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RESEARCH PAPER

Small Amounts of Sub-Visible Aggregates Enhance the Immunogenic Potential of Monoclonal Antibody Therapeutics

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ABSTRACT

Purpose Determine the effect of minute quantities of sub-visible aggregates on the *in vitro* immunogenicity of clinically relevant protein therapeutics.

Methods Monoclonal chimeric (rituximab) and humanized (trastuzumab) antibodies were subjected to fine-tuned stress conditions to achieve low levels (<3% of total protein) of sub-visible aggregates. The effect of stimulating human dendritic cells (DC) and $CD4^+$ T cells with the aggregates was measured *in vitro* using cytokine secretion, proliferation and confocal microscopy.

Results Due to its intrinsic high clinical immunogenicity, aggregation of rituximab had minimal effects on DC activation and T cell responses compared to monomeric rituximab. However, in the case of trastuzumab (low clinical immunogenicity) small quantities of aggregates led to potent $CD4^+$ T cell proliferation as a result of strong cytokine and co-stimulatory signals derived from DC. Consistent with this, confocal studies showed that stir-stressed rituximab was rapidly internalised and associated with late endosomes of DC.

Conclusions These data link minute amounts of aggregates with activation of the innate immune response, involving DC, resulting in T cell activation. Thus, when protein therapeutics with little or no clinical immunogenicity, such as trastuzumab, contain minute amounts of sub-visible aggregates, they are associated with significantly increased potential risk of clinical immunogenicity.

KEY WORDS anti-drug antibodies · biotherapeutics · $CD4^+$ T cell responses · humanized antibodies · immunogenicity

ABBREVIATIONS

ADA	Anti-drug antibody
BCR	B cell receptor
CBA	Cytometric bead array
DC	Dendritic cell
DLS	Dynamic light scattering
mAbs	Monocolonal antibodies
MHC	Major histocompatibility complex
MoDC	Monocyte derived dendritic cell
Tfh	Follicular helper T cell

INTRODUCTION

The presence of aggregates in biotherapeutics can have a critical effect on the biological activity of the molecule as well as immunogenicity *in vivo*. Patients treated with protein therapeutics can frequently develop anti-drug antibodies (ADA). These may be neutralising, and result in a loss of therapeutic effect, or induce serious side effects such as anaphylaxis and infusion syndromes. The mechanism for the development of high affinity IgG ADA in patients is poorly understood, as are the most important factors that contribute to the immunogenicity of these

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therapeutics. It has been suggested that aggregates induce a T-independent ADA response via crosslinking the B cell receptor (BCR) [1, 2], although evidence to confirm that protein aggregation can result in the highly ordered structures containing repetitively spaced B cell epitopes in the 1–10 nm range required to crosslink the BCR has not been presented [1, 3]. Furthermore, in humans T-independent antigens only stimulate the production of IgM [4]. T cell help is required for the production of isotype switched, somatically mutated antibodies that are characterised by high-affinity IgG antibodies typically observed in ADA responses. The effect of aggregates present in protein therapeutics on T cells has not been the focus of research and is critical to the understanding of the mechanisms involved in the induction of ADA responses in patients.

Clearly, the origin of the primary sequence is insufficient in itself to prevent the development of undesirable ADA responses in patients, as ADA responses are observed after recombinant human factor VIII treatment of moderate haemophilia A patients, i.e. individuals already expressing low levels or a complete lack of endogenous factor VIII [5]. Since tolerance to self-antigens is either incomplete or can be broken, research has focused on understanding the initial activation events that ultimately lead to the development of patient ADA. Manufacturing and product formulation processes, as well as handling of the product during shipment and storage pose certain stress factors for a protein, including freeze/thawing, pH shifts, heating and mechanical stress (stirring and shaking). This may result in the formation of protein aggregates with different properties, e.g. size, solubility, stability and chemical bonding. The presence of protein aggregates is known to facilitate potent immune responses via a variety of poorly understood mechanisms [6, 7]. Characterisation of protein aggregates has resulted in the identification of sub-visible particles that are usually present in biotherapeutic products, although the link between the presence of such particles and immunogenicity is not entirely clear [7]. It has been suggested that specific properties of sub-visible particles are required to initiate an immune response and much of this research has been based on inducing the development of sub-visible particles by artificially stressing proteins to induce aggregation. Chemical reactions including oxidation and deamidation are accelerated at elevated temperatures, which also have a direct effect on a protein's structure at the quaternary, tertiary and secondary levels and may result in unfolding and aggregation [8–10]. Aggregation resulting from mechanical stress, e.g. shaking, pumping or stirring during manufacturing and transport is well documented [6]. Aggregation through mechanical stress is mainly caused by surface-induced denaturation, but also cavitation effects have recently been described as critical driving forces for aggregation [11].

We have investigated the influence of small amounts of sub-visible aggregates on *in vitro* immune responses using two monoclonal antibodies (mAbs), rituximab (Rituxan) and trastuzumab (Herceptin), as model proteins. Both antibodies were artificially stressed to induce the formation of sub-visible aggregates of different properties at quantities that may be present in clinical material (after storage and handling), in contrast with earlier studies where highly aggregated mAb samples were created and tested for immunogenic potential *in vitro* [3, 12] or *in vivo* [13]. The stressed antibodies were characterized with an orthogonal analytical approach and compared for their immunogenic potential *in vitro*. The aggregate preparations were initially tested against large cohorts of healthy donors to study *in vitro* T cell activation followed by their effects on MoDC in a smaller cohort. These data show that protein therapeutics with a low risk of clinical immunogenicity may be associated with immune activation *in vitro*, even if only a minute fraction is aggregated into sub-visible particles. This occurs via increased DC activation and consequently T cell responses. Although other factors such as concomitant therapies and the therapeutic target are important to consider for immunogenicity *in vivo*, the role of aggregates and their association with an increased risk of immunogenicity *in vivo* should also be considered.

