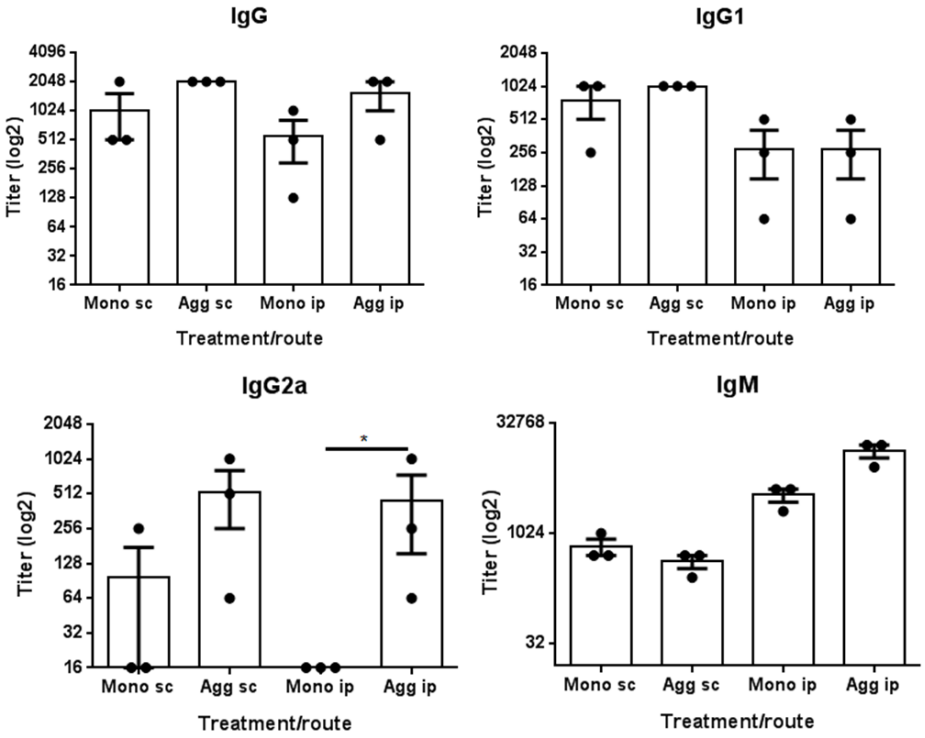
Although methods to predict immunodominant T cell epitopes can be useful to reflect the potential immunogenicity of a given biotherapeutic (Weber et al. 2009), other considerations, such as aggregate size and biophysical properties can also influence responses. Further work will be required to define more precisely the molecular mechanisms underpinning differential immune responses to monomer and aggregate, which could then lead to an enhanced ability to predict immunogenicity.

3.6 Acknowledgements

We thank Lorna Beresford for assistance with *in vivo* immunizations.

3.7 Supplementary data

Ip injections were used for administering immunogenic proteins in this study however this route is not typically used in patients for parenteral drug administration. Therefore, immunogenicity of the scFv monomer and aggregate were compared in BALB/c strain mice following sc or ip administration (250 µl at 1 mg/ml) on day 0 and 7, and serum isolated on day 14. No differences were observed in IgG titers. IgM titers appeared lower in sc immunized mice although this difference was not significant (Supplementary Fig. 3.1).



Supplementary Figure 3.1. Characterisation of immune responses to scFv following ip or sc routes of administration. Mice were immunized by ip or sc injection with monomer or heat aggregated scFv on day 0 and 7 and exsanguinated on day 14. Specific IgG subclass and IgM antibody expression in serum was assessed by ELISA. Doubling dilutions of serum samples (starting dilution 1 in 32 for IgG, 1in 128 for IgM) from scFv monomer (Mono) and aggregate (Agg) immunized animals (n=3) were analyzed against a scFv substrate (versus immunizing protein only results are shown). OD450 nm was measured; Data are displayed with respect to antibody titer (log2) calculated as the lowest serum dilution at which 3x the ELISA substrate blank OD450 nm reading was reached. Individual titers are displayed as

overall mean ± SEM. Statistical significance of differences in antibody detection between all sera groups against substrate was calculated using a one way ANOVA (*p<0.05).

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CHAPTER 4:

INFLUENCE OF *E. COLI* CHAPERONE DNAK ON PROTEIN IMMUNOGENICITY

4 Paper 3: Influence of *E. coli* chaperone DnaK on protein immunogenicity

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Running title: DnaK enhances protein aggregate immunogenicity

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4.1 Abbreviations

APC antigen presenting cell
CD circular dichroism
DEAE diethylaminoethanol
DLS dynamic light scattering
E.coli *Escherichia coli*
ELISA enzyme-linked immunosorbent assay
FDA Food and Drug Administration
GH growth hormone
HCP host cell protein
HSP heat shock protein
IgG immunoglobulin G
IgM immunoglobulin M
LCMS liquid chromatography-mass spectrometry
LPS lipopolysaccharide
NMS naive mouse serum
OD optical density
PBS phosphate buffered saline
ppm parts per million
scFv single chain antibody variable fragment
SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC-MALLS size exclusion chromatography coupled with multi angle laser light scattering
Th T helper

4.2 Abstract

The production of anti-drug antibodies can impact significantly upon the safety and efficacy of biotherapeutics. It is known that various factors, including aggregation and the presence of process-related impurities can modify and augment the immunogenic potential of proteins. The purpose of the investigations reported here was to characterize in mice the influence of aggregation and host cell protein (HCP) impurities on the immunogenicity of a humanized single chain antibody variable fragment (scFv), and mouse albumin. HCP impurities within a scFv preparation purified from *Escherichia coli* (*E.coli)* displayed adjuvant-like activity for responses to the scFv in BALB/c strain mice. The 70 kDa *E.coli* chaperone protein DnaK was identified as a key contaminant of scFv by mass spectrometric analysis. Preparations of scFv lacking detectable DnaK were spiked with recombinant *E.coli* DnaK to mimic the process related impurity. Mice were immunized with monomeric and aggregated preparations, with and without 0.1% DnaK by mass. Aggregation alone enhanced IgM and IgG2a antibody responses, but had no significant effect on total IgG or IgG1 responses. The addition of DnaK further enhanced IgG and IgG2a antibody responses, but only in the presence of aggregated protein. DnaK was shown to be associated with the aggregated scFv by Western blot analysis. Experiments with mouse albumin showed an overall increase in immunogenicity with protein aggregation alone, and the presence of DnaK increased the vigor of the IgG2a antibody response further. Collectively these data reveal that DnaK has the potential to modify and enhance immunogenicity when associated with aggregated protein.

4.3 Introduction

Biopharmaceuticals, and in particular therapeutic antibodies, currently make up some of the highest global sales of pharmaceutical products. For example, in 2015 the world's top selling drug was AbbVie's monoclonal antibody Humira (adalimumab) (Norman 2016). Biopharmaceuticals, including many antibody fragments that are currently in development (Spiess et al. 2015; Nelson and Reichert 2009), are primarily produced in non-human host cells, including *Escherichia coli (E. coli),* rodent cell lines, and yeast (Walsh 2010). During purification it is well established that small amounts of host cell protein (HCP), including intracellular proteins, will co-purify with the production biopharmaceutical (Hogwood et al. 2013). The presence of HCP in a final drug preparation is referred to as a process-related impurity (Shukla and Hinckley 2008), and such impurities may potentially affect immunogenicity, the production of anti-drug antibodies, and drug efficacy and/or safety.

Immunodetection based-methods, such as ELISA, which employ polyclonal antibodies raised against the whole HCP spectrum, are often used for monitoring HCP levels (Zhu-Shimoni et al. 2014). Most biologics submitted for approval to FDA contain less than 100 parts per million (ppm) HCP, as measured by ELISA. It cannot be guaranteed, however, that all HCPs are being measured and with sufficient sensitivity with this method.

HCPs themselves may be immunogenic but, additionally, can influence immune responses elicited by exposure to the biotherapeutic, by acting through an adjuvant-like mechanism (Bracewell et al. 2015). Such adjuvant activity could lead to the formation of anti-drug antibodies. HCP contaminants have been shown previously to act as an adjuvant by influencing the immunogenicity of biotherapeutics in the clinic (Wadhwa et al. 1999). For example, a recombinant form of human growth hormone (GH) Omnitrope was produced in *E.coli* and an early version resulted in the development of non-neutralising anti-GH antibodies in up to 60% of patients in clinical trials (Romer et al. 2007). The cause of immunogenicity was attributed to HCP

contamination and a further purification step was included to reduce the level of HCP impurities. As a result, immunogenicity was significantly reduced (Schneider et al. 2008). Thus, bacterial HCPs have the potential to influence immunogenicity of the biotherapeutic by having adjuvant-like activity. HCP contamination is also an issue for mammalian cell expression systems (Shukla and Hinckley 2008; Hogwood et al. 2014; Yuk et al. 2015). However, non-mammalian HCPs are more likely to pose a risk in patients, compared with their mammalian counterparts.

There is also evidence that HCPs can display adjuvant-like activity in vaccines. Heat shock proteins (HSPs) are molecular chaperones, and bacterial HSPs have been shown to augment adaptive immune responses (Atzingen et al. 2014). In vaccine development HSPs are now being exploited to improve efficacy (McNulty et al. 2013). For example, a novel vaccine strategy for *Neisseria meningitidis* utilises a bacterial HSP in a protein antigen-HSP complex that enhances antigen immunogenicity (Bailey et al. 2010). HSPs have also found application in cancer immunotherapy, where they are complexed with a tumour antigen, aiding the activation of anti-tumour immune responses (Nelson 2002).

The ability of HSPs to enhance immunogenicity emphasises the need for their identification and control in biotherapeutic formulations. HSPs have been identified in mAb preparations with high HCP content purified from CHO host cells (Jawa et al. 2016), and in a study by Schenauer *et al* (2012), detectable HCP were identified by using mass spectrometry in purified preparations of an Fc fusion biotherapeutic expressed in *E.coli*. The impact of these HCP on immunogenicity was not explored; however, within the twenty most abundant *E.coli* cell proteins in the preparation, three HSPs were identified: the 60kDa chaperonin GroEL, the chaperone ClpB and the 70kDa HSP DnaK. One of the key functions of HSPs is to bind hydrophobic regions on unfolded proteins to prevent aggregation and facilitate protein folding (Becker and Craig 1994). Since aggregates consist of partially unfolded proteins with exposed

hydrophobic regions, it would be anticipated that HSPs are likely to bind with high affinity to these regions. This is of particular relevance for biotherapeutics, where HSPs which are present as process related impurities, might bind to partially unfolded or aggregated proteins. It is widely acknowledged that aggregation itself can contribute to immunogenicity (Ratanji et al. 2014) and it is possible, therefore, that the presence of HSP could increase further their immunogenic potential.

We have previously shown that aggregates of a humanized single chain variable antibody fragment (scFv) caused a Th1-skewing of the immune response in BALB/c strain mice (Ratanji et al. 2016). The purpose of these investigations was to characterise the potential impact of bacterial HCP impurities on the immunogenic activity of protein biotherapeutics using the same system. To this end the scFv and mouse serum albumin were used as test proteins to generate aggregates, and the effect of addition of the *E. coli* HSP DnaK on immunogenicity was examined.

4.4 Methods

Single chain variable antibody fragment purification

A humanized single chain variable antibody fragment (scFv) (Edwardraja et al. 2010), was cloned into a pET-22b vector in Shuffle T7 express *E.coli* cells (New England Biolabs, Beverly, MA, USA). Transformants were cultured at 30°C to an optical density (OD) of 0.8 at 600 nm, induced with isopropyl β-D-1-thiogalactopyranoside and incubated overnight at 16°C. Cell pellets were resuspended, sonicated and centrifuged at 28700 g for 30 min. scFv was purified from supernatants using DEAE (diethylaminoethanol) Sepharose anion exchange chromatography, followed by Protein A affinity and size exclusion chromatography (Ratanji et al. 2016). Protein concentrations were determined by measuring the absorbance at 280 nm using an extinction coefficient of 58580 M^{-1} cm^{-1}.

For SEC-MALLS (SEC coupled with multi angle laser light scattering) analysis the SEC column outlet was connected to a Dawn Helios MALLS photometer (Wyatt, Santa Barbara, CA, USA) followed by an OptiLab T-rEX differential refractometer (Wyatt). Data were processed using the Wyatt-QELS software. Samples were processed by staff at the Biomolecular Analysis Facility at the University of Manchester.

SDS-PAGE and mass spectrometry

Protein samples were diluted in SDS-PAGE sample buffer (Biorad, Berkley, CA, USA) containing 1% 2-mercaptoethanol and heated for 3 min at 90°C. Samples were resolved on a pre-cast 10% acrylamide gel (Biorad) and stained using InstantBlue™ Coomassie protein stain (Expedeon, Swavesey, UK). *E.coli* host cell protein levels in scFv preparations were monitored by excising selected bands and analysis by liquid chromatography-mass spectrometry (LC/MS).

Data were processed using the statistical validation software Scaffold (version 3.0.04; produced by Proteome Software). Samples were processed by staff at the Biomolecular Analysis facility at the University of Manchester.

Generation of aggregates

scFv: Purified monomeric scFv was diluted to 1 mg/ml in Dulbeccos phosphate buffered saline (PBS) without Ca^{+2} or Mg^{+2} (Sigma-Aldrich, St Louis, Missouri) and heated at 40°C for 25 min. Aggregates formed were stable and did not dissociate into monomers when the temperature was subsequently decreased by refrigeration, and after storage at -80°C.

Mouse albumin: Lyophilized mouse albumin (MP Biomedicals, Santa Ana, CA, USA) was diluted to 5 mg/ml in PBS without Ca^{+2} or Mg^{+2} and stressed by heating at 60°C for 24 h. The solution was then diluted to 1 mg/ml in PBS. Aggregates formed were stable and did not dissociate into monomers following dilution and freeze/thaw after storage at -80°C. Protein concentrations of mouse albumin were determined from the absorbance at 280 nm using an extinction coefficient of 46030 M^{-1} cm^{-1}.

Monomeric preparations were passed through a 0.2 µm filter (Millipore, Billerica, MA, USA) and centrifuged prior to use in immunisations or assays. These protein preparations were confirmed to be monomeric using DLS.

Aggregate analysis: Dynamic light scattering (DLS)

Measurements of DLS were performed with a Malvern Zetasizer Nano ZS ZEN3600 (Malvern, Herrenberg, Germany), equipped with a 633 nm laser. Each sample (70 µl) was measured in a Suprasil® quartz cuvette (Hellma GmbH, Muellheim, Germany) with a path length of 3 mm and 200-2500 nm spectral range. Monomeric and stressed samples at 1 mg/ml were measured at 25°C to determine the volume-based average protein particle diameter in solution.

Aggregate analysis: Circular Dichroism

Far-UV circular dichroism (CD) was used to study the secondary structure of the protein before and after heat stress (see supplementary Figure 1). The

measurements were performed with a Jasco J-815 CD spectrometer in combination with a Jasco PTC-423S temperature controller (Jasco International, Tokyo, Japan) at 25°C. The samples were measured in quartz cuvettes (Hellma GmbH) with a path length of 1 mm. CD spectra were collected in a continuous scanning mode from 190 to 260 nm. The measurements were performed at a scanning speed of 50 nm/min, a response time of 2 s, a bandwidth of 1 nm, a sensitivity of 100m, steps of 0.5 nm, and an accumulation of 6 scans. Data were analyzed using the Spectra Analysis Software Dichroweb and reference data set SMP180 (Abdul-Gader et al. 2011). Data were converted to mean residue ellipticity by the software using a mean amino acid residue mass of 113.

Animal experiments

Female BALB/c strain mice were used for these experiments (Envigo, Bicester, UK). Mice were housed on sterilized wood bedding with materials provided for environmental enrichment. Food (Beekay Rat and Mouse Diet No1 pellets; B&K Universal, Hull, UK) and water were available *ad libitum*. The ambient temperature was maintained at 21 +/- 2°C and relative humidity was 55 +/- 10% with a 12 h light/dark cycle. All procedures were carried out in accordance with the Animals (Scientific Procedures) Act 1986, and approved by Home Office licence. Mice were immunized by intraperitoneal (ip) injection with 250 µl of 1 mg/ml protein (monomeric or aggregate) in PBS on days 0 and 7 and exsanguinated on day 14. In some experiments mice received an additional immunization on day 14 and were terminated on day 21. In some experiments protein preparations were ‘spiked’ with recombinant *E.coli* DnaK (Enzo Life Sciences, Farmingdale, NY, USA) at a 1 in 1000 dilution (0.1% by mass) relative to the immunizing protein. DnaK was added before and/or after the aggregation treatment. Individual and pooled serum samples were prepared and stored at -80°C until analysis.

Antibody ELISA

Plastic Maxisorb® plates (Nunc, Copenhagen, Denmark) were coated with 10 µg/ml scFv or 50 µg/ml mouse albumin in PBS overnight at 4°C. Where indicated in some experiments, protein preparations were spiked with lipopolysaccharide (LPS) from *E.coli* serotype 055:B5 (Sigma Aldrich) to achieve final LPS concentrations of 10 and 25 µg/ml. scFv coated plates were blocked with 2% (w/v) bovine serum albumin (BSA)/PBS (Sigma Aldrich) at 37°C for 30 min. Mouse albumin coated plates were blocked with 5% (w/v) skimmed milk proteins in 0.05% (v/v) Tween 20 in PBS for 1 h at room temperature. Doubling dilutions of serum samples were added (starting dilution 1 in 16, 1 in 32 or 1 in 64) in 1% BSA/ PBS for scFv experiments, or 2% skimmed milk proteins in 0.02% Tween 20 in PBS for mouse albumin experiments and incubated for 3 h at 4°C. Negative control naïve mouse serum (NMS) samples were analyzed concurrently. Plates were incubated for 2 h at 4°C with horseradish peroxidise (HRP) labelled sheep anti-mouse IgG diluted 1: 4000 (Cat no: AAC10P), goat anti-mouse IgG1 diluted 1: 2000 (Cat no: STAR132P), goat anti-mouse IgG2a diluted 1: 1000 (Cat no: STAR133P) (all Serotec, Kidlingtion, Oxfordshire) or goat anti-mouse IgM diluted 1: 6000 (Cat no: 62-6820; Invitrogen, Paisley, UK) diluted in 1% BSA/ PBS for scFv experiments, or 2% skimmed milk proteins in 0.02% Tween 20 in PBS for mouse albumin experiments. Plates were washed between incubations with 0.05% Tween 20 in PBS. Plates were incubated with substrate (1.6 mg/ml *o*-phenylenediamine and 0.4 mg/ml urea hydrogen peroxide in 0.5M citrate phosphate buffer [pH5]) for 15 min and reactions were stopped with 0.5M citric acid. Absorbance was read at 450 nm using an automated reader (ELx800; BioTek Instruments, Inc, Winooski, US), using Gen 5 1.10 software. Data are displayed as OD450 nm values ± SEM.

