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Infliximab aggregates produced in severe and mild elevated temperature stress conditions induce an extended specific CD4 T-cell response

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ABSTRACT

Aggregation has been widely described as a factor contributing to therapeutic antibody immunogenicity. Although production of high-affinity anti-drug antibodies depends on the activation of CD4 T lymphocytes, little is known about the T-cell response induced by antibody aggregates, especially for aggregates produced in mild conditions resulting from minor handling errors of vials. Large insoluble infliximab (IFX) aggregates produced in severe elevated temperature stress conditions have been previously shown to induce human monocyte-derived dendritic cell (moDC) maturation. We here showed that large IFX aggregates recruit *in vitro* a significantly higher number of CD4 T-cells compared to native IFX. Moreover, a larger array of T-cell epitopes encompassing the entire variable regions was evidenced compared to the native antibody. We then compared the responses of moDCs to different types of aggregates generated by submitting IFX to mild conditions of various times of incubation at an elevated temperature. Decreasing stress duration reduced aggregate size and quantity, and subsequently altered moDC activation. Of importance, IFX aggregates generated in mild conditions and not altering moDC phenotype generated an *in vitro* T-cell response with a higher frequency of CD4 T cells compared to native IFX. Moreover, cross-reactivity studies of aggregate-specific T cells showed that some T cells could recognize both native and aggregated IFX, while others responded only to IFX aggregates. Taken together, our results suggest that aggregation of antibodies in mild elevated temperature stress conditions is sufficient to alter moDC phenotype in a dose-dependent manner and to increase T-cell response.

1. Introduction

Immunogenicity has been described as a major concern to the clinical use of biotherapeutics, as it may be one of the causes for the loss of therapeutic response (Papamichael et al., 2019) as well as hypersensitivity reactions (Matucci et al., 2019). The detection of high-affinity and isotype-switched anti-drug antibodies in patient sera suggests that the activation of CD4 T cells is essential for their immunization against biotherapeutics (Krishna and Nadler, 2016; Nabhan et al., 2020b). Accordingly, CD4 T cells specific for biotherapeutics have been detected in patients with anti-drug antibodies (Cassotta et al., 2019; Hamze et al., 2017; Vultaggio et al., 2016). However, the mechanisms leading to

anti-drug antibodies onset remains poorly understood especially how CD4 T cells are activated. The preexistence of CD4 T-cell repertoires in healthy donors, specific for antibodies, such as infliximab (IFX), rituximab and adalimumab, has been well documented over the past years (Delluc et al., 2011; Schultz et al., 2017). T-cell epitopes have been identified on the variable fragments of native monoclonal antibodies, including IFX (Hamze et al., 2017) and rituximab (Hamze et al., 2017) or natalizumab (Cassotta et al., 2019; Meunier et al., 2019) and adalimumab (Cassotta et al., 2019). Engagement of the T-cell receptor (TCR) by binding to HLA/peptide complexes is the first required signal to activate T-cells but a second co-stimulatory signal provided by the recruitment of CD28 by CD80/CD86 molecules and other combinations expressed at

Abbreviations: DC, dendritic cells; IFX, infliximab; S, elevated temperature stress; moDC, monocyte-derived dendritic cells; Th, T helper.

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the surface of mature DCs is also a requisite to prime T-cells. Soluble molecules, including soluble antibodies, are not expected to generate inflammatory conditions and to provoke mature dendritic cells but rather to create appropriate conditions that would induce tolerance (Gahring and Weigle, 1989). In contrast, protein aggregates can act as danger signals for innate immune cells (mainly dendritic cells (DCs) and monocytes) and favor their activation and the initiation of an adaptive immune response leading to anti-drug antibody production (Gallais et al., 2017; Joubert et al., 2012; E. M. Moussa et al., 2016; Polumuri et al., 2018). Moreover, *in vivo* studies in wild-type and T-cell deficient mice have previously shown that ADA production in response to therapeutic proteins aggregates was T-cell dependent (Bessa et al., 2015; Fradkin et al., 2014, 2009). Cell-based assays have been developed to evaluate the role of antibody aggregates in the immunization process (Joubert et al., 2016, 2012; Morgan et al., 2019; Rombach-Riegraf et al., 2014). We have previously shown that large insoluble aggregates of IFX, induced by incubation at elevated temperatures, behave as danger signals and induce moDC maturation (Morgan et al., 2019). This activation was shown to be mediated through the Fc γ RIIa–Syk axis and enhanced T-cell proliferation in allogeneic DC/T-cell co-cultures (Nabhan et al., 2020a).