MATERIALS AND METHODS

Preparation of Aggregate Samples

Rituximab and trastuzumab antibodies were exposed to mechanical, heat and freeze/thawing stress. Several pilot studies were performed to optimize the stress conditions, such that a significant increase in sub-visible particles was obtained, while monomer loss was kept minimal (<3%) and no visible particles were observed. This led us to the following optimised methods. First, the antibodies were diluted in phosphate-buffered saline, pH 7.4 (PBS: 1.42 g $Na_2HPO_4.2H_2O$, 0.2 g KH_2PO4 8.12 g NaCl, and 0.18 g KCl per liter), to 1 mg/ml. The diluted antibodies were stressed by the following three conditions to generate small amounts of (mainly) sub-visible aggregates. For mechanical stress, 2 ml of antibody samples were stirred with Teflon®-coated stir bars in 5R glass type I vials (Sigma Aldrich, UK) at 200 rpm for 15–30 min at 25°C. For heat-induced stress, samples were placed in pre-heated thermomixer at 70°C for 10 min. Finally, to induce aggregation through freeze/thaw stress, samples were subjected to 10 freeze/thaw cycles of −80°C and 37°C.

Analysis of Unstressed and Stressed Samples

Monomer content was quantified by high performance size-exclusion chromatography (HP-SEC). A TSKgel4000 SWXL column (Tosoh Biosep, Stuttgart, Germany) was used for HP-SEC analysis with a mobile phase composed of 50 mM sodium phosphate, 150 mM

arginine, 0.025% NaN_3 at pH 7.0. Fifty µl of the formulations were injected and the flow rate was 0.5 ml/ml. UV detection was performed at 280 nm.

Sub-micron aggregates were analyzed by dynamic light scattering (DLS) performed with a Zetasizer Nano ZS (Malvern, Herrenberg, Germany). Five hundred µl of the formulations were analyzed in polystyrene half-micro cuvettes. Each sample was recorded three times with 10 sub runs of 10 s.

Micron particles in the size range from 1 to 200 µm were quantified by light obscuration using a SVSS-C with a HCB-LD 25/25 sensor (PAMAS, Partikelmess- und Analysensysteme GmbH, Rutesheim, Germany). Prior to each measurement the system was rinsed with at least 10 ml of filtered purified water until particle counts of less than 100 particles/ml>1 µm and less than 10 particles>10 µm were present. Results were calculated as mean value of three measurements of 0.3 ml and referred to a sample volume of 1.0 ml.

Fluorescent Labelling of Monomeric and Aggregated Antibody

For preparation of fluorescent-labelled rituximab aggregates for MoDC uptake experiments, Alexa Fluor 488 (A488) carboxylic acid, N-hydroxysuccinimide ester was obtained from Invitrogen (Merelbeke, Belgium). The fluorescent labelling of rituximab (rituximab-A488) was performed according to the manufacturer's instructions, using an IgG concentration of 10 mg/ml and a molar ratio of 4:1 (dye: IgG). A pH of 7.4 was chosen for the labelling buffer, in order to achieve selective labelling of the amine termini. The rituximab-A488 was dialyzed back to PBS using a Float-A-Lyzer® G2 (Spectrum, Rancho Dominguez, CA, USA) with a 100 kDa molecular weight cut-off membrane to remove excess dye, and the labelling ratio achieved was about two A488 labels per rituximab molecule.

Preparation of Peripheral Blood Mononuclear Cells (PBMC)

PBMC were isolated from healthy community donor buffy coats (from blood drawn within 24 h) obtained from the UK National Blood Transfusion Service (Addenbrooke's Hospital, Cambridge, UK) and according to approval granted by Cambridgeshire Local Research Ethics Committee. PBMC were isolated from buffy coats by Lymphoprep (Axis-shield, Dundee, UK) density centrifugation and $CD8^+$ T cells depleted using $CD8^+$ RosetteSep™ (StemCell Technologies Inc., London, UK). PBMC were then frozen and stored in liquid nitrogen until required.

In Vitro T Cell Assays

Where indicated healthy donors were selected based on broad representation of HLA-DR allotypes (40 donors for rituximab and 25 donors for trastuzumab). After donor selection, PBMC were thawed, counted, viability assessed and suspended in AIM-V® culture medium (Invitrogen, Paisley, UK) at 4–6 × 10^6 PBMC/ml. Bulk cultures were established for each donor where 1 ml of cell suspension was added to the wells of a 24-well plate (Corning Life Science, Amsterdam, NL) along with 1 ml of antibody in AIM-V® culture medium. Proliferation of $CD4^+$ T cells within the culture was measured on days 5, 6, 7 and 8 post-stimulation by gently re-suspending the cells in the bulk cultures followed by removal of 3 × 100 µl samples, which were transferred to a round bottomed 96-well plate for pulsing with 1 µCi/well tritiated thymidine (Perkin Elmer, Buckingham, UK). After 18 h the pulsed cultures were harvested onto filter mats (Perkin Elmer) using a TomTec Mach III cell harvester and counts per minute (cpm) for each well determined by Meltilex™ (Perkin Elmer) scintillation counting on a 1450 Microbeta Wallac Trilux Liquid Scintillation Counter (Perkin Elmer) in paralux, low background counting mode.

Generation of Monocyte-Derived Dendritic Cells

Monocytes were extracted from PBMC by negative selection with monocyte isolation kit II MACS beads (Miltenyi Biotech, Bisley, UK) according to the manufacturer's instructions. The $CD14^+$ monocytes were cultured at 1 × 10^6 cells/ml with 1000 IU/ml of IL-4 and GM-CSF (Peprotech, London, UK) in AIM-V® for 72 h to produce MoDC, which were stimulated with antibodies for a further 24 h and harvested before measuring cytokine secretion via cytometric bead array (CBA), protein uptake by phagocytosis (laser scanning confocal microscopy) or cell surface phenotype (flow cytometry).