Anti-DnaK Western blot

For Western blot analysis, protein samples were diluted in sample buffer (Biorad, Berkley, CA, USA) containing 1% (v/v) 2-mercaptoethanol and heated for 3 min at 90°C. Samples were resolved on a pre-cast 10% acrylamide gel and proteins transferred onto a nitrocellulose membrane. *E.coli* DnaK was detected using mouse anti-DnaK IgG1 antibody (Cat no: ADI-SPA-880; Enzo Life Sciences) diluted 1:10000. Blots were incubated with a HRP-labelled sheep anti-mouse IgG antibody (Serotec) diluted 1: 4000 and proteins visualized using enhanced chemiluminescence reagents (Thermo Scientific; Waltham, MA, USA).

Statistical analyses

Statistical analyses were performed using the software Graphpad Prism 6. Analysis of variance (ANOVA) was used to determine statistical significance of differences between more than two groups. Experiments were analyzed by non-parametric one way or two way ANOVA followed by the Tukey post hoc test, or Student's t-test (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

4.5 Results

Our previous work showed that a humanized scFv fragment, which was purified from an *E.coli* expression system, was an ideal test protein to study the effects of aggregation on the immune response (Ratanji et al. 2016). In the course of investigating protein purification, we analyzed the eluted fractions from Protein A affinity chromatography by SEC; separate peaks were observed (Fig. 4.1A). These fractions from SEC were subjected to SEC-MALLS analysis (SEC coupled to multi angle laser light scattering) to determine the mass of scFv in each of the peaks observed. The molecular mass estimate of Fraction M was approximately 26 kDa, and Fraction A was separated into three peaks on SEC-MALLS, which were approximately: 52 kDa, 80 kDa and 104 kDa (not shown). The higher molecular mass fractions eluted at positions consistent with the formation of dimers, trimers and tetramers of the scFv. For the purposes of immunological analyses, the higher molecular mass fractions were pooled and designated as Fraction A. The lowest molecular mass fraction from the SEC column was identified as monomer by SEC-MALLS, and designated Fraction M. Fractions A and M from SEC were run directly on denaturing SDS-PAGE for further characterization. After staining with Coomassie blue, protein bands from each fraction were identical in size, and only one band was observed in each case (data not shown). Incubation at 40°C for 25 min of both Fraction A and M resulted in the formation of aggregate populations that were apparently identical by DLS, with 2 µm (±1 µm) in mean diameter (Fraction A shown, Fig 4.1B) compared with a mean diameter of 7 nm for the monomeric untreated fraction. Aggregates were also stable following freeze/thaw, as verified using DLS.

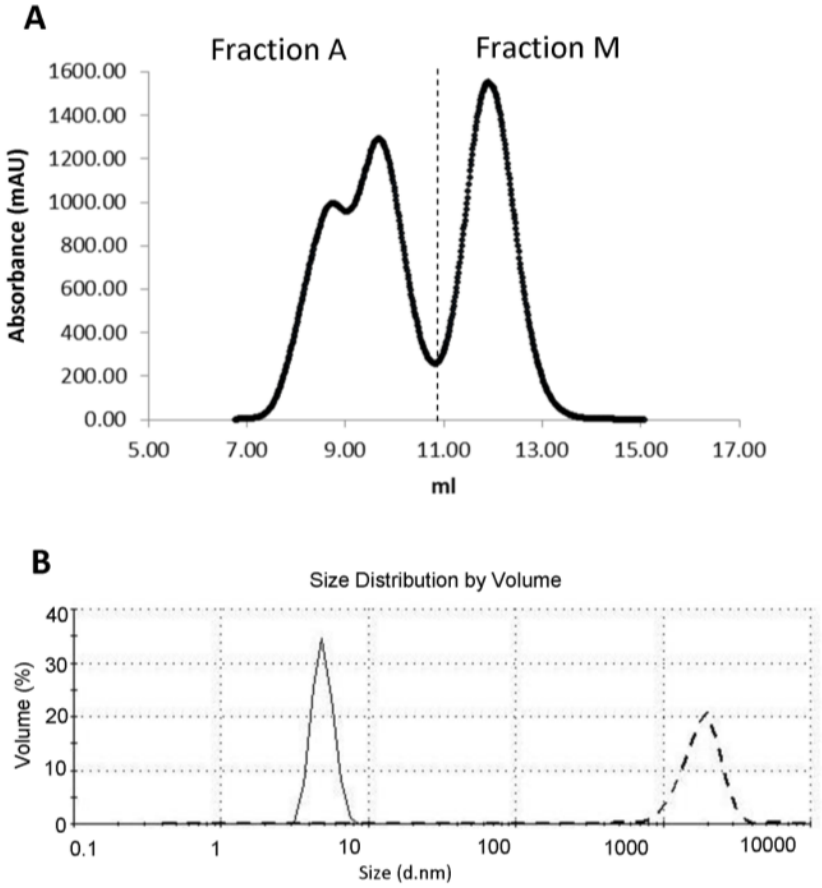


Figure 4.1.

scFv purification and aggregation. A) Pooled citrate elution fractions from Protein A affinity chromatography of scFv were fractionated by SEC using a 24 ml SD75 column, at a flow rate of 0.5 ml/min, and using PBS pH 7 as a column buffer. Absorbance at 280 nm is presented against the elution volume for a single representative run. B) The DLS profiles of 1 mg/ml Fraction A (oligomeric) scFv in PBS at pH 7 before and after heat treatment for 25 min at 40ºC are presented (the equivalent Fraction M size profile after heat treatment has been reported previously [22]). A change in protein particle diameter was observed between native (**-**) and heat treated (**- - -**) protein by DLS using the Malvern zetasizer.

Influence of aggregation on immunogenicity: presence of HCP

To investigate the impact of protein aggregation upon immunogenicity, scFv monomer (Fraction M) and aggregate (derived from Fraction A) preparations were

administered via ip injection to BALB/c strain mice on day 0 and 7, (n=5 per group) and sera isolated on day 14. The presence of anti-scFv IgG, IgG1 and IgG2a antibody in serum samples was analyzed by ELISA (Fig. 4.2). Antibody binding was analyzed against both monomer (Fraction M) and aggregated (Fraction A heat treated) protein substrates and compared with background activity detected in naive mouse sera (NMS) as a negative control.

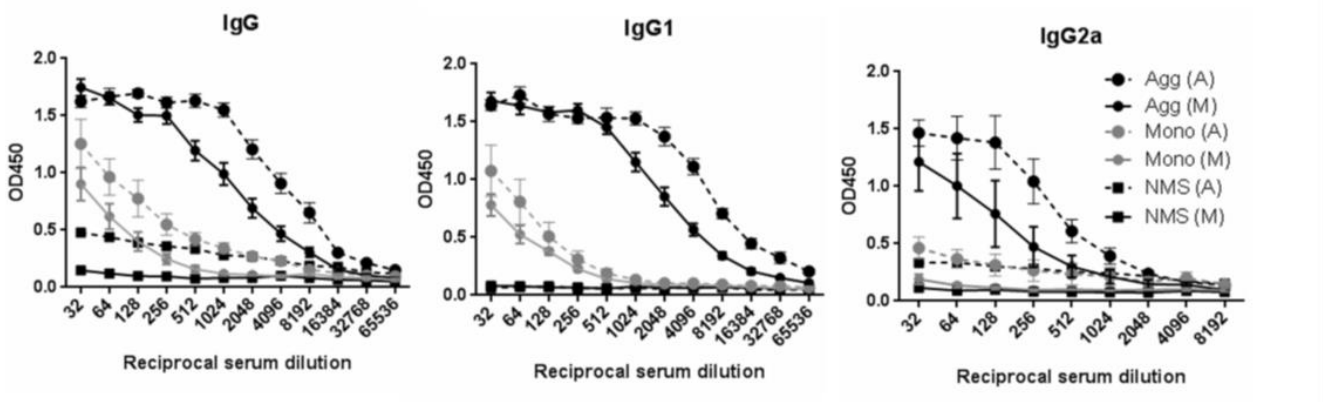


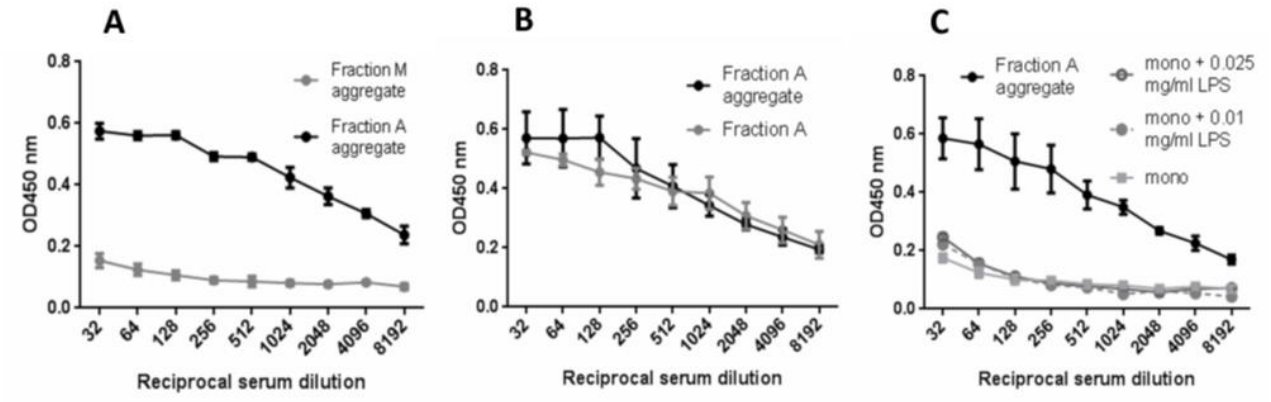
Figure 4.2. A comparison of scFv Fraction M and Fraction A aggregate immunogenicity. Fraction A scFv was subjected to heat treatment for 25 min at 40°C. Mice were immunized by ip injection with 250 µl of 1 mg/ml monomer (Fraction M) or heat aggregated (Fraction A) scFv on days 0 and 7 and serum isolated on day 14 (n=5 per group). Doubling dilutions of serum samples from scFv monomer (Mono) and aggregate (Agg) immunized animals and negative control naïve serum samples (NMS) were analyzed against both monomeric (M) and aggregated (A) scFv substrates by ELISA for IgG IgG1 and IgG2a anti-scFv antibody content. Data are displayed as OD450 nm (±SEM) for each reciprocal serum dilution (32 to 65536 for IgG and IgG1 antibodies; 32 to 8192 for IgG2a antibodies). Statistical significance of differences in antibody binding between all sera groups against each substrate were calculated using a one way ANOVA on the area under the curve (*p<0.05 **p<0.01, ***p<0.001). P values are cited in the results text where statistical significance was achieved.

Analysis of the serum dilution curves revealed that both forms of scFv induced detectable IgG and IgG1 antibody production compared with NMS samples, although only the aggregate provoked significant levels of IgG2a antibody. Indeed, for serum raised against the aggregated material, significantly higher binding was observed against both substrates compared with sera from monomer immunized mice, for total IgG and both subclasses (***$p<0.001$). Additionally, higher antibody binding against the aggregate substrate compared to monomer was observed for all serum samples, regardless of whether immunization was with the monomer or the aggregate, with a shift to the right of all titration curves to a broadly similar extent.

When plates were coated with monomeric protein, low background OD450 nm readings of less than 0.1 were recorded for each of the isotypes in the absence of serum (reagent blank samples) or following incubation with NMS. However, there were relatively high levels of binding of NMS against the aggregate substrate for IgG and IgG2a, but not IgG1, detection antibodies, with OD450nm readings of up to 0.5 recorded. Further ELISAs were carried out in order to assess whether the high background for NMS samples was due to aggregation *per se* (and hence exposure of neoepitopes following heat treatment) or whether it was due to a contaminant within the high molecular mass Fraction A. IgG antibody present in NMS against native oligomeric scFv from fraction A and the heat-treated protein was assessed (Fig. 4.3). Fraction M (monomer) was also aggregated to ~2 μm using heat treatment, and antibody binding analyzed. Furthermore, to identify whether lipopolysaccharide (LPS) present in the protein preparation might be responsible for the antibody binding activity of naive sera, 0.01 mg/ml monomeric protein was spiked with LPS at concentrations of 0.01 mg/ml and 0.025 mg/ml and used as ELISA substrates. IgG levels in naïve sera directed against aggregated Fraction A were significantly higher than against aggregated Fraction M (***$p<0.001$; Fig 4.3A). Furthermore, antibody binding against aggregated and non-aggregated Fraction A was virtually identical. No

change in antibody binding was detected when the monomeric substrate was spiked with LPS (Fig 4.3C). These results indicated the presence of contaminant(s) in the Fraction A that were absent from the monomeric Fraction M, to which there is naturally occurring antibody in naïve mice. Mass spectrometric analysis was therefore carried out on Fractions A and M to identify any *E.coli* proteins present.

Figure 4.3. Naïve mouse sera IgG antibody binding to scFv substrates. Plates were



coated with protein substrates at 10 µg/ml. Binding of IgG in naïve mouse sera to substrates was measured (n=3 independent analyses of different samples of pooled NMS ±SEM). Doubling dilutions of serum samples (starting 1/32) from naïve mice were prepared for incubation in the ELISA. Data are displayed as OD450 nm (±SEM) for each reciprocal serum dilution. Statistical significance of differences in IgG binding between substrates was calculated using a Students' *t*-test on the area under the curve **A)** IgG binding against heat-treated fraction A and fraction M. **B)** IgG binding against heat treated and native fraction A. **C)** IgG binding against monomer (mono) or Fraction A aggregate substrates, or monomer spiked with LPS to concentrations of 10 and 25 µg/ml. Statistical significance was calculated using one way ANOVA on the area under the curve (*p<0.05 **p<0.01, ***p<0.001). P values are cited in the results text where statistical significance was achieved.

A number of *E.coli* proteins were identified in Fraction A which were absent from Fraction M, of which the HSPs DnaK and GroEL were the most abundant. Given the documented effect of HSPs on adjuvanticity (Atzingen et al. 2014; McNulty et al. 2013), we hypothesized that these contaminants could be responsible for the enhanced IgG responses for Fraction A versus Fraction M aggregates presented in

Fig 2. The HSP protein DnaK was selected as a candidate to test this hypothesis. SDS-PAGE analysis of scFv spiked with recombinant *E.coli* derived DnaK demonstrated that a DnaK protein band was not visible below a concentration of 5 µg/ml (data not shown). Since a band from a low abundance contaminant would not be detectable when Fraction A was analyzed by SDS-PAGE/Coomassie staining, it was inferred that the DnaK concentration in Fraction A was less than 5 µg/ml. A dose level of 1 µg/ml, which provided a ratio by mass of 1 part DnaK to 1000 parts scFv, was therefore used for further experiments.

The ability of DnaK to bind selectively to aggregated, as compared with monomeric, scFv was analyzed by SDS-PAGE and Western blot (Fig 4.4A). After sedimentation of scFv aggregate plus recombinant DnaK, the HSP accumulated in the pellet (lane 4), with very little remaining in the supernatant (lane 6). Protein concentration was measured in the pellet and supernatant after centrifugation; protein was present in the supernatant at approximately 0.28 mg/ml, whereas the pellet resuspended in 100 µl had a concentration of approximately 3.2 mg/ml, therefore the pellet was enriched with the aggregate, to which DnaK was bound, with some scFv remaining in solution (for the Western blot, the pellet was resuspended in a volume of 30 µl so the whole sample could be run on a gel). Aggregate and monomer without recombinant DnaK contained no detectable DnaK by blot. A control with monomeric scFv plus DnaK which was not subject to aggregation failed to produce a pellet after centrifugation and the DnaK remained in the supernatant (lanes 11 and 13). The detection of DnaK in the monomer supernatant (lane 13) appears fainter than the aggregate pellet (lane 4) because the volume of supernatant was larger than the volume that the pellet was resuspended in.

The monomeric fraction of scFv from SEC was utilized to prepare the aggregate and this fraction was confirmed to be free from *E.coli* HCP contamination by mass

spectrometry, and by lack of binding of NMS in ELISA and anti-DnaK antibody in a Western blot.

Effect of DnaK and aggregation on immunogenicity of scFv

1 mg/ml monomeric scFv was spiked with DnaK (1 µg/ml; 1000:1) and aggregated by heat treatment for 25 min at 40°C. Monomer and aggregate protein preparations with and without the addition of DnaK, either before or after aggregation, were administered via ip injection (250 µg) to mice on day 0, 7 and 14 and sera isolated on day 21. IgG, IgG1, IgG2a and IgM antibody binding was analyzed in sera from immunized mice against monomeric scFv (Fig 4B). The results confirmed the baseline immunogenicity of the monomeric scFv observed previously (Fig 2) with respect to the production of total IgG, IgG1 and IgG2a anti-scFv antibody. An IgM response was also observed for monomeric scFv (Fig 4B). Addition of DnaK had no significant impact on the IgG or IgM response to monomer (see Table 4.1 for statistical analyses of differences). Aggregation was without effect on IgG1 anti-scFv antibody production, but significantly enhanced IgG2a and IgM antibody production (***$p<0.001$). The addition of DnaK enhanced the IgG2a anti-scFv response in aggregated preparations (**$p<0.01$), but was without effect on IgG1 or IgM antibody production. We infer that the increase in total IgG signal was due to an increase in IgG2a, as IgG1 antibody production was not up-regulated. The impact of DnaK on antibody binding was comparable when DnaK was added before or after heat treatment (total IgG: ***$p<0.001$ monomer vs aggregate with DnaK added before or after aggregation). The immunogenicity of DnaK alone at 1 µg/ml was also tested as a control and found to have no effect (data not shown). In subsequent dose response experiments the selective adjuvant effect of DnaK on anti-scFv IgG2a antibody responses was confirmed, and it was shown that doses below the 1 µg/ml concentration or 1: 1000 ratio (0.1 and 0.01 µg/ml) were insufficient to cause a marked impact on antibody responses (data not shown).