Nevertheless, ability of antibody aggregates to induce a specific T-cell response has to be explored in more details especially for small, soluble aggregates, which might be produced in mild stress conditions. Using MHC-Associated Peptide Proteomics assay (MAPPs), Rombach-Riegraf et al. showed that loading of stir-stress aggregated antibodies led to an increase of antibody-specific peptides associated with MHC class II molecules (MHC II) (Rombach-Riegraf et al., 2014). Moreover, antibody-specific peptides were augmented both in quantity and diversity with peptides only present with the aggregated form suggesting the emergence of new epitopes. However, these results have not been confirmed so far with functional assays using DCs and T cells.

Because of their high immunogenicity, aggregate content in drug vials is extremely controlled and limited as much as possible. However, drug vial content could be submitted to multiple stresses during their storage including shear and heat stresses, which might lead to formation of heterogeneous aggregates varying in size and solubility. These stresses might be severe due to poor handling or brief elevated temperature exposure, but in many cases, the contents of drug vials are subjected to mild stress conditions due to minor handling errors (Dill et al., 2020; Siska et al., 2020).

In this study, we used *in vitro* cell-based assays to evaluate the T-cell response directed against antibody aggregates produced in severe and mild stress conditions induced by exposure to elevated temperatures. IFX was chosen as a model as it is known to be immunogenic and to be prone for aggregation (Lerch et al., 2017). Aggregates of IFX were generated under different stress conditions and characterized. Amplitude and specificity of the generated T cell response to different aggregates were investigated and compared to the response to native IFX.

2. Materials and methods

2.1. Aggregate preparation

Lyophilized infliximab (Remicade®, IFX) was commercially available, and reconstituted to a final concentration of 10 mg/mL with sterile water. For a first set of experiments, aggregate induction was carried out by submitting IFX to an elevated temperature stress (S) at 55 °C for 24 h, as previously described (Morgan et al., 2019; Nabhan et al., 2020a). In a second set of experiments, IFX solution was diluted to a final concentration of 1 mg/mL with sterile water and then submitted to a S at 55 °C for 1 h (S1), 2 h (S2), 3 h (S3), 4 h (S4), 6 h (S6) or 24 h (S24). Aliquots of samples were then stored at –80 °C until use. Aggregate stability was checked by size measurements using size exclusion chromatography and flow imaging microscopy after freezing and thawing and no modifications were recorded following storage at –80 °C.

Endotoxin was quantified for each native or aggregated product by using a colorimetric assay (ToxinSensor, Genscript), according to the manufacturer's instructions. This assay kit can detect endotoxin concentration in the range of 0.01 to 1 endotoxin units per milliliter (EU/mL). Levels of endotoxin were considered acceptable under the limit of 0.25 EU/mL.

2.2. Characterization of native and aggregated infliximab

2.2.1. Size exclusion chromatography (SEC)

SEC-Fluorescence detection (FLD) experiments were carried out using a 1260 Agilent UPLC, and a 1260 LIF detector (Agilent Technologies). A column Agilent Bio SEC-3 (3 μ m, 300 Å, 4.6 \times 300 mm) was employed (Temperature 25 °C). The mobile phase (100 mM sodium phosphate, 150 mM NaCl, 5 % isopropanol, pH 7.0) was isocratically pumped at 300 μ L.min⁻¹. LIF detection was monitored by Fluorescence detector (λ_{ex} = 280 nm, and λ_{em} = 350 nm). Aggregate samples were centrifuged to remove visible and insoluble particles and then diluted ten-fold before injecting 20 μ L for separation. Sodium phosphate and sodium chloride were purchased from Fisher Scientific (Karlsruhe) and from Euromedex (Souffelweyersheim), respectively. HPLC grade isopropanol was obtained from Merck (Darmstadt).

2.2.2. Flow imaging microscopy

The aggregate size was studied by flow imaging microscopy, on a Flowcell FC200S+HR (Occhio). Briefly, this apparatus captured images of the sample flowing in a cell, and the software Callisto (Occhio) analyzed the shape and the size of each particle individually. A 100 μ m-thick spacer was used, to be sure that even the largest aggregates could pass through the cell and the samples were magnified 6 times. Before each analysis, a background calibration was performed on ultrapure water. Furthermore, the sample initially at 1 mg/mL was diluted in ultrapure water to a concentration equal to 0.5 mg/mL. The diluted sample was then deposited on top of the cell and pumped through it. From the captured images, size and shape information were extracted. As the aggregates looked anisotropic, "Length" was chosen as the most relevant size parameter for further descriptions. In brief, a Feret diameter is the distance between two parallel lines, both tangent to the particle shape. There are many Feret diameters for each particle, but the "Length" as defined in the software was the maximum Feret diameter. Polydispersity was given by the relative span index, which was calculated according to Span = $(d_{90} - d_{10})/d_{50}$ where d_{90} , d_{50} and d_{10} are the particle diameters determined respectively at the 90th, 50th and 10th percentile of undersized particles.