Cytometric Bead Array

Supernatants were collected from cultured cells and analyzed for cytokine production using BD CBA Enhanced Sensitivity Flex Set System (BD Biosciences, Oxford, UK), according to the manufacturer's instructions. Briefly, thawed culture supernatant and serially diluted standards were incubated with premixed capture beads for 2 h followed by additional 2 h with detection reagents. The sensitivity of detection was enhanced following a 1 h incubation with enhanced detection reagent. Cytokines were acquired by a BD Accuri C6 instrument (BD Biosciences). Data were analyzed using FCAP v3.01 software (Softflow, Inc, Minnesota USA).

Rituximab Aggregate Uptake by MoDC

Coverslips coated with MoDC (MoDC were initially grown on round coverslips) were transferred to 1 ml fresh AIM-V® medium and rituximab-A488 was added followed by incubation at 37°C or on ice (for control). At 0.5 h and 3 h the cells were washed three times with PBS followed by 15 min incubation on ice with BD Bioscience Cell Fix™. Cells were stained with HLA-DR PE (BD Bioscience) on ice for 30 min. Coverslips were inverted onto microscope slides with Vectashield (Vector Labs, Peterborough, UK) mounting medium. The stained cells were imaged with a Zeiss LSM 510 META laser scanning confocal microscope using a 40_NA 1.3 oil immersion objective. Single confocal slices were acquired to generate the data images. Three-dimensional reconstructions of confocal slices were made using Volocity software (Improvision, Perkin Elmer). Using image J software (http://rsbweb.nih.gov/ij/) the intensity across the width of the images was plotted for each wavelength (grey scale).

Data Analysis

For proliferation assays, a stimulation index (SI) was calculated as described previously [3]. Briefly, the counts/min for each donor treated with aggregated antibodies was divided by the background response. An SI≥1.9 was considered positive, based on a statistically derived cut point for the population tested for an adaptive response.

Statistical Analysis

Statistical analysis was carried out using GraphPad Prism Software version 6 (GraphPad Software Inc, California, USA). Two sample paired t-test or one way repeated measure ANOVA were carried out (corrected for multiple tests where appropriate).

RESULTS

In this study, therapeutic mAbs, stressed by a variety of pharmaceutically relevant methods to induce small amounts of aggregates, were used to investigate the effects of different aggregation conditions on *in vitro* T cell responses. Moreover, the effects of differently stressed IgG on protein uptake and activation of MoDC was studied. We chose two frequently used therapeutics for these studies; a chimeric monoclonal IgG, rituximab, and a humanized monoclonal IgG, trastuzumab.

Analytical Characterisation of Aggregated Monoclonal Antibodies

To test the effect of aggregate-containing therapeutics on T cell responses, monomeric antibody was exposed to stir, heat, or freeze/thaw stress conditions (Table I). The stress conditions were fine-tuned to ensure that for post-stress samples hardly any monomer was lost and the aggregates that were induced involved only a minor fraction of the total protein in the stressed samples. Therefore, these samples may reflect products that are administered to patients, e.g. after long-term or inadequate storage conditions or handling. Indeed, for all samples, hardly any loss of monomer was found after stress treatment, as determined by HP-SEC (Table I). For both antibodies, mechanical stress through stirring generated the highest level of micron-sized aggregates, as evidenced by light obscuration (increased levels of particles with a size of 1–10 μm) and by DLS (increased Z-average diameter/PDI score), indicating a very broad size distribution of aggregates in the nano- and low micron-size range. Heat-stressed aggregates for both rituximab and trastuzumab were mainly in the low nanometre-size range according to DLS (Table I). These aggregates were not detected by HP-SEC, although there was some monomer loss (Table I), indicating that they were too large or too sticky to pass through the HP-SEC column. Finally, freeze/thawing stress resulted in the formation of small (<10 μm) micron particles only for trastuzumab and not rituximab.

Aggregated Humanized Monoclonal Antibody Induces *in Vitro* T Cell Activation

The effect of the stressed and unstressed antibodies on *in vitro* T cell proliferation was assessed by ^{3}H-thymidine incorporation. PBMC depleted of $CD8^{+}$ T cells were isolated from healthy donors, selected for HLA diversity (supplementary Fig. 1), and stimulated with monomeric antibodies (40 donors for rituximab and 25 donors for trastuzumab). T cell proliferation was measured over days 5–8 of the *in vitro* culture, and the maximum proliferation of cells during this period (shown as stimulation index, SI) for each individual donor is shown in Fig. 1.

A significant increase in proliferation was detected for cells incubated with non-stressed rituximab compared to untreated cells in 4 donors tested (SI≥1.9) (Fig. 1). Non-stressed trastuzumab did not induce any significant $CD4^{+}$ T cell proliferation in any of the donors tested compared to the untreated cultures (Fig. 1).

Mean proliferation of $CD4^{+}$ T cells, and the frequency of donors responding, were increased in the presence of stir- and heat-stressed rituximab compared to the non-stressed

Table I Characteristics of IgG pre- and Post-Stress. [1] Besides Monomers, Only Dimers Were Detected; Values are Mean of Duplicate Measurements (Difference Between Upper and Lower Value of Duplicate Measurements in Brackets), [2] Mean Value ± Standard Deviation of Triplicate Measurements