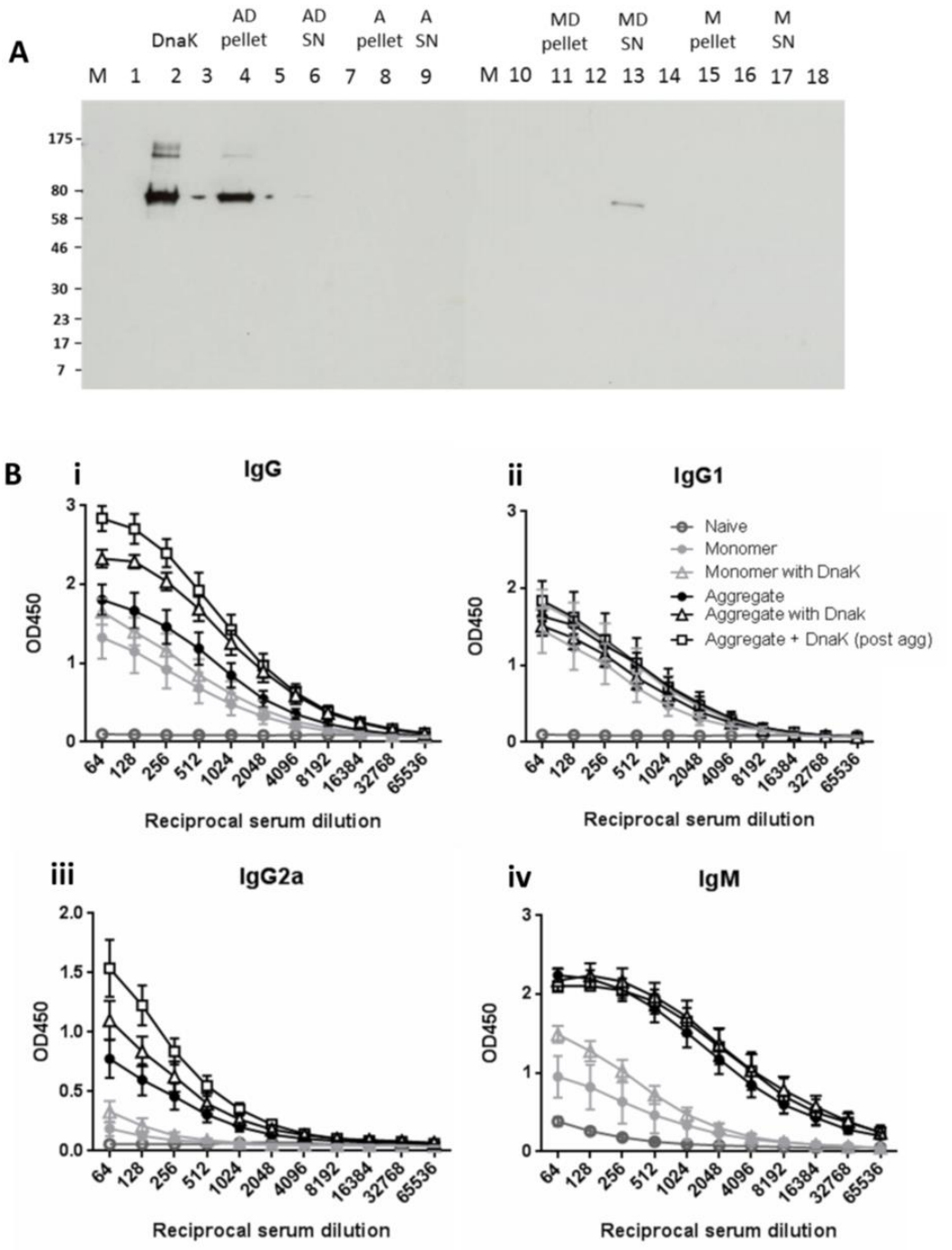


Figure 4.4. DnaK detection in scFv pellets and supernatants, and antibody responses to scFv monomer and aggregate spiked with DnaK. A) Western blot of DnaK samples added to monomeric and aggregated scFv. 0.5 µl DnaK (1 mg/ml) was added to 500 µl scFv (1 mg/ml) and either untreated (monomer), or heat treated at 40°C for 25 min followed by centrifugation at 13000rpm. Monomeric and aggregated scFv

without DnaK was also centrifuged and supernatants and pellets harvested. For monomeric samples, where pellets were not observed, supernatant was removed after centrifugation and 30 µl PBS added to the tube to dissolve any unseen pellet. Protein pellets resuspended in 30 µl PBS, 30 µl of supernatants, and 0.5 µg recombinant *E.coli* DnaK were analyzed by Western blotting using an anti-DnaK antibody. A protein marker lane (M) on each gel was used to determine the molecular mass. Lanes labelled 1, 3, 5, 7, 10, 12, 14, 16 and 18 were left blank in case of sample spill-over. Samples in corresponding lanes are as follows: 2) 0.5 µg DnaK 4) Aggregate with DnaK (AD) Pellet 6) AD Supernatant 8) Aggregate (A) pellet 9) A supernatant 11) Monomer with DnaK (MD) pellet 13) MD supernatant 15) Monomer (M) pellet 17) M supernatant **B)** ELISA analysis of antigen-specific antibody in sera. scFv at 1 mg/ml in PBS pH 7 was kept alone or spiked with DnaK at 1 µg/ml then subjected to heat treatment for 25 min at 40°C; another preparation was spiked with DnaK after heat treatment (post agg). Mice were immunized with 250 µl of these aggregate preparations by ip injection on days 0, 7 and 14 and serum isolated on day 21 (n=6 per group n3=aggregate + DnaK post agg). Doubling dilutions of serum samples from immunized animals and negative control naïve serum samples were analyzed against scFv substrate by ELISA for i) IgG ii) IgG1 iii) IgG2a and iv) IgM anti-scFv antibody content. Data are displayed as OD450 nm (±SEM) for each reciprocal serum dilution (64 to 65536). Statistical analysis of differences in antibody binding between all immunised groups is summarized in Table 4.1.

Adjuvant-like effect of DnaK on mouse albumin immunogenicity

Human proteins are used in a clinical setting, and trigger a much lower immune response in the absence of additional aggravating factors, such as aggregation. We therefore set out to investigate whether the adjuvant-like effect of DnaK could be reproduced with a murine protein. Mouse albumin was selected as a well characterized, commercially available 'self' protein. The adjuvant effect of DnaK on the response to mouse albumin, in monomeric and aggregated forms was then investigated in a similar fashion to scFv. A number of strategies were attempted to induce aggregation, including a range of temperature treatments, incubation times, stir stress, and the use of various buffers. Mouse albumin required harsher conditions than the scFv to reproducibly form aggregates: we identified heat treatment at 60°C for 24 h as the optimum condition, generating a maximum particle size of ~50nm, somewhat smaller than the scFv aggregates (Fig. 4.5A). Given the relatively high temperature required, the extent of unfolding of the protein was examined by CD analysis. Monomer and heat aggregates of both scFv and mouse albumin were analysed; whereas the scFv demonstrated little unfolding following aggregation, an overall reduction of about 50% in secondary structure content was observed after heat treatment of the mouse albumin (Fig. 4.5 B and C).

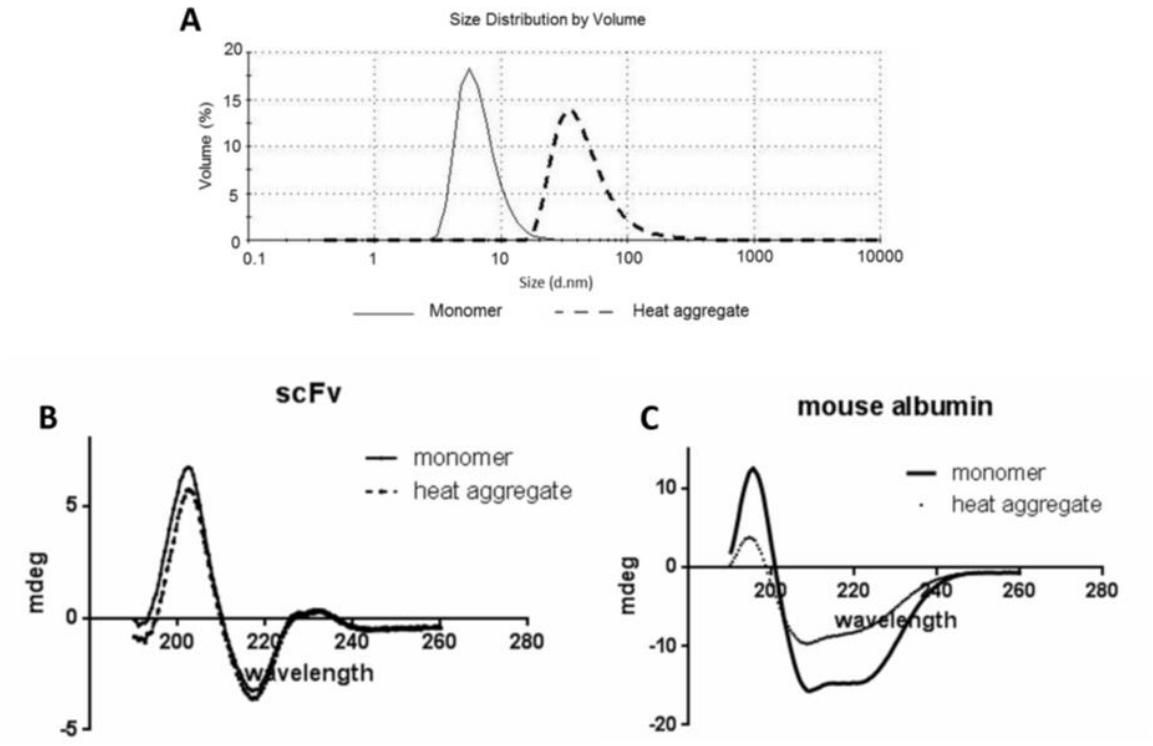
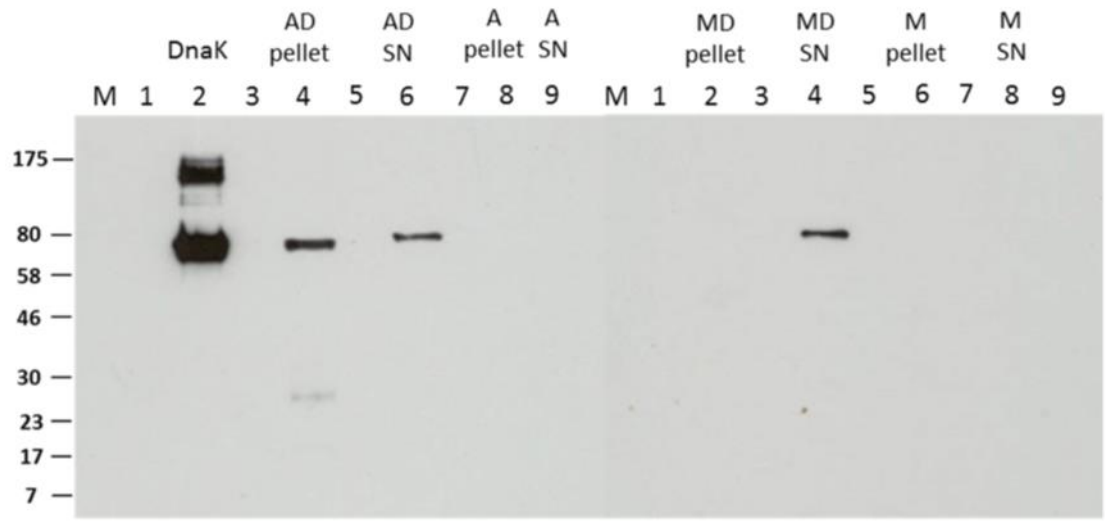


Figure 4.5. DLS and circular dichroism spectra of native and heat treated proteins. A) DLS of mouse albumin. Mouse albumin at 5 mg/ml in PBS pH 7 was heated for 24 h at 60°C, then diluted to 1 mg/ml in PBS. The mean particle diameter was measured by DLS before (i) and after (ii) the heat treatment. Circular dichroism measurements were recorded using a Jasco J-815 CD spectrometer at 25°C. **B)** scFv was measured at 1mg/ml in PBS as monomer, or after heat treatment at 40°C for 25 min (dashed line). **C)** Mouse albumin was measured as monomer at 1 mg/ml in PBS. Mouse albumin was stressed 5 mg/ml in PBS by heating at 60°C for 24 h; the solution was then diluted to 1 mg/ml in PBS before measurement (dotted line).

Recombinant *E.coli* DnaK was added into 1 mg/ml heat aggregated mouse albumin to a final concentration of 1 µg/ml, and its association with DnaK analyzed by Western blot (Fig. 4.6). As the mouse albumin aggregates (~50 nm) were much smaller than the scFv aggregates (~2000 nm), centrifugation at 13 000 rpm was insufficient to separate the aggregates and ultracentrifugation at 100 000 rpm was required. However, in this case the concentration of mouse albumin present in the supernatant after centrifugation was approximately 0.45 mg/ml, and 4 mg/ml in the pellet when

resuspended in a volume of 100 µl, indicating that only a proportion of the aggregate was fractionated into the pellet (which appeared visibly smaller than the pellet achieved with the scFv aggregate under the same conditions).

Figure 4.6. DnaK detection in mouse albumin pellets and supernatants. Mouse albumin



was heat treated at 60°C for 24 h at 5 mg/ml, then diluted to 1 mg/ml and kept alone, or 0.5 µl DnaK (1 mg/ml) was added to 500 µl aggregated mouse albumin. Mouse albumin was also diluted to 1 mg/ml and kept alone or with the addition of 1 µg/ml DnaK. Samples were subjected to ultracentrifugation at 100,000 rpm for 30 min, and supernatants and pellets harvested. For monomeric samples, where pellets were not observed, supernatant was removed after ultracentrifugation and 30 µl PBS added to the tube to dissolve any unseen pellet. Protein pellets resuspended in 30 µl PBS, 30 µl of supernatants, and 0.5 µg recombinant *E.coli* DnaK were analyzed by Western blotting using an anti-DnaK antibody. A protein marker lane (M) on each gel was used to determine the molecular mass. Lanes labelled 1, 3, 5, 7, 10, 12, 14, 16 and 18 were left blank in case of sample spill-over. Samples in corresponding lanes are as follows: 2) 0.5 µg DnaK 4) Aggregate with DnaK (AD) Pellet 6) AD Supernatant 8) Aggregate (A) pellet 9) A supernatant 11) Monomer with DnaK (MD) pellet 13) MD supernatant 15) Monomer (M) pellet 17) M supernatant.

Western blot analysis of DnaK content demonstrated that the HSP was present in both the pellet and supernatant in similar amounts, although the pellet fraction

appeared slightly larger (Fig. 4.6 lanes 4 and 6). The distribution of DnaK between supernatant and pellet fractions is therefore consistent with preferential binding of the HSP to albumin aggregate, in a similar fashion to the scFv aggregates in Fig 4.4. Aggregate and monomer without DnaK added contained no detectable DnaK by blot, and DnaK did not sediment under these conditions when it was added to the monomer, but remained in the supernatant (Fig. 4.6 lanes 11 and 13).

Mice were immunized with mouse albumin monomer or aggregate, with or without the addition of DnaK (at a 1 in 1000 ratio). In order to determine whether identical epitopes were present on both monomeric and aggregated proteins, parallel plates were coated with monomer or aggregate substrates. IgM, IgG, IgG1 and IgG2a antibody binding was analyzed in sera from immunized mice against both substrates, and compared with background activity detected in NMS (Fig. 4.7). As expected, there was little antibody detected in serum from monomer-immunized animals: IgG, IgG2a and IgM levels were identical to NMS, regardless of the presence of DnaK, and there was no difference between antibody binding to monomer or to aggregated substrates. Low levels of IgG1 antibody were detected, but this was independent of substrate used and was also unaffected by the presence of DnaK. Aggregation resulted in a more vigorous immune response for all antibody isotypes, but this was dependent on the substrate utilized in the ELISA, with aggregated substrate showing much higher antibody binding. Total IgG, IgG1 and IgG2a anti-mouse albumin antibody responses were enhanced by the presence of DnaK, most notably for IgG2a (*$p<0.05$; Table 4.1).