2.3. Generation of human monocyte-derived dendritic cells (moDCs)

Human peripheral blood mononuclear cells (PBMCs) were purified from buffy coats directly obtained from Etablissement Français du Sang (EFS Rennes, France). Healthy donors gave their written consent for the use of blood donation for research purposes. As previously described (Nabhan et al., 2020a), monocytes were isolated from the mononuclear fraction by magnetic positive selection with MidiMacs separation columns and anti-CD14 antibodies coated on magnetic beads (Miltenyi Biotec). Monocytes were cultured at 1×10^6 cells/mL in the presence of GM-CSF (550 U/mL) (Miltenyi Biotec, Cat# 130-093-868) and IL-4 (550 U/mL) (Miltenyi Biotec, Cat# 130-093-924) in RPMI-1640 25 mM HEPES GlutaMAX™ (Gibco, Thermo Fisher Scientific, Cat# 72400-021) supplemented with 10 % fetal calf serum, antibiotics (Pen Strep, Gibco, Thermo Fisher Scientific, Cat# 15140122) and 1 % pyruvate (Gibco, Thermo Fisher Scientific, Cat# 11360070) at 37 °C in humidified air containing 5 % CO₂. Within 4 days, monocytes differentiated into moDCs with an immature phenotype.

2.4. Treatment of moDCs & flow cytometric analysis of moDC maturation

On day 4, immature moDCs were stimulated with 100 µg/mL of native (IFX N) or aggregated IFX (S1, S2, S3, S4, S6, S24) or lipopolysaccharide (LPS, 25 ng/mL used as positive control; Sigma Aldrich) and incubated at 37 °C for 24 h. After 24 h of treatment, moDC viability and phenotype were analyzed by flow cytometry. Cell viability was assessed in preliminary experiments by propidium iodide (Thermo Fisher Scientific, Cat# R37108) staining, used at a final concentration of 625 ng/mL, on a small fraction of moDC cultures. Cell viability after treatment was never less than 80 %. For the phenotypic analysis, the surface labeling procedure was as follows: 2×10^5 cells per mL were washed in cold phosphate-buffered saline (PBS) supplemented with 0.5 % bovine serum albumin (BSA) and stained with monoclonal antibodies (mAbs) in the dark, on ice for 20 min. The following mAbs were used: allophycocyanin (APC)-labeled mouse anti-human CD86 (BD Biosciences, Cat# 555660); Phycoerythrin (PE)-labeled mouse anti-human CD83 (BD Biosciences, Cat# 556855). Appropriate isotype controls (mouse IgG1 κ , BD Biosciences) were used at the same concentration to determine non-specific staining. Cells were analyzed on an Attune Nxt (Invitrogen) using FlowJo software (version 10; FlowJo LLC), and we used a gating strategy to exclude dead cells, based on the FSC/SSC criteria. The data acquisitions were performed on a minimum of 10,000 living cells. Results were expressed as the relative fluorescence intensity (RFI), using the corrected mean fluorescence intensity (cMFI), as follows: $cMFI = MFI - MFI$ of isotype control; $RFI = cMFI$ of treated cells/ $cMFI$ of untreated cells).

2.5. Cytokine and chemokine quantification in cell culture supernatants

Meso Scale Discovery (MSD) multiplex assay was performed on culture supernatants according to the manufacturer's instructions to measure in duplicate IL-6, IL-8, IL-10, IL-12/23p40, CCL3 (MIP-1 α), CCL4 (MIP-1 β) and TNF- α . The quantification ranges were: IL-6: 0.49–2030 pg/mL, IL-8: 0.56–2060 pg/mL, IL-10: 0.94–3860 pg/mL, IL-12/23p40: 5.27–21,600 pg/mL, CCL3: 1.64–6720 pg/mL, CCL4: 0.43–1770 pg/mL, TNF- α : 0.89–3660 pg/mL.