Antibody	Stress condition	HP-SEC [1]		DLS [2]		Light obscuration (particles/ml) [2]		
		Recovery, relative to unstressed (%)	Monomer content (%)	Z-average diameter (nm)	PDI	>1 μm	>10 μm	>25 μm
Rituximab	Unstressed	100.0 (0.08)	99.1	10.2 ± 0.3	0.09 ± 0.02	2,989 ± 287	117 ± 58	22 ± 0
	Stir	103.1 (0.46)	99.1	1540 ± 134	1.00 ± 0.00	98,978 ± 2,125	60 ± 10	0 ± 0
	Heat	98.7 (0.02)	99.3	19.7 ± 0.4	0.38 ± 0.14	34,836 ± 1,314	133 ± 29	11 ± 19
	Freeze/thaw	100.5 (0.11)	98.9	10.1 ± 0.1	0.18 ± 0.01	4,511 ± 520	28 ± 19	0 ± 0
Trastuzumab	Unstressed	100.0 (0.20)	97.5	10.3 ± 0.1	0.14 ± 0.02	13,554 ± 904	17 ± 0	6 ± 10
	Stir	96.7 (0.24)	97.7	2203 ± 857	0.77 ± 0.24	896,544 ± 33,073	33,073 ± 67	0 ± 0
	Heat	96.9 (0.08)	97.6	28.7 ± 6.2	0.11 ± 0.01	12,982 ± 1,021	6 ± 10	0 ± 0
	Freeze/thaw	95.6 (0.17)	96.8	53.3 ± 30.1	0.14 ± 0.03	80,925 ± 10,404	278 ± 67	0

antibody (Fig. 1), although this was not statistically significant. In contrast, rituximab stressed by freeze/thawing did not induce any increase in proliferation, consistent with the absence of any measurable aggregate formation after stressing the rituximab material (Table I). Interestingly, $CD4^+$ T cell proliferation was significantly enhanced in the presence of all three stressed trastuzumab samples compared to the non-stressed sample (Fig. 1).

Overall, these data suggest that minute amount of aggregates generated by different stress conditions can induce activation of T cells *in vitro*. This effect was significant for trastuzumab but not rituximab, most likely because of the intrinsic immunogenic potential of unstressed rituximab which is not significantly enhanced by its aggregation.

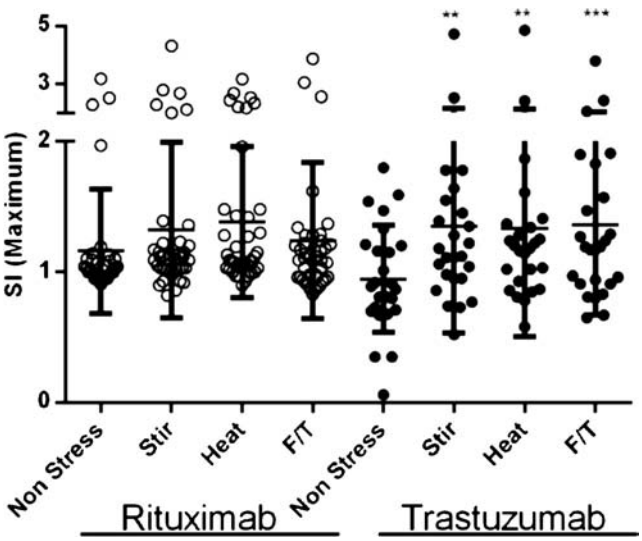

Fig. 1 Proliferation of $CD4^+$ T cells cultured in the presence of non-stressed or stressed rituximab and trastuzumab. Proliferation was measured by ^{3}H-thymidine incorporation and is expressed as maximum stimulation index (SI) from days 5–8. F/T – freeze thaw. Statistical significance was determined by Student's paired *t*-test by comparison to non-stressed antibody conditions; ** $p < 0.01$; *** $p < 0.001$.

Cytokine Production in $CD8^+$-Depleted PBMC Cultures Stimulated With Aggregated Humanized Antibody

Proliferation data indicated different magnitudes of $CD4^+$ T cell responses to stressed rituximab and trastuzumab. To examine whether such differences are reflected in cytokine production by PBMC, culture supernatants taken at day 4 of the *in vitro* T cell proliferation assays were assessed for the presence of inflammatory and anti-inflammatory cytokines (Fig. 2). Unstressed rituximab significantly increased TNF-α levels compared to the monomeric trastuzumab, confirming that rituximab is associated with increased immunogenicity compared to trastuzumab in its monomeric form (Fig. 2).

In-keeping with no significant increase in $CD4^+$ T cell proliferation, there was no significant difference in cytokines, including IL-2 and IFN-γ, produced in response to rituximab in the non-stressed form compared to the stressed conditions, although there was a trend towards increased TNF-α levels for the stressed samples. In contrast, all three stressed forms of trastuzumab induced significantly increased levels of IL-2 and IL-10 compared to non-stressed antibody. Although, incubation with stressed trastuzumab did not lead to increased levels of IL-21 in the entire cohort, 3 donors with increased $CD4^+$ T cell proliferation and IL-2 production in response to trastuzumab, also showed increased levels of IL-21. Consequently these individuals had a strong correlation between IL-21 secretion and increased IL-2 production.

Phenotype of Dendritic Cells Stimulated With Aggregated Antibody

We hypothesized that stimulation of MoDC with aggregates of antibodies would induce a change in DC phenotype, compared to non-stressed antibody. To examine whether proliferation of $CD4^+$ T cells in response to