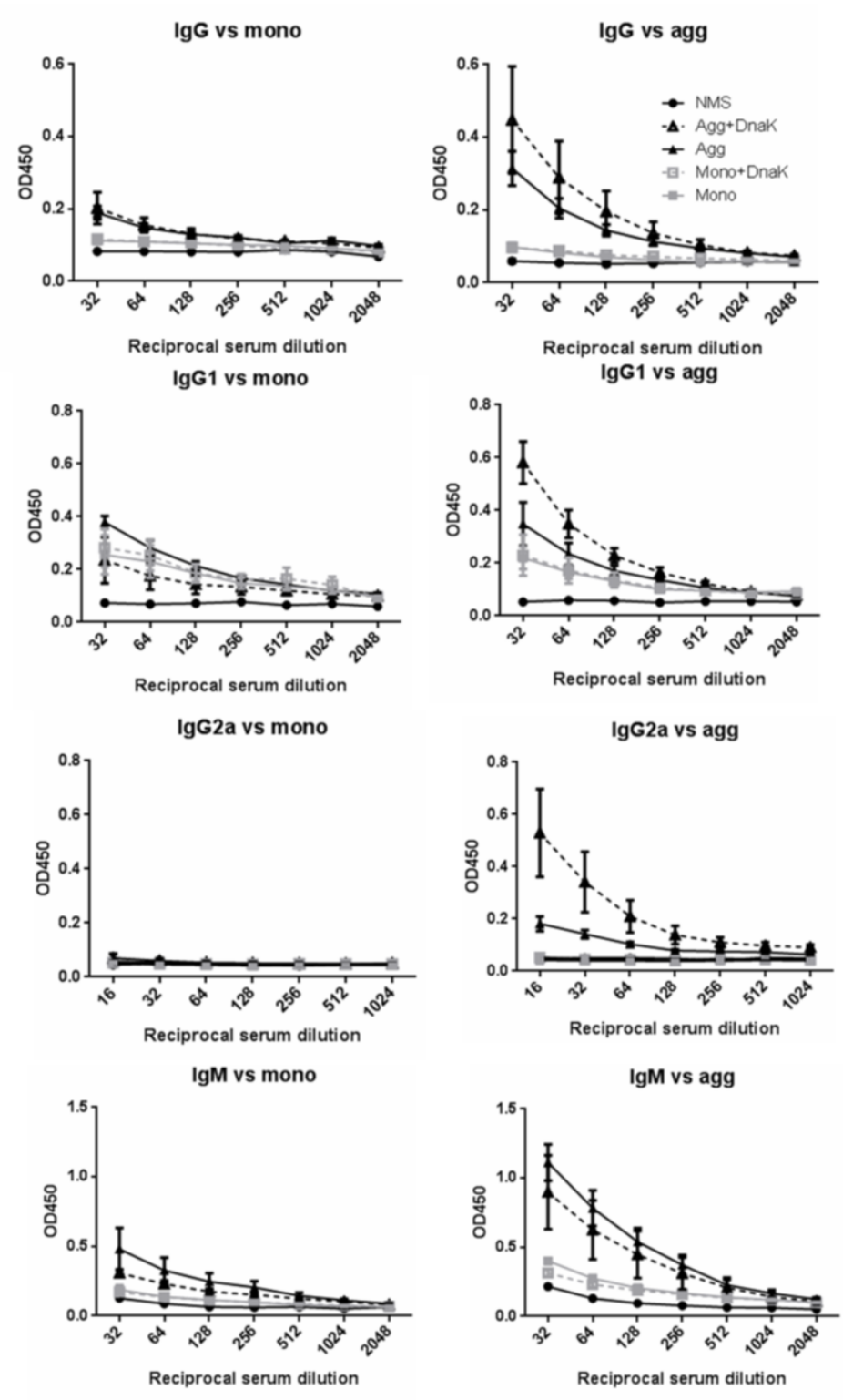

IgG vs mono
IgG vs agg
NMS
Agg+DnaK
Agg
Mono+DnaK
Mono
IgG1 vs mono
IgG1 vs agg
IgG2a vs mono
IgG2a vs agg
IgM vs mono
IgM vs agg
OD450
Reciprocal serum dilution

Figure 4.7. Characterization of immune responses to mouse albumin: comparisons of monomer and heat stressed aggregates. Mouse albumin at 5 mg/ml in PBS pH 7 was subjected to heat treatment for 24 h at 60°C, then diluted to 1 mg/ml in PBS. Mice were immunized by ip injection with 250 µl of 1 mg/ml monomer or heat aggregated mouse albumin, with or without the addition of DnaK at 1 µg/ml on days 0, 7 and 14 and serum isolated on day 21 (n=3 per group). Doubling dilutions of serum samples from scFv monomer (Mono) and aggregate (Agg) immunized animals and negative control naïve serum samples were analyzed against both scFv substrate proteins (vs monomer [left] and vs aggregated protein [right]) by ELISA for IgG, IgG1, IgG2a and IgM anti-scFv antibody content. (i) Data are displayed as OD450 nm (±SEM) for each reciprocal serum dilution (1 in 32 to 2048 for IgG, IgG1 and IgM antibodies; 1 in 16 to 1024 for IgG2a antibodies). Statistical analysis of differences in antibody binding against the aggregated substrate between all immunized groups is summarized in Table 4.1.

Table 4.1.

Statistical analysis of antibody quantification by serum dilution

Antibody isotype	**P value**	**Condition 1 (immunizing material)**	**Condition 2 (immunizing material)**
scFv + DnaK (Figure 4)			
Total IgG	***p<0.001	monomer with or without DnaK	aggregate with DnaK
Total IgG	**p<0.01	aggregate	aggregate with DnaK
IgG1	No significant differences between immunized groups		
IgG2a	***p<0.001	monomer	aggregate with or without DnaK
IgG2a	**p<0.01	aggregate	aggregate with DnaK
IgM	***p<0.001	monomer with or without DnaK	aggregate with or without DnaK
Mouse albumin (vs aggregate substrate only; Figure 7)			
Total IgG	*p<0.05	monomer	aggregate
Total IgG	***p<0.001	monomer with DnaK	Aggregate with DnaK
IgG1	No significant differences between immunized groups		
IgG2a	***p<0.001	monomer with or without DnaK	aggregate with or without DnaK
IgG2a	*p<0.05	aggregate	aggregate with DnaK

IgM	*p<0.05	monomer with or without DnaK	aggregate

For antibody data presented in Figures 4 and 7, statistical significance of differences in antibody binding between all immunized sera groups were calculated using a one way ANOVA applied to the area under the titration curve (*p<0.05, **p<0.01, ***p<0.001). Results are presented where values of p<0.05 were achieved between two immunization conditions.

4.6 Discussion

Preparations of biotherapeutic proteins inevitably contain HCP impurities. For the purposes of clinical administration, difficulties arise in deciding on an acceptable level of HCP impurities. Such considerations need to be based on evidence that, at particular levels, HCP may impact on therapeutic efficacy and/or safety. To date the focus has been primarily on the identification and quantification of HCP, the aim being controlling the levels of these proteins (Reisinger et al. 2014), and evaluation of their potential role as antigens (Zhu-Shimoni et al. 2014). Here we sought to address a somewhat different question regarding the adjuvant properties, rather than the immunogenic potential, of HCP. For this purpose we chose to consider the potential of HCP impurities to display adjuvant properties and modify and/or enhance the immune response to aggregated and non-aggregated third party proteins.

Initially, we observed that an oligomeric aggregate fraction of a humanized scFv resulted in a more vigorous anti-scFv response than did the monomer. Further analysis identified the presence of *E.coli* HCPs, including HSPs, in the oligomeric population. It is well established that protein aggregation has the potential to contribute to immunogenicity (Ratanji et al. 2014). Furthermore, the principal function of HSPs is to mediate the correct folding of proteins inside the cell and therefore, by nature, they bind to partially unfolded proteins (Becker and Craig 1994). We also noted that the ability of HSPs to enhance the immunogenicity of a co-administered

antigen is well-documented, with the successful use of HSPs in vaccine development (McNulty et al. 2013). We accordingly selected the *E.coli* HSP DnaK, as a candidate HCP impurity, which had been detected in the oligomeric scFv, and studied this as an individual HCP impurity. However, it is possible that the presence of multiple HCP may have a synergistic effect, and could explain why any adjuvant effect of DnaK alone might not be as strong as the effect of combined HCP impurities. We examined the effects of DnaK on the immunogenicity of aggregated forms of two different proteins. One, a scFv, was selected because we have characterised its immunological responses previously and it displays a relatively vigorous baseline immunogenicity (Ratanji et al. 2016). We also studied mouse albumin, as an exemplar protein for *in vivo* immunogenicity assessment as it would be treated as 'self' by the BALB/c mice.

The requirement of HSP binding to peptides in order to elicit immunogenicity has been shown previously: for example, HSP70-associated peptides derived from cancer tissues elicited tumour specific immunity (Udono et al. 1993; Srivastava et al. 1998). We therefore examined the ability of DnaK to bind to aggregates; Western blot analysis demonstrated that DnaK co-sediments with scFv aggregates, and was therefore bound to aggregates rather than remaining in solution. It is proposed that heat treatment of the scFv exposed hydrophobic regions on the protein, leading to aggregation and also enabling binding of the HSP. Binding studies by Western blot using mouse albumin were also consistent with DnaK binding preferentially to aggregates. Incomplete sedimentation of the mouse albumin aggregates may be due to their small size; however, detection of DnaK in the pellet does show that DnaK was bound to the aggregated protein. It is possible that the higher temperature used for heat treatment of mouse albumin compared with the scFv resulted in a more complete unfolding and denaturation of the protein structure. A CD spectrum of the aggregated

material supports this hypothesis and indicates a more disrupted secondary structure after stress treatment of the mouse albumin compared with the scFv aggregate.

Antibody analysis of serum samples from mice immunized with scFv monomer or aggregate (free from DnaK, as verified by mass spectrometry analysis), alone or spiked with recombinant *E.coli* DnaK, highlighted differences in the IgG2a response between monomeric and aggregated scFv. This was expected as we have previously shown that subvisible sized aggregates of scFv promote a Th1-skewed response (Ratanji et al. 2016). It is possible that the Th1 skewing is an effect of differential antigen processing and presentation, and this effect is also consistent with the hypothesis that the repetitive epitopes formed by aggregation can mimic characteristics of microbes and viruses (Kastenmueller et al. 2011). The anti-scFv response was higher still with the addition of DnaK, but in aggregated preparations only, where an increase in anti-scFv IgG and IgG2a was observed. This suggests an amplification of the Th1-skewed response with the presence of DnaK, as there was no difference in IgG1 between immunised groups. Importantly, the increased response occurred regardless of whether DnaK was added to the scFv preparation before or after heat treatment, indicating that the presence of DnaK during protein unfolding was not necessary for DnaK binding to the aggregates. This suggests that once the therapeutic has begun to aggregate, endogenous HSP may also associate with the unfolded protein. Furthermore, a dose response experiment showed that the adjuvant effect observed can be titrated out, as scFv spiked with DnaK to 1pp10000 resulted in anti-scFv responses that were comparable to aggregate alone.

In these experiments a dose of 250 µg scFv was used in mice, and so, for groups immunized with DnaK, each dose provided 0.25 µg of DnaK. Even after sophisticated purification steps, low levels of HCP may still remain in a final purified biotherapeutic (Doneanu et al. 2012), although it is important to note that the ratio of DnaK: scFv used in these experiments (1:1000) was higher than would be expected in most

biotherapeutic preparations used clinically. Accepted limits of HCP are generally 1-100ppm (Doneanu et al. 2012). However, 1ppm HCP could result in 0.1 µg HCP in a 100 mg dose (biotherapeutics given to patients can reach doses well above 100 mg [Leveque et al. 2005; Castro et al. 2014]). HSPs, by nature, bind with high affinity to partially unfolded proteins with exposed hydrophobic segments and aggregates (Doyle et al. 2013; Bukau and Horwich 1998) and so, even at very low levels will bind preferentially to any aggregates present and therefore have the potential to affect protein immunogenicity.

The scFv experiment was repeated using mouse albumin as the antigen. Mouse albumin was chosen as it is a commercially available murine antigen that could be aggregated within the subvisible size range. It also differs both in size and structure to the scFv, so was ideal to study as an alternative protein. Using a mouse antigen also allows investigations to mimic more closely the situation in humans where patients elicit an immune response to human derived biotherapeutics. Antibody analysis of sera from mouse albumin-immunized mice highlighted increased binding of aggregate immunized mouse sera to the aggregated protein substrate, and little or no binding to the monomeric substrate. This suggests that the heat treatment at 60°C required for mouse albumin aggregation resulted in the formation of immunogenic neo-epitopes that were not recognized by sera from monomer immunized mice. While there was no clear adjuvant effect observed with the addition of DnaK at the level of total IgG, the IgG2a response was higher with the addition of DnaK to the aggregate (*$p<0.05$), indicating that the Th1 skewing, which we have also observed with other aggregates, is enhanced when DnaK is present. An increase in IgM was also observed with aggregation of both the scFv and mouse albumin, with no effect when DnaK was present. This was not expected after a three week immunisation protocol as IgM production is a primary response, and the IgG analyses demonstrated that class-switching had occurred. It is possible that the persistence of IgM after three

immunizations may be due to immature B cell activation caused by antigen repetitiveness on the aggregates.

The adjuvant effect observed with DnaK contamination of scFv and mouse albumin aggregates is consistent with observations made by other investigators. It is well documented that HSPs are capable of exerting an adjuvant-like effect (Singh-Jasuja et al. 2001), although this is the first demonstration, to our knowledge, that it can amplify responses to protein aggregates. It is difficult, at this stage, to speculate on the immunological mechanisms which might underpin this effect. It is possible that DnaK interacts directly with receptors on immune cells, enhancing uptake of the aggregate. It has been reported that HSPs interact with and activate the immune system via TLRs on antigen presenting cells (APCs) (Enomoto et al. 2006; Mukai et al. 2009). For example, HSP70 has been shown to stimulate cytokine and chemokine production in dendritic cells (Lehner et al. 2004), and it has been speculated that this stimulation is due to HSP70 interaction with the CD40 receptor, to which mycobacterial and human HSP70 are known to act as alternative ligands (Wang et al. 2001; Becker et al. 2002). Other receptors present on APCs that have been implicated in HSP interaction include CD91, scavenger receptors, c-type lectin receptors and LOX-1 (Calderwood 2007). It is also possible that aggregation itself enhances antigen uptake, and the presence of DnaK within the complex somehow enhances processing and presentation once inside the APC.

This study only investigated the effect of exogenous bacterial HSP contamination on protein immunogenicity. It is possible that endogenous HSP in patients may also interact with aggregates once administered. Exogenous HSP in the biotherapeutic formulation or endogenous HSP *in vivo* could, in theory, complex with aggregates and thus be presented to the host immune system in a more ‘immunogenic’ form. Clinical reports demonstrate that HSP levels are elevated in the plasma of patients with certain illnesses such as dyslipidaemia (Ghayour-Mobarhan et al. 2005),

coronary heart disease (Zhang et al. 2010), prostate cancer (Abe et al. 2004) and neurodegenerative disease (Son et al. 2015).

The data here provide evidence that low levels (1pp1000) of the HSP DnaK can act as an adjuvant to a co-administered antigen. Clearly further investigations will be required to establish the degree to which this phenomenon has implications for the clinical use of protein therapeutics. Nevertheless, these results highlight important areas that might form the focus of future work. For example, it will be important to determine whether, and to what extent, other HSPs have a similar potential to exert adjuvant-like activity.

In conclusion, we report that while aggregation alone can increase the immunogenicity of a mouse protein, and selectively enhance the IgG2a response to a humanized scFv, the addition of DnaK to either protein can enhance further the IgG2a response. Based on these results, it is proposed that the association of certain HSPs with proteins may contribute importantly to the enhanced immunogenicity displayed by aggregated proteins, possibly via promotion of Th1-type immune responses.

4.7 Acknowledgements

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CHAPTER 5:

SUPPLEMENTARY CHAPTER

5 Supplementary Chapter

5.1 Abbreviations

BM	bone marrow
ConA	Concanavalin A
DC	dendritic cell
DEAE	diethylaminoethanol
E.coli	*Escherichia coli*
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorting
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
GM-CSF	granulocyte/macrophage-colony stimulating factor
HCP	host cell protein
IL	interleukin
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
OVA	ovalbumin
PBS	phosphate buffered saline
PI	propidium iodide
scFv	single chain variable antibody fragment
SEC	size exclusion chromatography

5.2 Introduction

In the initial stages of the work described in this thesis, it was necessary to optimise the purification protocol for the single chain variable antibody fragment (scFv) antibody fragment to be used in biophysical analysis experiments (chapter 2), and subsequent immunogenicity studies (chapters 3 and 4).

To supplement the scFv antibody data in chapter 4 (Figure 4.2), a proliferation assay was carried out using splenocyte cultures taken from the same experiment. These data were not presented in chapter 4, and are shown here instead. It was also necessary to optimise the antigen-specific proliferation and cytokine secretion by splenocyte cultures. To find conditions for a sensitive and reproducible assay, antigen dosing concentrations, foetal calf serum (FCS) concentrations in culture medium, and the addition of bone-marrow-derived dendritic cells (BMDC) to co-culture with splenocytes at different ratios were tested. It was also necessary to characterise membrane marker expression on BMDC cultures to demonstrate that cells were sufficiently DC like.

In chapter 4 the monomer was derived from Fraction M and the aggregate from Fraction A in initial experiments. The direct comparison between Fraction M monomer and the Fraction A (dimer/trimer and tetramer) before and after aggregation was not made in chapter 4. It was necessary to make this comparison in order to determine the impact of the possible contaminants in Fraction A on the immune response, as well as the further impact of aggregation of this material.

Finally, ovalbumin (OVA) was heat aggregated using a high temperature (Chapter 2). A sighting study using this material for immunisation was carried out, followed by antibody analysis of serum samples.

5.3 Methods

Single chain variable antibody fragment purification

A humanised scFv (Edwardraja et al. 2010), was cloned into a pET-22b vector in Shuffle T7 express *E.coli* cells (New England Biolabs, Beverly, MA, USA). Transformants were cultured at 30°C to an optical density (OD) of 0.8 at 600 nm, induced with isopropyl β-D-1-thiogalactopyranoside and incubated overnight at 16°C. Cell pellets were resuspended, sonicated and centrifuged at 28,700 g for 30 min. scFv was purified from supernatants using DEAE (diethylaminoethanol) Sepharose anion exchange chromatography, followed by Protein A affinity chromatography and size exclusion chromatography (described in more detail below).

Protein A chromatography

A HiTrap rProtein A column (GE Healthcare, Uppsala, Sweden) was equilibrated in binding buffer (50mM TrisHCl 25mM NaCl, pH 8.5). Samples were applied at a flow rate of 2 ml/min (optimised to 1ml/min). Supernatants from approximately 5 g cells were applied per run on a 5 ml column (optimised to supernatants from approximately 3.5g cells per run on a 5ml column). The column was washed with 10 column volumes of binding buffer. Bound protein was eluted with 0.1M Na/citrate buffer pH 3.5 (flow rate of 1 ml/min optimised to 3 ml/min). Eluted sample was neutralised to pH 7 with 1.5M Tris base (Tris was added dropwise during elution step in optimised method, rather than after the elution was completed). Eluted sample pooled from 3-5 Protein A chromatography steps was concentrated to approximately 3 ml and subjected to analytical size exclusion chromatography (SEC). N.B. HCP contamination of the scFv was more likely with overuse of the protein A column, if re-used over approximately 20 times.

SEC and SEC-MALLS

SEC was carried out using an AKTA FPLC system (GE Healthcare). SEC was carried out using a SD75 24 or 120ml column (GE Healthcare) in 20mM NaH2PO4/NaOH 150mM NaCl pH 7 at a flow rate of 0.5 or 1 ml/min and collected in 0.5 or 1 ml fractions. Monomeric, and higher molecular weight peaks were pooled separately. Protein concentrations were determined by measuring the absorbance at 280 nm using an extinction coefficient of 58580 M^{-1} cm^{-1}. For SEC-MALLS (SEC coupled with multi angle laser light scattering) analysis the SEC column outlet was connected to a Dawn Helios MALLS photometer (Wyatt, Santa Barbara, CA, USA) followed by an OptiLab T-rEX differential refractometer. Data were processed using the Wyatt-QELS software. Samples were processed by staff at the Biomolecular Analysis Facility at the University of Manchester.

SDS-PAGE

Protein samples were diluted in SDS-PAGE sample buffer (Biorad, Berkley, CA, USA) containing 1% 2-mercaptoethanol and heated for 3 minutes at 90°C. Samples were resolved on a pre-cast 10% acrylamide gel and stained using InstantBlue™ Coomassie protein stain (Expedeon, Swavesey, UK).

Generation of aggregates

scFv: Purified monomeric scFv was diluted to 1 mg/ml in Dulbeccos phosphate buffered saline (PBS) without Ca^{+2} or Mg^{+2} (Sigma-Aldrich, St Louis, Missouri) and stressed by heating at 40°C for 25 min. To induce stir-stressed aggregates 1 ml of 1 mg/ml purified scFv was stirred with an 8x2 mm Teflon stirrer bar in a 5 ml glass tube for 6 h.

OVA: Thermal stress; Lyophilised OVA (Sigma-Aldrich) was reconstituted in PBS to a concentration of 10 mg/ml and heated at 80°C for 1.5 h.

Analysis of aggregates

Measurements of DLS were performed with a Malvern Zetasizer Nano ZS ZEN3600 (Malvern, Herrenberg, Germany) equipped with a 633 nm laser. Each sample (70 µl) was measured in a Suprasil® quartz cuvette (Hellma GmbH, Muellheim, Germany) with a path length of 3 mm and 200-2500 nm spectral range. Monomeric and stressed samples at 1 mg/ml were measured at 25°C to determine the volume-based average protein particle diameter in solution.

Animal experiments

Female BALB/c strain mice were used for these experiments (Envigo, Bicester, UK). All procedures were carried out in accordance with the Animals (Scientific Procedures) Act 1986, and approved by Home Office licence. Mice were immunised by intraperitoneal (ip) injection with 250 µl of 1 mg/ml protein (monomeric or aggregate) in PBS on days 0 and 7 and exsanguinated on day 14. In some experiments mice received an additional immunisation on day 14 and were terminated on day 21. Spleens and serum were isolated for evaluation.

Generation and culture of murine bone marrow derived DC

Murine bone marrow (BM) derived DC (BMDC) were generated using a previously described method (Lutz et al. 1999). Briefly, BM was extracted by flushing the femurs and tibias with PBS. The cell suspension was centrifuged at 112 g for 5 min. The pellet was then resuspended in warmed culture medium (RPMI 1640, GIBCO, Paisley, UK), supplemented with 10% FCS (PAA laboratories, GmbH, Austria) containing 400 µg/ml penicillin/streptomycin, 292 µg/ml L-glutamine, 0.05 mM 2-mercaptoethanol and 20 ng/ml granulocyte macrophage-colony stimulating factor (GM-CSF) (Miltenyi Biotech, Bisley, UK). Viable cell counts were performed by trypan blue exclusion (0.5%, Sigma-Aldrich). Cells were cultured at approximately $2x10^6$ cells per 100 X 15mm petri dish at 37°C in a humidified atmosphere of 5% CO_2 in air.

Medium was refreshed every 3 days, and cells harvested by gentle agitation. BMDC were used in co-culture with splenocytes on day 7.

Splenocyte-BMDC co-culture

Single cell suspensions of splenocytes were prepared by mechanical disaggregation. Red blood cells were lysed by incubation in 0.85% ammonium chloride for 3 min. Splenocytes were washed and resuspended in culture medium, supplemented with 10% FCS containing 400 µg/ml penicillin/streptomycin, 292 µg/ml L-glutamine and cultured in 96 well round bottomed tissue culture plates at either 4.5 x 10^5 or 3x10^5 cells per well alone, or with BMDC (3 x 10^5 splenocytes with 3 x 10^4 BMDC for the 10:1 ratio, or 20 x 10^4 splenocytes with 10 x 10^4 BMDC for the 2:1 ratio). Triplicate wells per individual mouse were cultured with monomeric or aggregated protein at 100 µg/ml, or with the T cell mitogen Concanavalin A (ConA; Sigma-Aldrich) as a positive control at 2 µg/ml, or with an equal volume of medium alone for negative control wells and cultured at 37^{o}C in a humidified atmosphere of 5% CO_2 between 24 to 168 h. Supernatants were harvested by centrifugation.

^{3}H-Thymidine incorporation assay: In parallel, aliquots of cells were pulsed with 0.2 MBq ^{3}H- thymidine (^{3}HTdR) (PerkinElmer, *Waltham, MA, USA)* per well 24 h before harvesting. Cells were harvested onto glass fibre filter mats with a multichannel semi-automated harvesting device (Titertek, Skatron AS, Lierbyen, Norway). Incorporation of ^{3}HTdR was measured as disintegrations per minute (DPM) in a liquid scintillation cocktail (Fisher Scientific, Loughborough, UK). Standard error of mean (SEM) was calculated from averages of three replicate wells.

Cytokine enzyme-linked immunosorbent assay (ELISA): Interferon-γ

Splenocyte culture supernatants were tested for Interferon-γ (IFNγ) protein using specific ELISA Duosets from R&D Systems. (*R&D Systems,* Minneapolis, US). The

lower limit of accurate detection was 31.25 pg/ml. ELISAs were performed following the manufacturer's instructions.

Analysis of BMDC membrane marker expression using flow cytometry

BMDC cell pellets were resuspended in 1ml of cold FACS buffer. Aliquots of 100 µl were transferred into the wells of a round-bottomed 96-well plate. Cells were stained with rat anti-mouse MHC antibody (2.5 µg/ml), rat anti-mouse CD86 antibody (Cat no: 553689), rat anti-mouse CD80 antibody (Cat no: 553368), rat anti-mouse CD40 antibody (Cat no: 550285), rat IgG2a isotype control antibody (Cat no: 559073) (all 10 µg/ml; BD biosciences, San Jose, CA, USA), or rat anti-mouse CD11c antibody (Cat no:MAB6950; R&D Systems), and incubated on ice for 45 min. Cells were washed with 200 µl FACS buffer, centrifuged at 300g for 5 min at 4°C, blotted and aspirated. This wash was repeated 3 times. Cells were stained with 100 µl/well STAR69, a goat anti-rat IgG fluorescein isothiocyanate (FITC)-labelled polyclonal antibody (10 µg/ml; AbD Serotec, Kidlington, UK) and incubated on ice for 45 min. Plates were washed and pellets resuspended in 200 µl sodium azide buffer. Samples were analysed using a FACScalibur machine and CellQuest Pro software (BD biosciences). 2 µl of propidium iodide (PI) was added to each sample prior to analysis to gate out non-viable cells. 10, 000 viable cells were acquired from each sample.

5.4 Results

Optimisation of the scFv purification protocol

A humanised scFv antibody fragment was chosen as the main protein to be used in immunogenicity studies. The purification protocol initially yielded a 50% monomer, 50% oligomer mixture (Fig. 5.1A). In order to improve monomer yield, the protocol was optimised. Steps taken to optimise the method included reducing the on-column protein load, and reducing the proteins exposure to low pH citrate by increasing the flow-rate and immediate pH neutralisation of elutions.

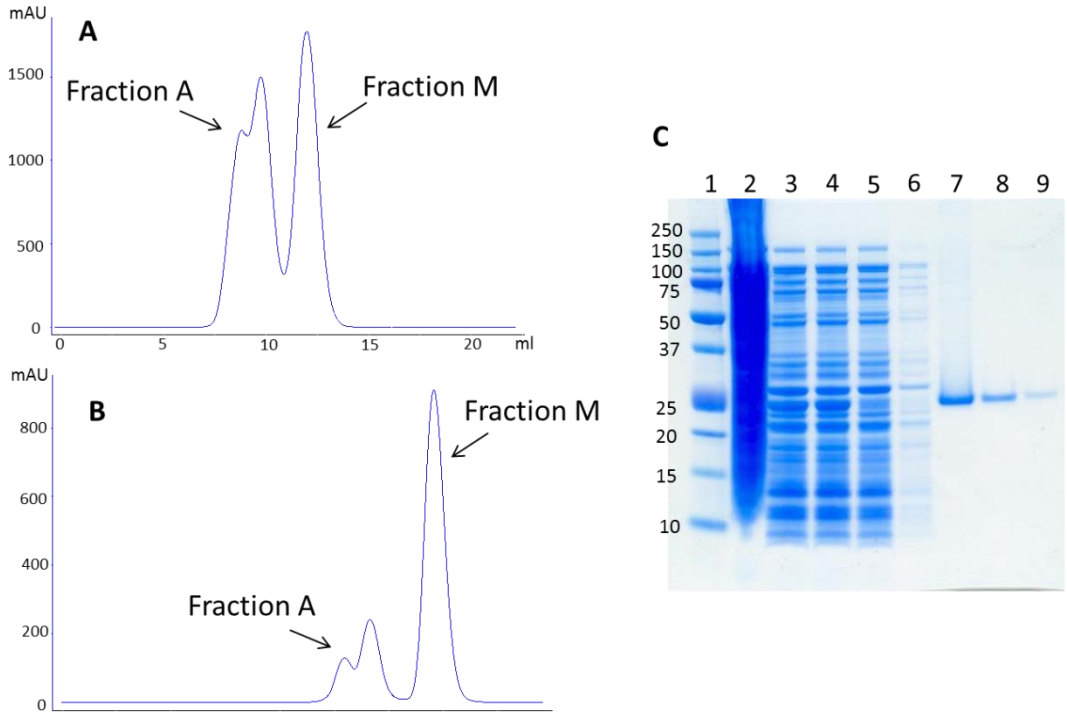

Figure 5.1. **scFv purification optimisation** **(A)** Size exclusion chromatography (SEC) plot using the original purification method (with a 24 ml SEC column) **(B)** SEC plot using the optimised purification method (with a 120 ml SEC column) **(C)** Using the optimised method, batches of human scFv expressing *E coli* cells were grown to stationary phase and the cells were lysed and sonicated. The protein was purified firstly by, DEAE anion exchange, secondly by protein A affinity chromatography and finally SEC. Samples were taken at each step during the purification process. 15 µl of sample was added to 5 µl Laemmli sample buffer and run on a SDS gel followed by staining with Coomassie blue. Samples in each lane are identified as follows: **(1)** Molecular weight markers (250 to 10

kDa) **(2)** Cell lysate **(3)** After cell lysis and sonication **(4)** After DEAE anion exchange **(5)** Protein A column, unretained flow through **(6)** Protein A binding buffer wash **(7)** Protein A citrate buffer elution **(8)** Monomer (Fraction M) following SEC **(9)** Oligomers (Fraction A) following SEC.

Changes in the purification protocol improved the yield of monomer in comparison with the oligomeric scFv, where a 50% yield of monomer was improved to approximately 75% monomer. The monomeric peak (Fraction M; right, singular peak) and oligomeric peaks (Fraction A; left, double peak) both appeared as a single band after SDS-PAGE and Coomassie staining (Fig. 5.1C), and were collected separately for immunogenicity studies. In initial experiments Fraction M was retained as the monomer, and Fraction A was heated for 25 min at 40°C to achieve subvisible aggregates of ~ 2 µm. In subsequent experiment, both materials were retained as their initial states as well as aggregated for comparison.

Immunogenicity of scFv Fraction M monomer and Fraction A aggregate

In addition to the antibody data presented Chapter 4, which showed an increase in overall immunogenicity with Fraction A aggregate when compared with monomer, a proliferation assay was carried out with splenocyte cultures from these animals. Monomer (Fraction M) and aggregate (Fraction A) protein preparations (250 µl at 1 mg/ml) were administered via i.p injection to mice on day 0 and 7, (n=5 per group). On day 14 spleens and serum were collected on an individual animal basis. A single cell suspension of splenocytes was prepared and cultured in the presence of cell culture medium alone (negative control), 2 µg/ml ConA (positive control) or with monomeric or aggregated scFv protein at 100 µg/ml. The proliferation response at time points from 24 to 168 h was measured by ^{3}H-thymidine incorporation.

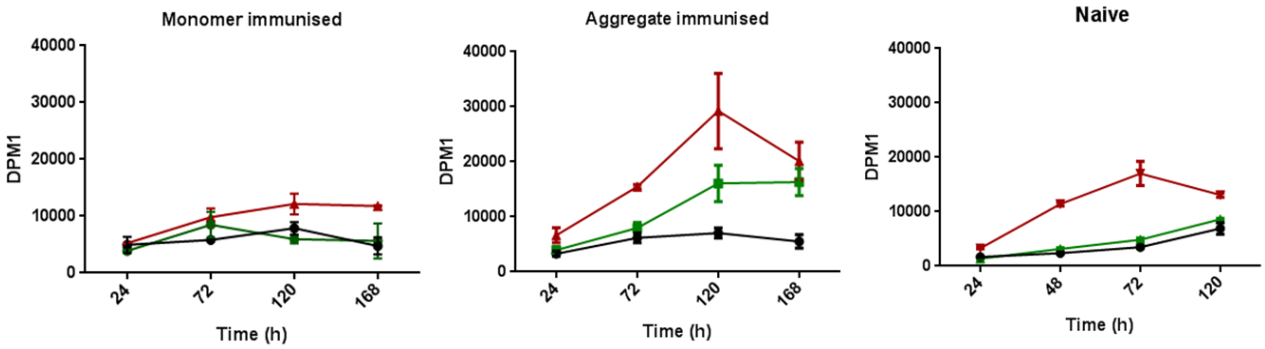

Figure 5.2. Splenocyte culture ^{3}H-thymidine incorporation following *ex vivo* scFv challenge. Splenocytes from immunised mice (n=5 per group) and 3 naive mice were cultured at 4.5x10^{5} cells/well. Splenocytes were challenged with 100 µg/ml monomer (green), 100 µg/ml aggregate (red) or the same volume of medium alone (black). ConA was also added at 2 µg/ml as a positive control (data not shown). Cells were pulsed with ^{3}H-thymidine 24h before harvesting at 24, 48, 72, 120 or 168 h and β scintillation counting. Each culture condition was performed in triplicate for splenocytes derived from each individual animal and a mean value calculated. Group mean proliferation measured as disintegrations per minute (DPM) is shown ±SEM for n=5 animals. The statistical significance of differences between the various culture conditions vs medium alone at each time point was calculated using a two way ANOVA. **A)** Monomer immunised mice **B)** Aggregate immunised mice **C)** Naive mice (time point up to 120h only). Naïve mouse experiments were done contemporaneously. *Due to an infection in some wells from the immunised mice, a minimum of 3 wells (each one from a different mouse) and maximum of 15 wells per data point (3 wells per mouse) are presented.*

In splenocyte proliferation assays, there is always some background response to medium, but specific protein stimulates a response above this background level in cells from primed mice. Splenocytes from the naive mouse proliferated at a low level in response to medium or monomer, but culture with aggregate increased proliferation. Splenocytes from monomeric immunised mice were unresponsive to challenge with both monomer and aggregate, as proliferation was not significantly different to media at all time points. Although levels were not significantly different, the co-culture with the aggregate nonetheless resulted in the highest levels of thymidine incorporation for these cells. Splenocytes from aggregate immunised mice

were more responsive, with an increase in proliferation in response to both monomer and aggregate, but with the aggregate inducing a more profound proliferative response (Fig. 5.2). Splenocytes proliferated in response to the positive control ConA which is not shown here (see Fig. 5.4A for a representative ConA response).

Splenocyte ^{3}H-thymidine incorporation assay optimisation

It was shown using mass spectrometry that Fraction A contained higher levels of *E.coli* host cell proteins (Chapter 4), which could be having and adjuvant effect, therefore mice were immunised with monomer and aggregate derived from Fraction M only. The host cell proteins present in Fraction A might also have been responsible for the higher levels of proliferation observed in response to this material for splenocytes isolated from monomer and aggregate immunised mice as well as for naïve mice. The proliferation assay as described above was not sensitive enough to show reproducible antigen specific proliferation, so experiments were carried out to optimise the assay. For all of the optimisation experiments mice were immunised on day 0 and 7 and 14, and exsanguinated on day 21. The additional immunisation was included to improve proliferation assay sensitivity, as the added immunisation resulted in a more robust antigen-specific proliferative response.

Firstly, the scFv dose in culture was tested at 1, 10 and 100 µg/ml in order to determine the concentration that was most effective at inducing antigen specific splenocyte proliferation.

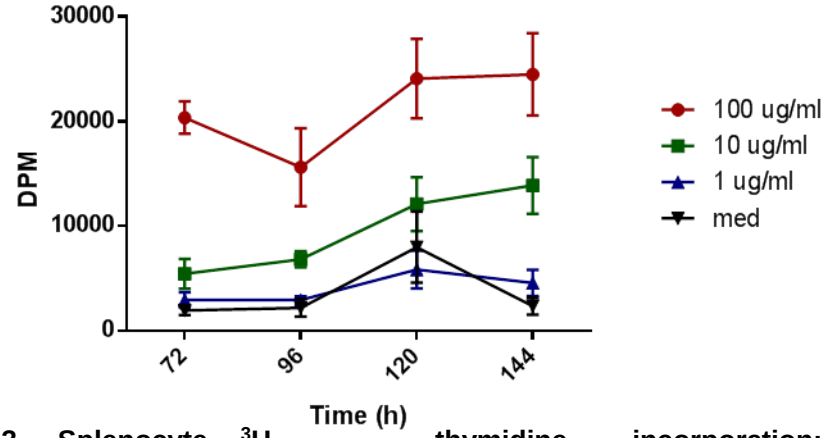

Figure 5.3. Splenocyte ^{3}H-thymidine incorporation: dose response to scFv. Splenocytes from scFv monomer immunised mice (n=3 per group) were cultured at approximately 4.5x10^{5} cells/well. Splenocytes were cultured in medium and treated with 100, 10, and 1 µg/ml monomeric scFv or medium alone. Cells were pulsed with ^{3}H-thymidine 24h before harvesting at 72, 96, 120 and 144 h and β scintillation counting. Each culture condition was performed in triplicate for splenocytes derived from each individual animal and a mean value calculated. Group mean proliferation measured as disintegrations per minute (DPM) is shown ±SEM.

The dose response experiment illustrated that 100 µg/ml scFv in culture was needed for optimal splenocyte proliferation. There was some background proliferation to medium, peaking at 120 h, and the 1 µg/ml dose was no different to medium with some increase in response to 10 µg/ml although this was maximal at 100 µg/ml. The kinetics is also important, as previous data suggested that measuring proliferation at a very early (24 h) time point, was definitely suboptimal. The high dose seems to reach a plateau quite quickly i.e. at ~72 h. At lower concentrations (10 and 1 µg/ml) proliferation readings were considerably lower. In addition to the scFv dosing concentration, the concentration of FCS in medium was tested at reduced concentrations (2.5 and 5% FCS). The proliferative response to the positive control, mitogen ConA, and antigen specific response to scFv, was tested in media supplemented with reduced FCS. Optimal proliferation levels were observed with the 10% FCS media groups, with reduced proliferation in the presence of both ConA and scFv in the presence of the lower FCS concentrations. The baseline proliferation was unaffected by the FCS concentration, rather, it was the antigen-specific proliferation

and the ConA induced proliferation that were affected, particularly at the later time points. At an even lower FCS concentration (2.5%) a more marked inhibition of ConA induced proliferation was observed.