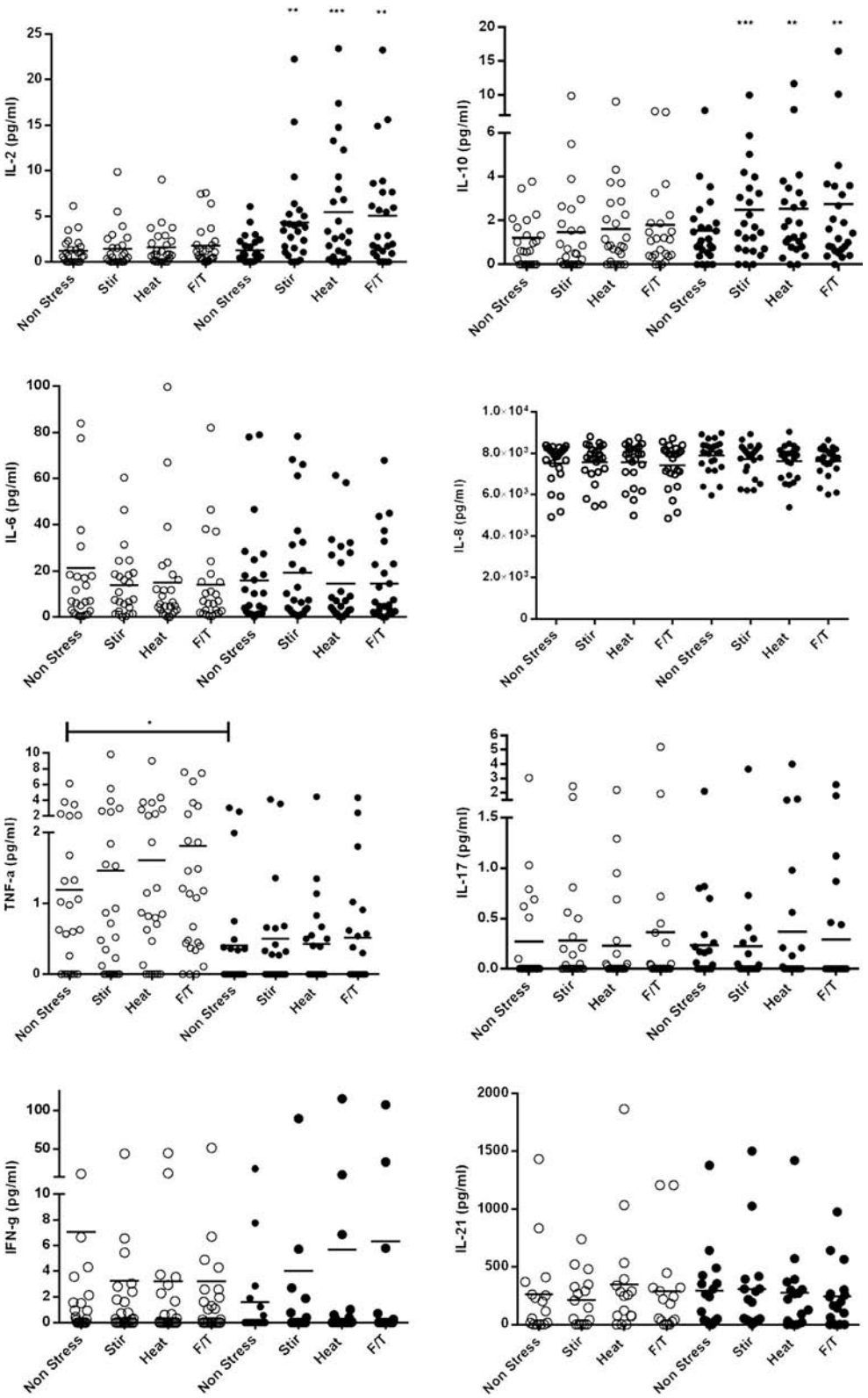

Fig. 2 Cytokine production by $CD4^+$ T cells cultured in the presence of non-stressed or stressed rituximab and trastuzumab. Culture supernatant was collected on day 4 and analysed by CBA. Statistical significance was determined by Student's paired *t*-test by comparison to non-stressed antibody conditions; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

aggregated trastuzumab would correlate with DC activation, DC were generated from 4 donors that showed $CD4^+$ T cell proliferation in response to stressed trastuzumab. MoDC, derived from $CD14^+$ monocytes, were generated from each of the 4 donors and stimulated with each of the stressed samples. After 24 h, cells were surface phenotyped for antigen presenting and co-stimulatory molecules (Table II).

Table II Phenoyping of MoDC Following Treatment With Aggregated Samples. MoDC Were Treated With non-Stressed or Stressed Rituximab or Trastuzumab Antibodies for 24 h. Cells Were Washed and Stained for Cell Surface Expression of Various Receptors. Expression Levels on $CD11c^+$ Gated Cells Were Evaluated Using LSRFortessa™ and MFI Values Were Calculated Using FACSDiva™ Software. Table Shows Mean MFI, Values in Bold Were Significantly Increased on Stressed Antibody-Treated MoDC Relative to Cells Treated With non-Stressed Antibody, Italic Values Were Significantly Decreased, by one Tailed Paired Student's *t*-Test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). An Example Histogram is Shown in Supplementary Fig. 2 and raw Data for MFI Values are Shown in Supplementary Table 1

Marker	Rituximab				Trastuzumab			
	Monomeric	Stir	Heat	Freeze/Thaw	Monomeric	Stir	Heat	Freeze/Thaw
CD80	404	*300***	182	268	212	162	301	254
CD86	1203	*1280**	*1053***	*945***	987	**1141***	**1073***	1106
HLA-DR	573	545	578	*528**	511	**531***	516	525
CD40	187	179	171	*160****	209	208	221	216
CD83	194	201	188	*174**	183	197	187	187
CD209	1472	1431	1250	*1169**	1228	1342	1251	1341

MoDC treated with aggregates of trastuzumab generated by stirring or heat treatment had significantly increased expression of CD86 (Table II), and stir-stress aggregated trastuzumab also induced higher surface expression of HLA-DR, consistent with their maturation. All other markers for DC activation (CD40, CD80, CD83 and CD209) were not significantly altered by stressed trastuzumab treatment compared to non-stressed samples. Rituximab did not induce a significant increase in any of the markers when stressed samples were compared to unstressed antibody, if anything markers were lower, suggesting that despite the MoDC being less activated by aggregated rituximab, the intrinsic immunogenicity of rituximab was able to overcome this.

Cytokine Profile of Dendritic Cells Stimulated With Aggregated Antibody

Supernatants taken from the cultures of MoDC treated with stressed therapeutics described above, i.e., in the absence of T cells, were analyzed for the presence of cytokines to assess if differently stressed antibodies alter cytokine profiles specifically produced by DC.