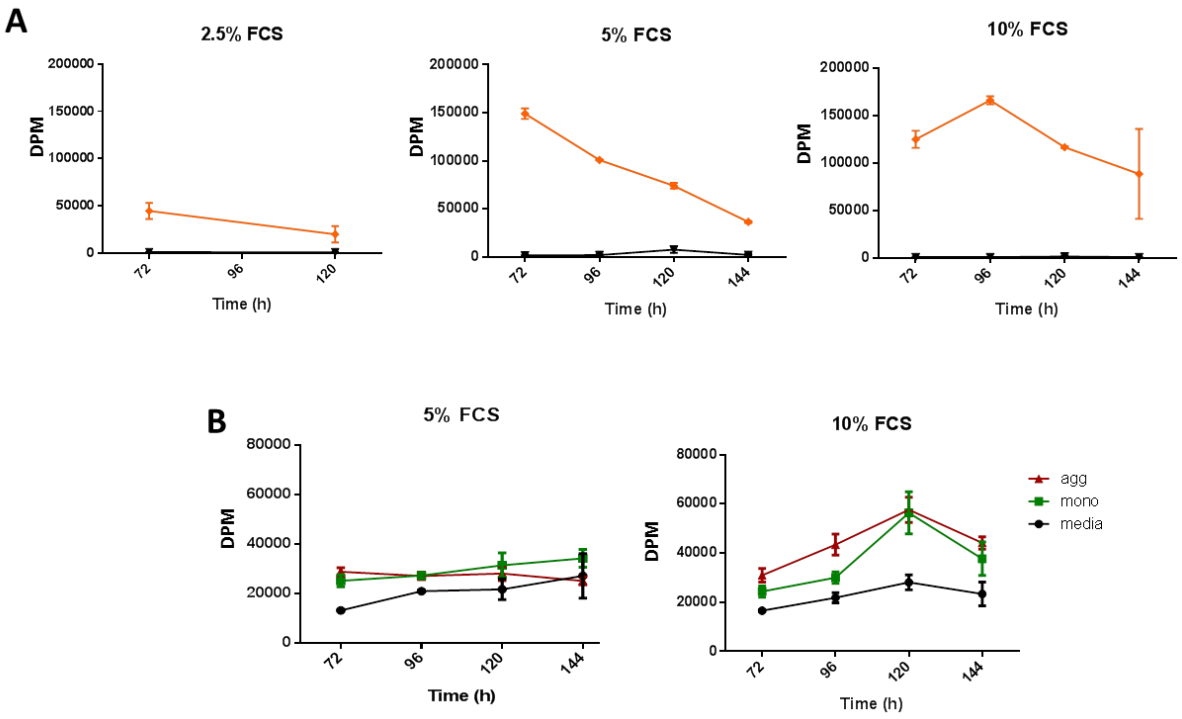

Figure 5.4. The effect of FCS concentration on splenocyte proliferation. Splenocytes from monomer immunised mice (n=3 per group) were cultured at approximately 4.5x10^5 cells/well. **A)** Splenocytes were cultured in medium supplemented with 2.5, 5 or 10% FCS, and treated with ConA at 2 µg/ml (orange) or medium alone (black). **B)** Splenocytes were cultured in medium supplemented with 5 or 10% FCS and cultured with 100 µg/ml monomeric (mono) or aggregated (agg) scFv or medium alone. Cells were pulsed with ^{3}H-thymidine 24h before harvesting at 72, 96, 120 and 144 h and β scintillation counting. Each culture condition was performed in triplicate for splenocytes derived from each individual animal and a mean value calculated. Group mean proliferation measured as disintegrations per minute (DPM) is shown ±SEM.

Robust and reproducible results could still not be attained using the proliferation assay with splenocytes only. In an attempt to achieve a robust reproducible assay with good sensitivity, naïve BMDC were tested in co-culture with splenocytes from mice immunised with monomeric, heat aggregated or stir aggregated scFv.

BMDC are DC-like with regards to membrane marker expression

It was important to characterise the expression of membrane markers on BMDC that were used in co-culture with splenocytes. Membrane markers MHCII, CD80, CD86, CD40 and CD11c were examined by flow cytometry (Fig. 5.5). It was shown that the majority of day 7 BMDC expressed MHCII (~78%), CD80 (~50%) and CD86 (~60%) (Fig. 5.5B and C). In addition, around 30% of these cells express CD40. Therefore these results suggest that BMDC express the appropriate machinery for antigen presentation. As ~77% of these cells expressed the DC marker CD11c, it was proposed the majority of these cells are DC.

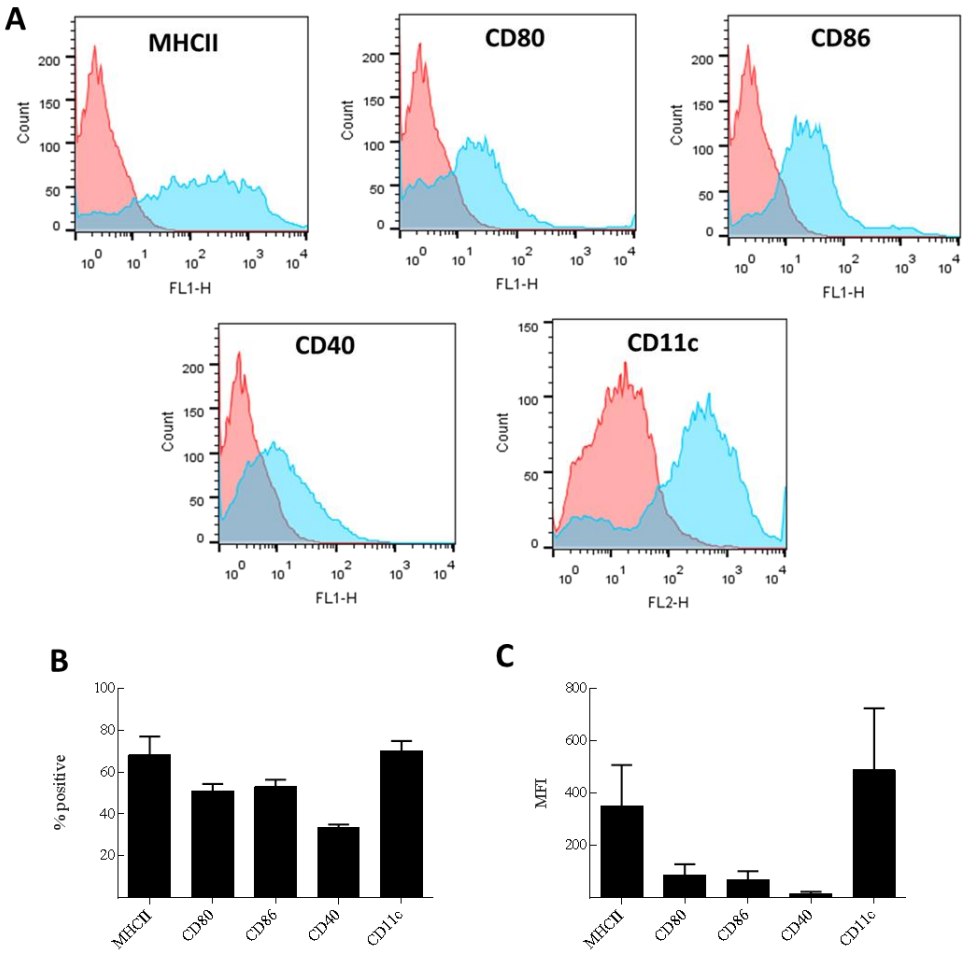

Figure 5.5. Characterisation of bone marrow-derived dendritic cell membrane marker expression. The expression of membrane markers MHCII, CD86, CD80, CD40 and CD11c was analysed on unstimulated viable day 7 BMDC by flow cytometry. 10, 000 cells were acquired for each sample. Data are shown as A) representative histograms where red histograms represent isotype controls, and with respect to B) the percentage of positive cells for each marker and C) The mean fluorescence intensity (MFI; arbitrary units). Data shown are mean ±SEM (n=3 independent experiments).

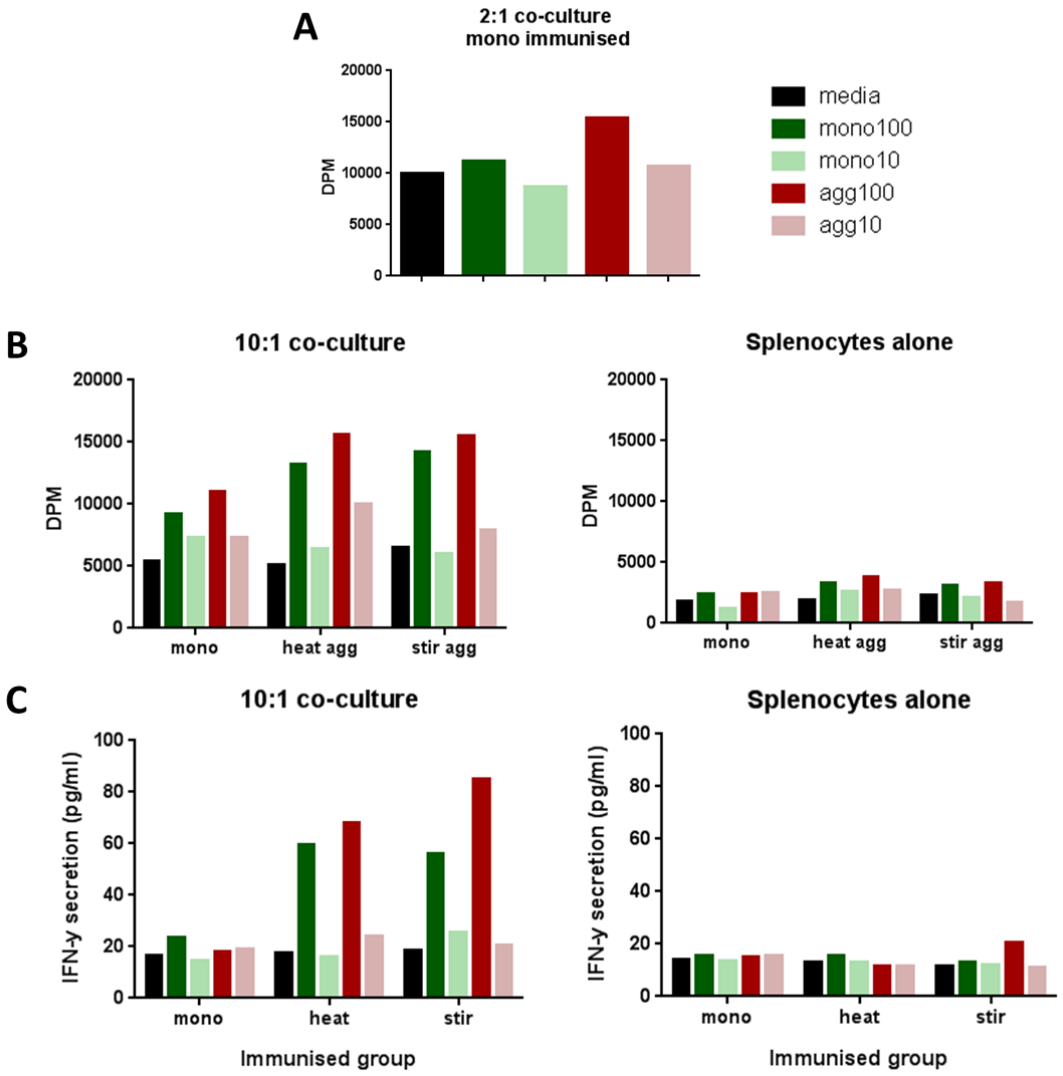

Figure 5.6. The effect of BMDC co-culture with splenocytes on antigen specific proliferation and IFNγ secretion. Splenocytes from monomer (mono), heat (heat) or stir aggregated (stir) scFv immunised mice, were cultured alone and in co-culture with day 7 BMDC and challenged with 100 µg/ml monomeric scFv, heat or stir aggregated scFv or with media alone (splenocytes were pooled from 3 animals per group). **A)** Splenocytes from monomer immunised mice were cultured 2:1 with BMDC and challenged with 100 and 10 µg/ml monomeric or heat aggregated scFv, or medium alone. **B)** Splenocytes from mice immunised with monomer, heat aggregated, or stir aggregated scFv were cultured 10:1 with BMDC and challenged with 100 and 10 µg/ml monomeric or heat aggregated

scFv, or medium alone. Cells were pulsed with ^{3}H-thymidine 24h before harvesting at 72 h and β scintillation counting. Each culture condition was performed in triplicate and a mean value calculated. **C)** Splenocytes were treated as in (B); here supernatants were harvested at 72h and analysed for the presence of IFNγ by cytokine specific ELISA.

The use of BMDC in co-culture with splenocytes did improve assay sensitivity in comparison to splenocytes alone (Fig. 5.6). A single time point of 72 h was chosen based on previous experiments. Proliferation of DC alone was tested and shown to be very low with no change in the presence of antigen (not shown), so both DC and splenocytes are needed together for proliferation. The background level of proliferation does increase with the addition of DC, but this is acceptable, as antigen-specific proliferation is substantially enhanced, further than the background level. The 10:1 ratio of splenocytes to BMDC provided the best level of antigen specific proliferation without increasing the background level of proliferation too much. These optimised conditions were used for further assays (presented in Chapter 3). The IFNγ secretion profile was similar to the proliferation assay in that enhanced antigen-specific secretion was observed in aggregate immunised mouse cultures, however, the difference was not seen in monomer immunised cultures.

Fraction A immunogenicity: Native state vs aggregate

In Chapter 4, it was shown that aggregated Fraction A scFv was more immunogenic than Fraction M. Fraction A was originally used for the aggregated protein, as it showed equivalent purity to the monomer by SDS-PAGE analysis. Antibodies were present in naïve sera against Fraction A, but not Fraction M, which indicated the presence of impurities; this was confirmed by mass spectrometric analysis of Fraction A and M. An *in vivo* experiment was carried out to compare the immunogenicity of Fraction A in its native oligomeric form, with a similar level of impurity to previous batches, with Fraction A after heat aggregation, and Fraction M monomer. Monomer (Fraction M), native Fraction A, and Fraction A aggregate protein preparations (250

µl at 1 mg/ml) were administered via i.p injection to mice on day 0 and 7, (n=3 per group). On day 14 serum were collected on an individual animal basis. IgG binding to a scFv substrate in individual serum samples was assessed using ELISA.

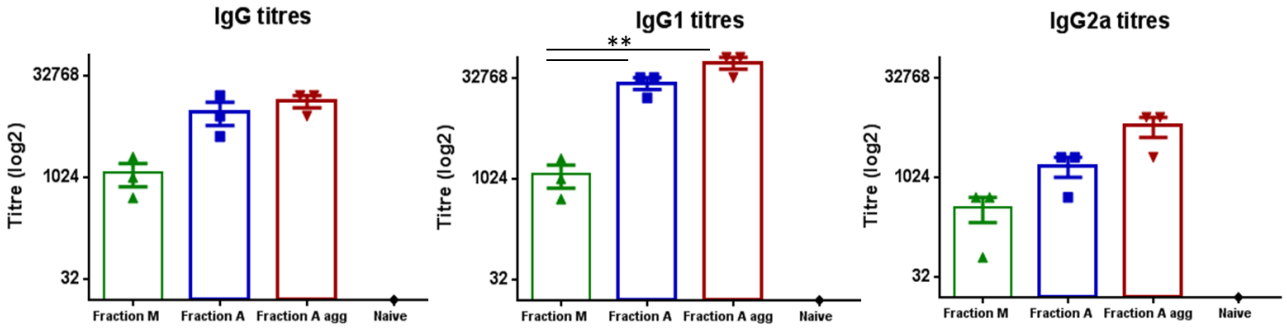

Figure 5.7. Antibody responses to scFv Fraction M, Fraction A and Fraction A aggregate. IgG antibody binding to monomeric scFv substrate was assessed by ELISA. Substrate (monomer) was plated at 10 µg/ml. Serial dilutions of sera from immunised mice (n=3 per group), or naïve pooled serum from a 1/64 starting dilution were prepared for incubation in the ELISA. Mean reciprocal titres are displayed (serum dilution at which substrate conversion of 3 x background [reagent blank] was achieved). One way ANOVA was used for statistical analysis.

Fraction A was more immunogenic than Fraction M, with significantly higher IgG1 titres in both Fraction A and Fraction A aggregate immunised mouse groups. In the experiment presented, Fraction M was used as the substrate. Fraction A was also used as a substrate in concurrent experiments (not shown), here, overall antibody binding levels were higher, but the trends between immunised groups were the same. IgG2a levels did not reach statistical significance but were increased with Fraction A, and further still with aggregation.

Immunogenicity of heat aggregated ovalbumin

In order to compare monomer and aggregate immunogenicity of ovalbumin (OVA), heat treatment was initially employed (Chapter 2, Fig. 2.3). OVA required heating at

80°C to achieve aggregation within the subvisible size range. A sighting study with monomer and heat aggregated OVA preparations was carried out, and antibody binding in sera analysed against both monomeric and aggregated substrates.

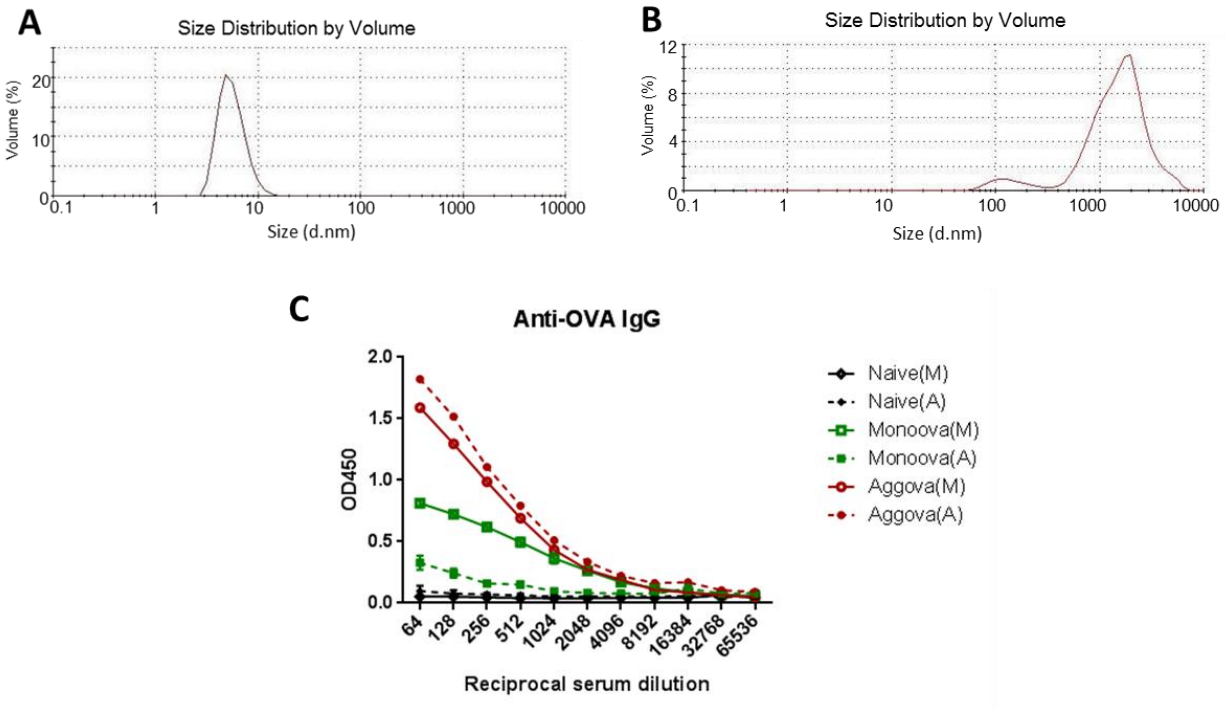

Figure 5.8. Characterisation of immune responses to OVA: comparison of monomer and heat stressed aggregates. OVA at 10 mg/ml in PBS pH 7 was subjected to heat treatment for 1.5 h at 80°C. The mean particle diameter was measured by DLS at 1mg/ml before (A) and after (B) the 80 °C incubation. C) Mice were immunised by i.p injection with 250 µl of 1 mg/ml monomer or heat aggregated scFv on days 0 and 7 and serum isolated on day 14 (n1 for aggregate, n3 for monomer; 1 contemporaneous serum sample, 2 historical serum samples). Doubling dilutions of serum samples from immunised animals and negative control naïve serum samples were analysed against both OVA substrate proteins (versus monomer [M] and vs aggregated protein [A]) by ELISA for IgG anti-OVA antibody content.

Serum from the aggregate immunised mouse bound to both monomeric and aggregated substrates. The response to the aggregate was considerably more vigorous than the response to the monomer. However, sera from monomer immunised mice bound monomer more avidly, with decreased binding to aggregated substrate, indicating that epitopes recognised by serum from the monomer-

immunised mice were altered with heat treatment. Naïve sera did not bind to either monomeric or aggregated OVA substrates.

5.5 Discussion

The original scFv purification method resulted in an equal yield of fraction A and fraction M; this indicated that fraction A scFv was in the early stages of aggregation and had been subjected to stress leading to partial unfolding and self-association. To optimise the method and improve monomer yield, the load of sample on the Protein A column per run, and flow rate for loading the sample was reduced, as scFv was found remaining in the unbound flow-through due to overloading of the column. The concentration of scFv on the resin was therefore too high, potentially contributing to self-association of protein molecules. By reducing the protein load, more scFv was retained on the column. Low pH is another factor that can contribute to protein aggregation (Talley and Alexov 2010). Most proteins are stable in their folded states within a narrow pH range, and low pH can contribute to aggregation by affecting surface charges and resulting in pH induced partial unfolding. A low pH citrate buffer at pH 3.5 was required for dissociation of scFv from the Protein A resin. If the scFv was in this buffer for a prolonged time the protein was more likely to destabilise and self-associate; by neutralising pH at the earliest opportunity this risk could be reduced. Therefore, the flow rate of citrate buffer through the column was increased from 1ml/min to 3ml/min so that the elution step was more rapid. Secondly, low pH citrate elutions were neutralised to pH 7 dropwise with Tris immediately rather than after the elution step was completed. As a result of the changes made the yield of Fraction M was improved so that only approximately a fifth of the total scFv purified was oligomeric. Along with the association of impurities in Fraction A, this made the need to increase the yield of Fraction M more important, as Fraction A could not be used for further immunogenicity studies.

Antibody data presented in Chapter 4 showed increased immunogenicity of the Fraction A scFv aggregate compared with the scFv monomer. A splenocyte proliferation assay from the same *in vivo* experiment also illustrated increased immunogenicity of Fraction A aggregate at the cellular level. The difference in sensitivity between antibody ELISAs and the splenocyte proliferation assay was highlighted here; whereas a robust immune response was demonstrated at an antibody level with monomer immunisation, the same was not demonstrated with the proliferation assay. Splenocytes were used as a source of lymphocytes to obtain a sufficient cell number for the assays; however, after ip exposure the draining lymph nodes are perhaps more likely to be activated than the spleen and may be a source of lymphocytes that would respond more effectively to antigen. Furthermore, proliferation is more of a snapshot, whereas antibody in serum is cumulative.

In order to enhance proliferation assay sensitivity, FCS was tested at reduced concentrations as it was thought that the high FCS concentration might mask or reduce the uptake of scFv, as FCS proteins would be present in excess compared with the antigen of interest; However the response to both positive control conA and scFv stimulation was abrogated with reduced FCS. It is likely that splenocytes cultured in lower FCS concentrations were not in optimal conditions for cell growth and viability. The highest tested concentration of scFv (100 µg/ml) provided the optimal level of proliferation, which is understandable as increased availability of antigen in culture would allow more antigen uptake and presentation.

The use of BMDC in co-culture with splenocytes was then investigated. Phenotypic characterisation of day 7 BMDC was consistent with previous reports using the same protocol and strain of mice (Englezou et al. 2015; Dearman et al. 2009), as the majority of BMDC displayed a DC phenotype expressing relatively high levels of MHC class II and the DC associated marker CD11c. Expression levels of other DC

associated markers CD80, CD86 and CD40 were also consistent with the majority of cells being DC.

The use of BMDC at a ratio of 10 splenocytes to 1 BMDC proved to be successful in providing enhanced assay sensitivity for both the proliferation assay and also cytokine secretion (IFN-γ). This work is consistent with previous work where BMDC have been used in this type of assay (Sun et al. 2013; Mackenzie-Dyck et al. 2015), and confirms that BMDC co-culture provides a useful system to study protein immunogenicity in an *ex vivo* setting. It is likely that the BMDC provided enhanced antigen presentation to supplement the antigen presenting cell population already present within the splenocyte preparations.

The comparison of Fraction A immunogenicity in native oligomeric, and heat aggregated forms indicated that increased immunogenicity was due to the composition of Fraction A and not heat aggregation. This *in vivo* data is consistent with data presented in Chapter 4, which suggests that host cell protein impurities within Fraction A were responsible for increased immunogenicity.

The immunogenicity of the scFv was reported in Chapter 2 after both heat and stir stress, however, only stir aggregated OVA was tested. This was due to the fact that it was not possible to aggregate OVA by heat stress within the subvisible size range without disrupting antibody binding. Antibody data indicates a disruption of epitopes such that sera from monomer immunised mice could no longer bind to the aggregated substrate in an ELISA. The aggregates also resulted in a higher overall immune response, suggesting that aggregates were more immunogenic and that resulting antibodies were cross-reactive to both aggregate and monomer. These data are consistent with the formation of neo-epitopes resulting from a partial unfolding of protein structure with stress at a high temperature of 80°C. The results highlight the

need to keep stresses used in such studies similar to those encountered during bioprocessing when investigating the immunogenicity of biotherapeutics.

5.6 References

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CHAPTER 6:
DISCUSSION

6 Discussion

6.1 General discussion

6.1.1 Revisiting the background and initial aims

Since the release of Eli Lilly's recombinant human insulin, Humulin, in 1982, there has been a steady increase in the use of protein therapeutics in the clinic (Jefferis 2012; Aggarwal 2014; Ryu et al. 2012). A distinct advantage of biotherapeutics over traditional small-molecule drugs is that they are highly specific and, therefore, have a reduced potential for off-target effects and toxicity. However, despite the rapid growth in the market, and clinical advantages compared with small-molecule drugs, there are challenges associated with biotherapeutic development. One important challenge is the ability of biotherapeutics, including those with fully human protein sequences, to induce immune responses and the formation of anti-drug antibodies (ADAs). Such ADAs can impact upon the safety and efficacy of a drug. The first fully human sequence-derived antibody, Panitumumab was approved in 2006 (Jakobovits et al. 2007), and the formation of ADAs has since been observed with other 'fully' human antibodies, including Adalimumab and Golimumab (Radstake et al. 2009; Kay 2008).

This ability of human proteins to elicit an immune response in humans is interesting, and taken at face value it is somewhat of an immune paradox, as one would expect a fully homologous protein to be completely tolerated by the human immune system. Whilst the amino acid sequence of a protein therapeutic can be fully human, or identical to an endogenous human protein, biotherapeutics originate from host cells that have been manipulated to produce the protein, and are essentially 'foreign'. During bioprocessing, from protein expression to formulation and storage, there are a number of factors that can affect the immunogenic potential of a biotherapeutic. For example, there is a considerable potential for heterogeneity of biotherapeutics due to

post-translational modifications (PTMS). These cover a range of effects, including disulphide bond formation, oxidation, deamidation, sulphation and glycosylation (Jefferis 2016). For example, glycoform profiles can be dependent upon the species cell line or cell type (Yu et al. 2012; Hossler et al. 2009), and there is a possibility for the addition of sugar residues that are effectively foreign to humans.

There are well-documented cases where immunogenicity has resulted in undesirable effects such as hypersensitivity reactions, or serious adverse events such as pure-red cell aplasia in patients (Casadevall et al. 2002; Chung et al. 2008, Li et al. 2001; Wadhwa et al. 2015). Therefore, biotherapeutic immunogenicity has become an area of increased regulatory scrutiny. While there are many parameters that contribute to immunogenicity, one particular factor that has gained a strong focus is protein aggregation, which is known to have the potential to enhance protein immunogenicity (Rosenberg 2006). Aggregate immunogenicity can be caused by the formation of neo-epitopes, or structural changes that result in enhanced antigen recognition, processing or presentation. A loss of structural integrity caused by unfolding and denaturation can result in the exposure of 'non-self' forms and the formation of ADAs. Protein aggregate structure could also play a role in differential interaction with immune cells involved in initiating or regulating immunological responses.

Extensive studies have been conducted to understand protein unfolding and aggregation during bioprocessing and storage (Cromwell et al. 2006; Obrezanova et al. 2015), and to minimise aggregation where possible i.e. with excipients in biotherapeutic formulations (Liu et al. 2013). However, the mechanisms through which aggregation impacts on immunogenicity, and the important biophysical characteristics of protein aggregates in this respect, are poorly understood. Therefore, the aim of the work described in this thesis was to begin to characterise the relationship between biophysical properties of protein aggregates and the

magnitude and character of induced immune responses. For this purpose an experimental model based on BALB/c strain mice was employed.

6.1.2 Biophysical analyses of protein preparations

In Chapter 2 a number of biophysical techniques were used to analyse stressed protein preparations. Results demonstrated that the measured biophysical properties varied for each protein and with different stress conditions. For each test protein a variety of approaches were used to generate subvisible-sized aggregates. These included heat treatments for varying times and temperatures, stir stress for different time lengths, and at varying protein concentration and solution volumes. It was shown that tailored stress conditions were needed to aggregate different proteins, and that aggregate properties were also different.

Results obtained from particle sizing methods also varied, depending on the technique used. This is not surprising as it is known that there are biases between particle counting instruments in the 1-100 µm size range, and that characterisation with some light obscuration and flow imaging methods is only feasible for chemically homogenous particles (Ripple and Hu 2016). It is also known that particle shape can impact upon subvisible particle sizing measurements (Cavicchi et al. 2015). Furthermore, in addition to the limitations that have been previously discussed, the presence of buffers or excipients in formulations such as polysorbate (Singh et al. 2012) can also interfere with biophysical methods.

In the research phase of biotherapeutic development, the ability of a candidate drug to withstand bioprocessing related stresses such as purification, lyophilisation and long term storage are tested. Biophysical techniques are used to analyse the properties of biotherapeutic preparations, including particle size, so that changes to the protein sequence can then be made to improve the drug development potential if

properties are not desirable (Houde and Berkowitz 2015). Biotherapeutics then require further characterisation as they progress through the development process. Biophysical techniques also play a key role in comparability studies for biosimilar products to show similarity between the innovator product and a biosimilar (Berkowitz et al. 2012). There is currently no regulatory guidance regarding which analytical techniques should be used in characterising the biophysical properties of therapeutic protein formulations, although it is recognised as being an important part of biotherapeutic development (Houde and Berkowitz 2015). Collectively, the results from Chapter 2 highlighted that there are limitations associated with some of the biophysical techniques used for biotherapeutic characterisation, and that results from orthogonal techniques can yield varied results. It was concluded that the use of multiple techniques in conjunction can provide a more comprehensive analysis of the biophysical properties of aggregates.

It was also hypothesised that aggregated preparations of the test proteins would elicit different immune responses *in vivo* compared with their monomeric counterparts. This was investigated in the experiments presented in Chapter 3.

6.1.3 Characterising the immunogenicity of subvisible protein aggregates

In Chapter 3 a series of experiments are described that were conducted to investigate the basis for enhanced immunogenicity of aggregates of two proteins; a scFv and OVA in BALB/c strain mice. Subvisible-sized aggregates were chosen for investigation in order to reflect concerns over immunogenicity of protein particles in this size range (Ahmadi et al. 2015; Zoells et al. 2012; Carpenter et al. 2009). It was demonstrated that protein aggregates within the subvisible size range promoted a Th1 skewing of the immune response, as well as an increased IgM antibody response. The results from these experiments imply differential antigen processing

or stimulation of immune cells by the aggregated state. One hypothesis is that aggregation affects antigen uptake and processing by APCs, and there is some evidence to support this in the literature (Purohit et al. 2006; Joubert et al. 2012). In order to explain why aggregation might enhance or modify the immune response to proteins, it can be speculated that the development of an immune response to aggregates is due to molecular mimicry. Viruses often have repetitive structures, and it has been hypothesised that aggregates can mimic the multiple antigen copy distribution characteristics of pathogenic microbes and viruses (Kastenmueller et al. 2011; Rosenberg 2006). The data presented in this thesis are consistent with this hypothesis.

In the experiments presented in this thesis efforts were made to stress proteins so that they formed aggregates within the desired subvisible size range, but in some cases it was not possible to find the appropriate stress conditions to achieve this. In order to standardise the size of protein particles, foreign micro or nano-particles can be used, which could provide an alternative experimental strategy for investigating the impact of particle size on immunogenicity. This approach also has relevance for biotherapeutic immunogenicity as protein particles can form in solution by adsorption to micro and nano-particulate contaminants during bioprocessing (Bee et al. 2009). Potential sources of these particles include syringes, and water for reconstitution. Particles of set sizes are available commercially, these particles can be made from gold, glass or polystyrene, for example, and there are methods for conjugating or adsorbing proteins to these particles (Aubin-Tam 2013; Xiang et al. 2013). Soluble protein antigen can be adsorbed onto foreign micro/nanoparticles in order to study protein particle immunogenicity within specific size ranges. For example, mouse mAb adsorption onto glass micro-particles, which were 1-2 µm in size, were reported to result in slightly higher antibody levels in BALB/c and C57BL/6J strain mice when compared with the soluble antigen (Shomali et al. 2014). However, increased

immunogenicity was attributed to an increase in IgG1 in BALB/c mice and IgG3 in C57BL/6J mice. These responses contrast with our results which showed a stronger IgG2a response with aggregation (Ratanji et al. 2016). This may be due to a difference in antigen processing with the protein aggregates compared with protein adsorbed onto a foreign particle. There is also an inherent Th1/Th2 bias of different inbred strains of mice which might affect murine immunogenicity experiments. For example, C57BL/6 and BALB/c strain mice are proto-typical Th1 and Th2-biased mouse strains respectively (Watanabe et al. 2004). In our experiments BALB/c mice were used, so a Th2 response might be expected, however, a Th1 skewed response was observed with aggregation. The Th1 response was not pure, as a robust IgG1 antibody response was still observed with monomeric and aggregated proteins, but aggregation enhanced IgG2a responses.

The IgM response in the study described above was, however, consistent with our results and showed an increased IgM response against the micro-particle adsorbed mAb, compared with the soluble antigen. T cell-independent antibody responses can be generated when B cells alone recognise epitopes and respond by producing predominantly IgM antibodies. Studies have shown a correlation between repetitive epitopes and T cell-independent B cell activation (Fehr et al. 1998; Dintzis et al. 1983; Bachmann et al. 1995). Therefore, it is possible that repetitive epitopes present on aggregated proteins and protein adsorbed micro-particles can activate B cells independently of T cells, resulting in IgM production, potentially by cross-linking B cell receptors. It is also possible that aggregated proteins interact more effectively with antigen presenting cells to enhance a T cell-dependent response and IgG class-switching. It can be speculated that the mechanism of Th1 skewing is at the level of recognition by cell surface receptors on APCs. For example, there is evidence that activation of particular toll-like receptors (TLRs) can result in a Th1 skewing of cytokine profiles (Netea et al. 2005), and it is possible that aggregates could act as a

ligand to these receptors. A simultaneous activation of innate and adaptive immune responses would account for the aggregate induced increase of both IgM and IgG2a antibodies observed in our experiments. It might be the case that the IgM and IgG2a epitopes differ, and this could potentially be investigated using a peptide-microarray approach to determine whether IgM and IgG2a antibodies in sera bind to similar or separate epitopes; this microarray approach has previously been used to assess immunogenic epitopes on Infliximab (Homann et al. 2015). The data also indicate that more than one mechanism contributes to the increased magnitude and differential character of induced immune responses (see Figure 6.1). It has been reported in the literature that aggregated protein can enhance IgG1 levels in BALB/c mice, which differs from the results reported here (Freitag et al. 2015). Therefore, it is likely that, owing to the wide range of properties that aggregates possess (including, for example, size, morphology and hydrophobicity), there exists more than one mechanism through which aggregates might affect the magnitude and character of immune responses. Our studies focused on aggregates within a fairly narrow size range, and it is possible that, were similar experiments conducted with these same proteins stressed to different sizes and with different methods, the measured immunogenicity profiles may be different.