Multiplex CBA analysis for IL-1β, IL-6, IL-8, IL-10 and IL-12 showed no significant changes in culture supernatants when non-stressed rituximab and non-stressed trastuzumab treated MoDC were compared, nor stressed rituximab-treated MoDC compared to non-stressed rituximab (Table III). Stir- and heat-stressed trastuzumab did induce significantly higher levels of IL-10 in the culture supernatants of MoDC compared to non-stressed trastuzumab (Table III). Stressed trastuzumab induced no significant changes in IL-1β, IL-6 and IL-12 levels, although levels of IL-8 were reduced in response to freeze/thawed trastuzumab.

Enhanced Uptake of Aggregated Antibody by MoDC

Since stir-stressed rituximab had a wide range of aggregate sizes in the sub-micron and micron size range (Table I), we compared the uptake of stir-stressed rituximab and unstressed rituximab by MoDC, using fluorescently labelled rituximab (rituximab-A488) and MoDC generated in the same way as

Table III Mean Cytokine Production by MoDC Following Treatment With Aggregated Samples. MoDC Were Treated With non-Stressed or Stressed Rituximab or Trastuzumab Antibodies for 24 h. Following the Incubation, Supernatant from the Cultures was Collected and Frozen at −80°C Until use. Levels of Cytokine Production Were Evaluated by Cytokine Bead Array Analysis and Concentration was Calculated in pg/ml Using FCAP Software. Table Shows Mean Cytokine Concentration (pg/ml), Values in Bold Were Significantly Increased for Stressed Antibody-Treated MoDC Relative to Cells Treated With non-Stressed Antibody Controls, Italic Values Were Significantly Decreased by one Tailed Paired Student's *t*-Test. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Raw Data for Cytokine Concentrations are Shown in Supplementary Table 2

Cytokine	Rituximab				Trastuzumab			
	Monomeric	Stir	Heat	Freeze/Thaw	Monomeric	Stir	Heat	Freeze/Thaw
IL-1B	1411	1424	1669	1448	1516	1446	1320	1202
IL-12	8	21	5	8	39	10	0	3
IL-10	836	959	834	773	720	**904***	**827***	715
IL-8	693605	677216	700453	691715	704575	678019	657762	*649358**
IL-6	1738	1990	1756	1651	2860	1561	1436	1102