Whilst the use of foreign particles can be useful to control size distributions of protein particles and to study the effect of foreign particles in biotherapeutic preparations, results cannot be used to show directly how protein aggregation might influence the immune response. For example, it is not known how coated particles behave *in vivo,* and how antigen recognition, internalisation, processing and presentation are affected by presence of the particle in addition to the antigen. Further experiments with fully protein aggregates in the same size range would be needed to verify whether the particle size was responsible for any change in immunogenicity.

In attempting to understand the differential immunogenicity displayed by protein monomers and aggregates, information can also be drawn from research in the field of vaccinology, where a protective immune response is required to promote host resistance to pathogenic microorganisms. There have been several reports on the effect of particle size on vaccine efficiency. These reports are addressed and summarised in a recent review (Benne et al. 2016). Particle size, shape and rigidity can all affect vaccine effectiveness (Huang et al. 2011, Champion and Mitragotri 2009), and it is possible to control these variables by altering manufacturing conditions (Cohen et al. 2009; Kumar et al. 2015). For example, it has been shown that smaller (<50nm) sized particles can reach the lymph nodes more effectively (Reddy et al. 2007), whereas larger particles are better retained within the lymph nodes (Oussoren et al. 1997). There is also evidence of differential skewing of immune responses to vaccines with differently sized particles with some studies suggesting that particles of around 50 nm in size trigger an optimal Th1 response (Mottram et al. 2007; Fifis et al. 2004; Joshi et al. 2013). Knowledge from the field of vaccinology can therefore serve to inform the biotherapeutics industry, where immunogenicity is unwanted, and to guide research on biotherapeutic immunogenicity. Similarly, knowledge gained from biotherapeutics research can inform vaccinology. For example, our experiments demonstrated a Th1 skewing of the immune response with aggregation in the subvisible size-range; since, in most instances, a Th1 response is desired against vaccine antigens, this information could be useful in vaccine design.

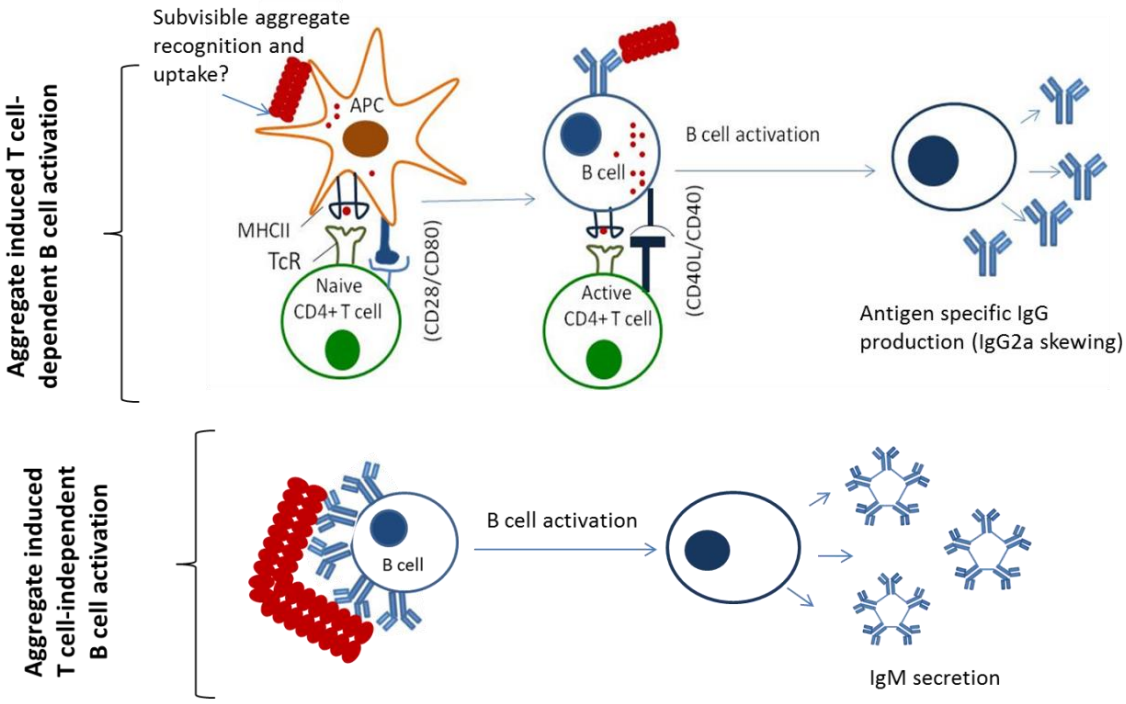

Figure 6.1. Proposed mechanisms for enhanced IgG2a/IgM production with subvisible protein aggregates. T cell-dependent activation: The aggregate (red) is recognised by cell surface receptors on APCs, internalised, processed and presented on major histocompatibility complex (MHCII) followed by $CD4^+$ T cell recognition via the T cell receptor (TcR). Activated T cells then stimulate B cells through TcR:MHCII interaction, and also cytokine secretion. B cells are stimulated to secrete IgG, with a bias towards IgG2a. T cell-independent activation: Repetitive epitopes presented by aggregates stimulate B cell activation and transient IgM production.

6.1.4 Impact of aggregation and HCP impurities on protein immunogenicity

In these experiments *E.coli* host cell proteins (HCPs) were identified in scFv preparations and shown to have an adjuvant-like effect. The presence of HCPs in biotherapeutic formulations is monitored due to their potential effects on drug safety and/or efficacy. Commonly, conventional analytical methods, such as HPLC and 1D/2D PAGE, have been used to measure HCP levels (Bracewell et al. 2015), but these are too insensitive and non-specific to identify low abundance HCPs. Immunodetection based-methods, such as ELISA, are often used for monitoring HCP

levels. However, the assessment of individual HCP components by ELISA is impractical, and investigators have instead employed polyclonal antibodies raised against the whole HCP spectrum (Zhu-Shimoni et al. 2014). It cannot be guaranteed by using this method that all HCPs are being measured with sufficient sensitivity. Mass spectrometry has been applied successfully to measure a wider range of impurities with sensitivity and specificity (Reisinger et al. 2014), allowing the identification of low abundance HCPs in complex mixtures and therefore complementing the results obtained using ELISA methods, although quantification can be a problem with this method.

Regulatory guidance documents highlight the need for the detection and quantitation of HCP in final drug products. For example, according to International Conference on Harmonisation (ICH) guidelines Q6B, the use of a sensitive assay to detect HCP, such as an immunoassay should be used (ICH, 1999). The guidelines also acknowledge that technologies for HCP detection are continuously being developed and that new techniques should be used where appropriate. The World Health Organisation (WHO) guidelines also state that HCPs should be identified and evaluated qualitatively and/or quantitatively (WHO, 2013). Since there is no set limit for HCPs, the limit must be determined on a case by case basis. However, as a general rule, HCPs must not exceed a threshold of approximately 100 parts per (pp) million (Eaton 1995; Doneanu et al. 2012).

Interestingly, the data in this thesis showed that the presence of a single HCP, the *E.coli* heat shock protein (HSP) DnaK at 1 pp thousand did not impact upon immunogenicity of the monomeric protein, but that the same level of DnaK in conjunction with protein aggregation did result in an adjuvant-like effect. It is known that HSPs can have adjuvant-like properties, though it is not known what the mechanism is behind this effect. It has been speculated that the HSP adjuvant-like effect is mediated by binding to cell surface receptors of the immune system (Wang

et al. 2001; Calderwood 2007). However, it may be that HSPs have more of an influence on antigen processing. The fact that the HSP impurity elicits a change in immunogenicity only in the presence of aggregate indicates that inherent aggregate structural properties are required for differential antigen recognition and uptake and that, once internalised in the APC, the presence of a HSP confers additional immunogenicity. This is potentially mediated by HSPs influencing the processing and presentation of the aggregate antigen; there is some evidence in the literature for the involvement of intracellular HSPs in influencing antigen processing (Zugel and Kaufmann 1999; Constant et al. 1994; Kunisawa and Shastri 2006).

6.1.5 Predicting immunogenicity

The objective of the experiments in this thesis was not to develop a method for predicting whether a particular protein (whether monomeric or aggregated) will have the potential to provoke an immune response in humans. The aim was rather to use an experimental system that would allow an investigation into whether aggregation of proteins *per se* influences the magnitude and/or character of induced immune responses. The data presented in this thesis has value in pointing to the general mechanisms behind aggregate immunogenicity, which might operate in a similar way in humans; however, this information cannot be applied to improving the prediction of biotherapeutic immunogenicity of a given protein. Prediction of a protein's immunogenic potential is, however, an important part of biotherapeutic development, and is discussed in more detail below.

The detection and characterisation of antibodies in serum from patients is the best way to assess biotherapeutic immunogenicity. This can be done using screening and confirmatory assays, and is important in understanding the efficacy and safety of a drug (Wadhwa et al. 2015). However, prediction of immunogenicity at the early stages

of development before pre-clinical and clinical trials is important to avoid adverse events and reduce attrition of drug candidates in clinical development. Unfortunately, there is no standardised or single method to predict immunogenicity at this stage, though there has been a substantial investment in the development of approaches that might meet this need.

Epitope prediction strategies, which identify immunodominant parts of the antigen (i.e. those likely to be recognised by B or T cells), are attractive in biotherapeutic development. T-cell epitope prediction is being used early in drug development (Lundegaard et al. 2012; Jawa et al. 2013), and can involve the use of sequencing mass spectrometry to identify portions of a protein that contribute to a T-cell response (Yu et al. 1998). There are a number of companies that now offer immunogenicity screening services to the biopharmaceutical industry, including epitope prediction tools; for example, ProImmune offers assays that allow the identification of epitopes presented by HLA molecules to T cells. EpiVax is also an informatics company that offers *in silico* immunogenicity services which involve screening protein sequences for putative T cell epitope clusters and identifying amino acid sequences which contribute to the immunogenic potential of epitopes. Lonza also provides immunogenicity assessment services, which includes Epibase, an *in silico* T-cell epitope prediction technology to predict the binding affinity for peptide sequences (Sampei et al. 2013; Van Walle et al. 2007).

While approaches used for T-cell epitope identification can be useful in predicting immunogenicity, these methods predict linear epitopes that are wholly determined by the primary amino acid sequence. However, conformational epitopes are formed by a complex of loops and chains in the secondary structure of a protein, bringing together amino acid residues that can be far apart in the linear sequence (Forsstrom et al. 2015). B cell epitope prediction includes both linear and conformational epitopes, and, as approximately 90% of B cell epitopes are conformational, this is

therefore a more complex and challenging task. A structural approach can be taken to predict B cell epitopes, where if the 3D structure is known or can be modelled, then surface epitopes can be predicted using an algorithm based approach (Habibi et al. 2015; Zhang et al. 2011).

The propensity of a protein to aggregate can also be predicted using computational methods (Tsolis et al. 2013; Chennamsetty et al. 2009). There are several computational tools to predict aggregate prone regions in proteins; these can use sequence compositions, secondary structure and conformational predictions to determine the risk of aggregation (Tartaglia et al. 2008; Trovato et al. 2007; Obrezanova et al. 2015).

Immunogenicity prediction tools are useful in that they can allow for the improvement of candidate biotherapeutics by removing high risk sequences so that there is a reduced risk of immunogenicity issues arising, and they may progress further in clinical development. However, these methods cannot guarantee low or no immunogenicity, and do not take into account many of the other contributory factors that can influence the immune response. Furthermore, there is no generally accepted suite of prediction tools or methods that is used consistently in biotherapeutic development.

The experiments described in this thesis focused on increasing an understanding of the factors that affect the immunogenicity of therapeutic proteins and, in particular, the impact of protein aggregation. To this end, foreign proteins were studied in a BALB/c strain mouse model so that a baseline level of immunogenicity with the monomer could be compared with aggregated preparations. However, there is a clear difference between using mice as a predictive tool for human immunogenicity, and using mice to improve our understanding of the factors that can influence the elicitation of an immune response. When mouse studies are used for the prediction

of immunogenicity of biotherapeutics that are to be used clinically, there are obvious limitations with using traditional mouse models. For example, human proteins are foreign to mice and thus immunogenicity is expected due to the protein sequence (Jawa et al. 2013). In order to investigate the potential of a biotherapeutic formulation to be immunogenic in patients, transgenic 'humanised' mouse models that are tolerant to a human protein can be used. This approach has been used in a number of studies that were able to demonstrate the potential for aggregated biotherapeutic preparations to break tolerance and induce ADA (Abdolvahab et al. 2016; Filipe et al. 2012; Fradkin et al. 2009; Bi et al. 2013). The different mouse models that have been used for predicting protein immunogenicity, and challenges associated with these models are discussed in more detail in a recent review (Jiskoot et al. 2016).

6.2 Future work

Progress has been made in understanding the differences between monomer and aggregate immunogenicity, and work presented in this thesis illustrates that aggregation can impact on both the magnitude and character of immune responses, and that HCP may serve to enhance further the immunogenic potential of aggregated proteins. However, it is clear that there is still much work to be done in elucidating the mechanisms behind aggregate immunogenicity, and understanding the biophysical properties required for aggregates to have an impact on the immune response.

A skewing of the character of the immune response was demonstrated by analysing IgG isotypes in serum, and the cytokines IFN-γ and IL-4 in cell culture supernatants from immunised mice. The serum antibody responses measured in this thesis are largely a cumulative marker, given the half-life of IgG, but cellular assays can be more appropriate for looking at cellular markers/cytokines. The character of the immune response could be further characterised using lymphocyte cultures from immunised

mice. Here, cell surface markers could be analysed by flow cytometry, and secretion of a wider range of cytokines could be measured with ELISpot techniques. It might also be useful to further investigate the kinetics of immune responses to monomeric and aggregated proteins by measuring *in vivo* responses at different time points after immunisations, to determine if there are differences between kinetics of the response against monomer and aggregate. There is also much to be done in understanding the basis for altered immunogenicity to aggregates at a cellular/molecular level i.e. what receptors are involved in recognising aggregates more effectively.

I have also postulated in this thesis that the profile of an induced immune response is dependent upon aggregate properties such as size range, hydrophobicity, structure and morphology. For example, it is possible that certain size ranges have more risk inherent than others. It would, therefore, be informative to study the immunogenicity of a wider range of proteins, including monoclonal antibodies, which have been stressed to create aggregates with varied properties. In order to understand accurately the relationship between individual aggregate properties such as particle size or morphology, and immunogenicity, it is required that investigations change only a single parameter. Where multiple variables are changed, the direct effect of one parameter is difficult to determine with confidence. However, the ability to control a single aggregate parameter such as particle size, while keeping others the same is often not possible due to the nature of aggregation and propensities of different proteins to aggregate under different conditions.

From a manufacturing and regulatory perspective, it would be beneficial to understand what characteristics of aggregates can result in increased immunogenicity, so that limits can be set. In order to address industrial concerns regarding the percentage of aggregates that might be acceptable in biotherapeutic formulations, it would be useful to use a humanised mouse model that is tolerised to a human biotherapeutic, and so does not respond to the monomeric protein

therapeutic. The therapeutic at different ratios of monomer to aggregate (v/v) could be administered to these mice; i.e. ranging from 100% aggregate to 1% aggregate and 99% monomer. This type of approach may be useful in guiding limits for the acceptable amount of aggregate in drug preparations for clinical use, as it may indicate that aggregation below a certain level will not impact immune responses.

The work conducted in this thesis on HCP impurities demonstrated an adjuvant-like effect of the *E.coli* HSP DnaK in aggregated protein preparations. DnaK was studied individually as a candidate HCP contaminant, although in reality, biotherapeutic preparations are likely to contain more than one HCP, including other HSPs (Schenauer et al. 2013). For example, *E.coli* HCPs were profiled using mass spectrometry in a therapeutic Fc fusion protein preparation, purified from *E.coli* host cells; results demonstrated the presence of numerous HCPs which included the HSPs 60kDa chaperonin and DnaK (Schenauer et al. 2012). While DnaK was shown to have an adjuvant-like effect, it is possible that there may be synergy, or additional immunogenic effect with multiple HCPs in aggregated preparations. For example, different HCPs may influence antigen recognition, processing or presentation by different mechanisms. It would therefore be interesting to look at the effect of multiple HCPs on protein aggregate immunogenicity.

To further investigate the effect of aggregation and HSP impurities on immunogenicity, an *in vitro* system such as a dendritic cell (DC) cell line, or primary DC, could be used to observe whether the presence of HSPs affects antigen processing or DC maturation. If any effect is observed in an *in vitro* system, inhibitors could be used to determine if the effect can be abrogated by the blockade of cell surface receptors that might be implicated with HSP binding. For example, TLR9 activation has been shown to stimulate isotype switching to IgG2a (Jegerlehner et al. 2007), and TLR7 has been implicated in the activation of Type 1 immune responses

in mice (Fang et al. 2012), so the effect of TLR7/9 inhibition could be investigated. The effect of HSPs on intracellular signalling could also be explored further.

I have speculated in this thesis that elevated HSP levels in patients with certain conditions such as lupus (Dhillon et al. 1994), dyslipidaemia (Ghayour-Mobarhan et al. 2005) and coronary heart disease (Zhang et al. 2010) may contribute to the increased immunogenicity of biotherapeutic aggregates. In order to address this experimentally, a disease mouse model with elevated serum HSP levels could be used. The immunogenicity of aggregated proteins in these mice could be compared with responses seen in healthy 'low HSP' mice. Using this approach, it may be possible to show that an increase in endogenous HSP contributes to enhanced immunogenicity of biotherapeutic aggregates, and allow more accurate identification of patients who may be at increased risk of developing ADA.

6.3 Concluding remarks

The broad aim of this thesis was to begin to characterise the relationship between the biophysical properties of protein aggregates and the magnitude and character of induced immune responses. From the data presented it was shown that different stress conditions are required to aggregate proteins within a similar size range, and that subvisible aggregates promote a Th1 skewing of the immune response, in addition to an increase in antigen specific IgM. Together, these results point to differential antigen recognition and/or processing when a protein is in the aggregated state. Although further work is required to understand the underlying mechanisms of aggregate immunogenicity, the data described herein add to the knowledge and provide a basis for future experiments. The work conducted in this thesis also identified that DnaK can enhance the immunogenicity of aggregated proteins. While DnaK was the only HCP tested in these experiments, the data indicate that HSPs, as

a class of HCPs, potentially contribute to biotherapeutic aggregate immunogenicity by having an adjuvant-like effect.

CHAPTER 7: BIBLIOGRAPHY

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