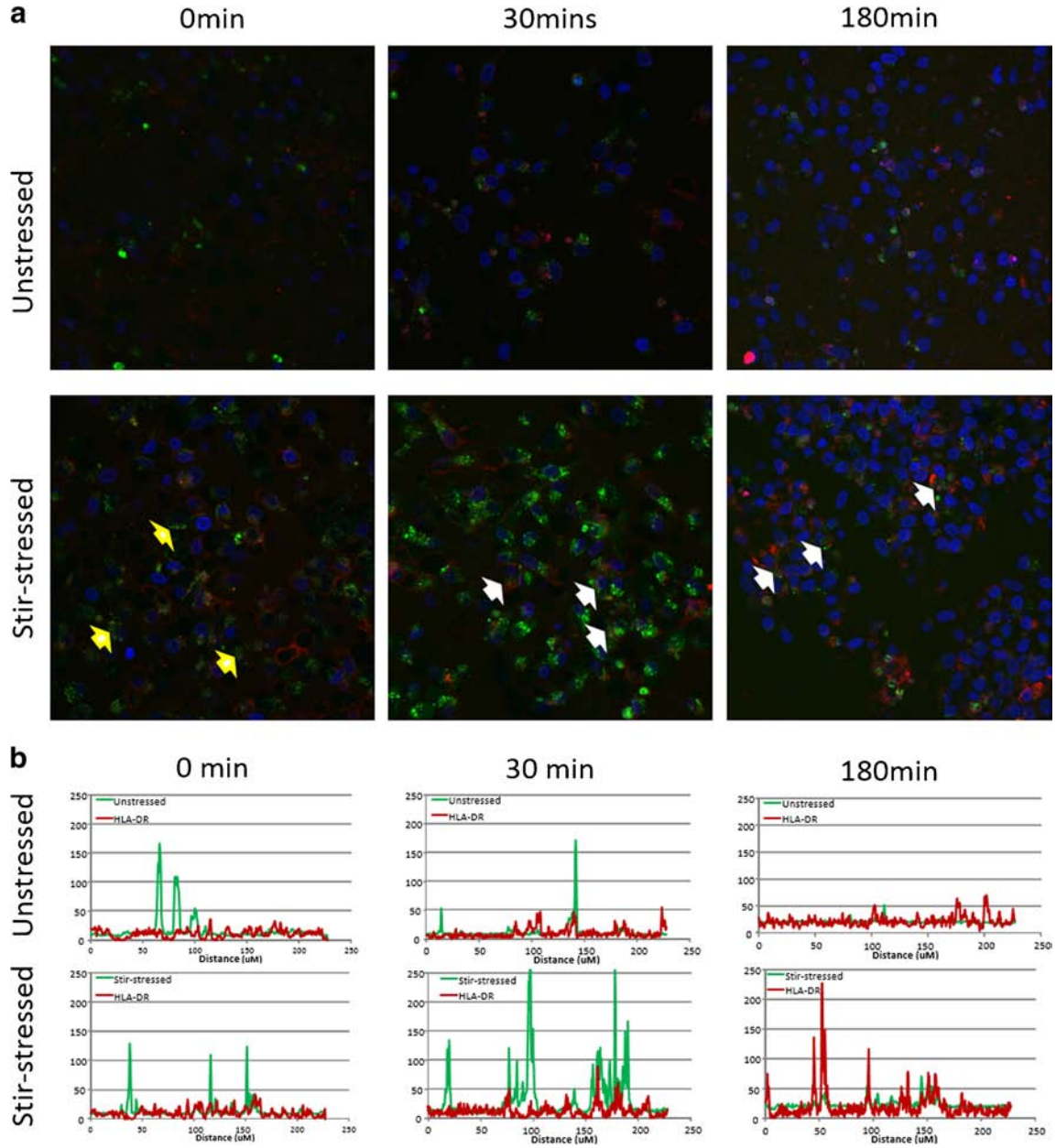

Fig. 3 Uptake of stir-stressed rituximab by MoDC. Stir-stressed rituximab was labelled with Alexa-fluor 488 (*green*) and incubated with cultures of DC. Uptake of the stir-stressed rituximab and its co-localisation with the endosomal pathway (identified by intracellular staining for HLA by PE conjugated anti-HLA antibody, in red) was measured by confocal microscopy, at 0, 30 and 180 min. Confocal images (**a**) and plots showing the intensity across the image (**b**) are shown for each time point. The nucleus is shown by DAPI staining in confocal images (*blue*). Yellow arrows highlight punctate staining with Alexa-fluor 488, white arrows highlight areas of Alexa-fluor 488 and PE co localisation.

described in the above studies, but grown on slides. Labelling monoclonal IgG with low amounts of fluorescent label, as done here, does not have a major influence on the physical or chemical characteristics of the molecule or its sensitivity to stress, as reported previously [14, 15]. Confocal microscopy of live cells loaded with unstressed rituximab (Fig. 3a) showed diffuse staining of rituximab (green) at time 0, with low amounts of punctate staining at 30 min with some overlap with HLA-DR (red), and no overlap at 3 h. This was confirmed by the image line transects showing very little increase in green intensity and few overlapping peaks in both the green and red channels (Fig. 3b). In contrast, cells loaded with stir-stressed rituximab (Fig. 3a) showed punctate staining at time 0 (yellow arrows in Fig. 3a) with significant overlap with HLA-DR by 30 min (white arrows in Fig. 3a). This overlap remained at 3 h, although levels of rituximab appear reduced, likely due to cleavage of the fluorochrome from the aggregate during antigen processing. This is confirmed by the image line transects showing large peaks in the green channel intensity which significantly overlap peaks in HLA-DR in the red

channel intensity (Fig. 3b). These results indicate that stressed rituximab is efficiently taken up by MoDC and rapidly accumulate in late endosomes, which are associated with antigen presentation.

DISCUSSION

For protein therapeutics the formation of aggregates can occur at multiple stages during manufacturing, storage or handling. These aggregates are heterogeneous, dynamic and sometimes reversible by nature, therefore characterizing these qualities and their impact on the immunogenicity of protein therapeutics is a major interest in the development of new biologics.

Here we have shown that stressed mAb products containing low (<3% of total protein content) levels of protein aggregates can induce immune responses *in vitro*, by activating innate and adaptive pathways, and that the increased immunogenicity of these stressed products is dependent on the type of antibody as much as the physical characteristics of the aggregates formed. Two monoclonal antibodies, rituximab and trastuzumab, were selected based on their differences in inherent immunogenicity observed in the clinic. Rituximab has documented clinical immunogenicity in rheumatoid arthritis patients [16] whereas trastuzumab has a reported low rate of clinical immunogenicity [17]. In keeping with these data, the monomeric rituximab was associated with a 16% T cell proliferation response rate in the donor cohort tested and induced significantly higher TNF-α response by PBMC compared to trastuzumab. In contrast, the non-stressed trastuzumab did not induce any significant T cell proliferative and PBMC cytokine responses in the donor-cohort tested, consistent with the low rate of clinical immunogenicity described for this antibody.

Stressed rituximab did not have a significant effect on $CD4^+$ T cell responses when compared to monomeric rituximab, likely due to its inherent immunogenicity such as that observed in patients treated for rheumatoid arthritis where 11% of patients develop ADA [16]. Indeed the presence of MHC class II restricted T cell epitopes in the variable domain [18] are likely to mask any effects of aggregates since monomeric antibodies were still in vast excess in the stressed samples. In contrast, monomeric trastuzumab induced a lower $CD4^+$ T cell proliferative/cytokine response than monomeric rituximab which is consistent with lack of $CD4^+$ T cell epitopes (MB, unpublished observations). However, once stressed and regardless of the stress method to create minute amounts of sub-visible aggregates, trastuzumab became a potent inducer of $CD4^+$ T cell proliferation and cytokine production by human PBMC. As $CD4^+$ T cell epitopes have not been described for trastuzumab, the observed increased $CD4^+$ T cell proliferation may reflect a bystander T cell response, in which T cells are activated independent of TCR engagement in response to costimulation from DC induced by the stimulation of innate immune receptors. In support of this, our data showed that the presence of low levels of stress-induced aggregates of trastuzumab resulted in significantly increased production of the T cell stimulatory cytokine IL-2. Increased IL-2 levels correlate with the enhanced levels of T cell proliferation observed with aggregated, compared to non-stressed, trastuzumab. This suggests that in the presence of a suboptimal antigen-specific $CD4^+$ T cell response, the majority of $CD4^+$ T cells are being non-specifically activated and producing IL-2 in response to aggregate-activated MoDC. This hypothesis is supported by previous studies in which blocking of TLR2 and TLR4 (as well as FcγRI and FcγRIII) reduced cytokine secretion from PBMC stimulated with stress induced aggregates [3]. Indicating that aggregates bind to pattern recognition receptors on DC resulting in a highly activated phenotype that can potentially activate bystander T cells. It is of note that levels of IL-2 were lower in rituximab-treated cultures compared to those treated with stressed trastuzumab. We hypothesise that the rapid burst of IL-2 production induced by the intrinsic immunogenicity of rituximab [18] had already occurred by day 4 [19], when supernatant was collected from cell cultures. In contrast, aggregated trastuzumab-specific IL-2 production is likely to occur later, thus allowing its detection. Further studies would be required to determine if there are significant differences in the cytokine kinetics between the two responses, i.e. intrinsic immunogenicity *versus* immunogenicity driven by aggregates.

In some donors we found that IL-21 secretion was increased in response to stressed trastuzumab compared to the monomer. IL-21 is mainly produced by activated $CD4^+$ T cells, including Th2, Th17 and follicular helper T cells (Tfh). Secretion of this cytokine by Tfh cells is essential for differentiation of B cells into antibody secreting plasma cells [20, 21] and production of ADA. Although the source of IL-21 was not identified, Tfh cells are present in the peripheral blood in healthy controls [22] and thus may be the source of IL-21 in our study.

In addition to its immune-stimulatory effect, IL-21 has been shown to be a potent inducer of IL-10 cytokine [23]. We did observe significantly increased concentrations of IL-10 following treatment with aggregated trastuzumab, compared to the monomer, although this did not correlate with IL-21 production at the level of individual donors. The increased production of IL-10 may be attributable to MoDC, since IL-10 levels were significantly increased in trastuzumab-treated cultures of MoDC alone. This is in keeping with previous reports, where an increase in IL-10 was observed in PBMC cultures treated with antibody aggregates [24, 25], suggesting that a source of IL-10 production in those studies was monocyte-derived cells. The influence of IL-10 induction by

stressed antibody therapeutics on the developing T cell response, in terms of tolerance, remains to be determined, however IL-10 has been demonstrated to inhibit DC maturation [26, 27] and cytokine release [28] by these cells, resulting in T-cell anergy [29, 30].

Stir- and heat-stressed trastuzumab were shown to have a direct effect on APC maturation phenotype compared to its monomeric form, with significant up-regulation of the T cell co-stimulatory molecule CD86 (and for stir-stressed trastuzumab also the HLA-DR antigen-presenting molecule). Levels of other co-stimulatory molecules were also increased, albeit not significantly. This suggests that APC treated with aggregates of trastuzumab are much more likely to activate $CD4^+$ T cells in antigen-dependent or independent manner, which is in agreement with a recent report utilizing different therapeutic mAbs [12]. The APC phenotype did not differ amongst monomeric and stressed rituximab samples, which is likely due to the intrinsic immunogenicity associated with monomeric rituximab and as such the additive effects of aggregates is not observed.

Furthermore, the majority of the cytokines secreted by mAb-treated MoDC were not significantly different both within antibody treatments and between monomeric rituximab and trastuzumab. Whereas 24 h treatment of MoDC with mAbs was sufficient to detect differences in MoDC phenotypes, differences in cytokine levels in MoDC culture supernatants may be hard to detect at these early time points, and in future studies, analysis of multiple time points may be required to identify other differences.

Internalization of aggregates was studied using fluorescently tagged aggregates of rituximab. These studies showed efficient uptake of aggregates by MoDC, and subsequent presentation was strongly suggested by the presence of aggregated rituximab within late endosomes, as evidenced by its co-localization with HLA-DR [31]. Up-regulation of co-stimulatory molecules on the surface of DC, and their ability to present peptides derived from aggregated antibodies, could strongly suggest a role of DC in activation of immune responses against aggregates [12]. *In vivo*, following activation of DC in the presence of pro-inflammatory cytokines, DC migrate to the lymph nodes where T cell priming takes place, potentially resulting in T cell-dependent B cell activation in germinal centres and subsequent development of ADA [32, 33]. Understanding the mechanisms by which aggregated antibodies are internalised by DC is important, as the identification of the type of receptors involved may provide mechanisms to bypass the immunogenicity of aggregates. For example Joubert *et al.* demonstrated the involvement of toll like receptors in the uptake of aggregates [3].

Many therapeutics are dosed subcutaneously and can result in unwanted immunogenicity [34], this is proposed to happen via lymph node resident DC during a primary response [35, 36], and upon subsequent exposure subcutaneous DC and epidermal Langerhans cells play an important role in presentation of antigens to $CD4^+$ T cells [37].

The physicochemical properties of an aggregate (including but not limited to size, hydrophobicity and solubility), are dependent on the type of stress a protein is subjected to [7, 13, 24, 38, 39]. In the present study we exposed two mAbs to pharmaceutically relevant stress conditions, i.e., heat stress, stir stress and freeze/thaw stress to create low levels of aggregates, that may mimic products that are potentially administered to patients, e.g., after improper storage or handling procedures. In previous studies we have shown that aggregates induced by heat or mechanical stress are more conformationally changed and more hydrophobic than aggregates created by freeze/thaw stress [13, 38]. In the current study the intentionally low aggregate contents were too low to allow a proper conformational analysis of the aggregates. Moreover, the presence of polysorbate in the products did not permit an accurate differentiation of the products by fluorescent dye-based analysis (results not shown). Whilst our *in vitro* studies demonstrate that minute amount of aggregates can lead to immune activation, and can provide valuable information on risk of such aggregates on clinical immunogenicity, *in vitro* assays are limited in their ability to address long term development of ADA and cannot replace clinical studies [40].

In summary, our data from $CD4^+$ T cell proliferation, MoDC phenotyping and cytokine analysis suggest that only a minor fraction of a protein therapeutic has to be aggregated to induce T cell activation, which in turn could lead to anti-drug antibody formation. This is in line with a study by Fradkin *et al* [41] , who showed that the presence of as little as 20 ng sub-visible protein aggregates in otherwise 100% monomeric (according to HP-SEC) murine growth hormone led to antibody production in mice. In the case of trastuzumab these responses may be predominantly a product of bystander T cell activation resulting from strong cytokine and co-stimulatory signals derived from monocytic cells (e.g., monocyte-derived DC). In contrast, for rituximab, the presence of immunogenic aggregates may not necessarily have an additive effect on the epitope specific T cell response that is already being observed to monomeric antibody.

Conflict of interest The authors declare no conflicts of interest.

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