2.6. Autologous co-cultures of dendritic cells and naïve CD4 T cells

Cells were isolated from healthy donors' blood. MoDCs were differentiated from monocytes as described above. Autologous naïve CD4 T cells were isolated from PBMCs by negative selection using specific microbeads (Miltenyi Biotec). Percentage of CD4+CD45+CCR7+ (>90 %) was evaluated using flow cytometry (Attune Nxt, Invitrogen); the following mAbs were used: APC-labeled mouse anti-human CD4 (BD Biosciences, Cat# 555349), FITC-labeled mouse anti-human CD45RA (Miltenyi Biotec, Cat# 130-113-927) and FITC-labeled mouse anti-human CCR7 (Miltenyi Biotec, Cat# 130-120-608). For T-cell stimulation, moDCs were loaded overnight with 100 µg/mL of native (N) or aggregated (S1) IFX in the presence of LPS (1 µg/mL; Sigma Aldrich) to induce efficient presentation in AIM-V medium (Gibco, ThermoFisher Scientific, Cat# 12,055,091). MoDCs were then washed and 10^4 cells were added to 10^5 autologous naïve CD4 T cells per round-bottom microwell containing 200 µL Iscove's modified Dulbecco's medium (IMDM, Lonza, Cat# BE12-726F) supplemented with 10 % human AB serum (Lonza), 10 000 U/mL rh-IL-6 and 10 ng/mL rh-IL-12 (R&D Systems) and 5 µg/mL primocin (Invivogen). T cells were re-stimulated on days 7, 14 and 21 with autologous moDCs loaded with LPS and IFX N or IFX S1 in the presence of rh-IL-2 (10 U/mL) and rh-IL-7 (5 ng/mL) (R&D Systems). T-cell stimulation with moDCs loaded with KLH (Keyhole Limpet Hemocyanin, Imject™ ThermoFisher Scientific) was used as positive control. Specificity of T-cell response was investigated using an IFN- γ ELISpot assay on day 27.

2.7. IFN- γ ELISpot assay

96-well nitrocellulose plates (Merck Millipore) were coated with a capture mouse IgG1 anti-human IFN- γ monoclonal antibody (Mabtech, Cat# 1-D1K) and saturated with IMDM supplemented with 10 % human AB serum. Protein-specific T-cell lines were then co-cultured with moDCs loaded with different forms of IFX, overlapping pools of synthetic peptides (Pepscan) or individual peptides or KLH with or without MHC class II blocking antibodies (anti-human HLA-DP (clone B7/21; Abcam), DQ (clone Tü169; Biolegend) and DR α (clone L243; Biolegend), each at 10 µg/mL) or unloaded moDCs (as negative control). After 16 h of co-culture, biotinylated mouse IgG1 anti-human IFN- γ monoclonal antibody (Mabtech, Cat# 7B6-1) was added for 2 h. Spots were revealed by the addition of extravidin conjugate and BCIP substrate (Sigma Aldrich). The number of spots was determined using the AID ELISPOT Reader system (Mabtech). A well was considered positive, containing antigen-specific T cells, A positive value was assigned to culture wells with a spot count that was two-fold higher in the presence of the protein than in its absence (unloaded moDCs). A minimum of 25 spots/well is required for analysis to be valid. Antigen-specific naïve CD4 T-cell frequency was estimated using the Poisson distribution according to the following formula: $Frequency = -\ln(\text{Negative wells}/\text{Total wells tested}) / (\text{Naïve CD4 T cells per well})$.

2.8. Statistical analysis

Differences between groups were evaluated with the Kruskal-Wallis (for moDC maturation experiments) or Wilcoxon signed-rank test (for specific naïve CD4 T-cell frequencies) (Prism software, GraphPad). *P* values below 0.05 were considered to denote statistical significance.

3. Results

3.1. Aggregation increases specific CD4 T-cell response to infliximab peptides

As we previously showed that IFX aggregates produced in severe stress conditions behave as an immune danger signal towards moDC (Morgan et al., 2019; Nabhan et al., 2020a), we first investigated the ability of such aggregates to mount an *in vitro* T-cell response. Weekly and for four weeks, we stimulated CD4 T cells derived from PBMCs of 12 healthy donors with autologous moDCs loaded overnight with either native or aggregated IFX. Each independent T-cell line (a "cell-line" represents CD4 T cells present in a single well) was evaluated for its specificity to native or aggregated IFX. Each healthy donor gave rise to a variable number of native or aggregate IFX-specific T-cell lines, however IFX aggregates induced the activation of a greater number of specific CD4 T cells compared to native IFX (Fig. 1a). T-cell lines that were found to respond to either native or aggregated IFX were then evaluated for their specificity using pools of 45 overlapping peptides encompassing the entire variable regions (VH and VL) of IFX and individual peptides, as previously described (Hamze et al., 2017). Here we showed that aggregate-specific T-cell response was sustained by 17 different IFX peptides found in the variable regions of both light and heavy chains while CD4 T-cell response specific for native IFX was only detected with 5 IFX peptides (Fig. 1b).

3.2. Elevated temperature stress induces a time-dependent formation of micrometer-size infliximab aggregates

To evaluate the role of size and quantity of antibody aggregates on DC and specific T-cell response, diluted IFX (1 mg/mL) was submitted to different durations of stress through incubation at an elevated temperature (S) at 55 °C. Increasing times of stress (1, 2, 3, 4, 6 and 24 h) were applied to test the stress duration effect on IFX aggregation. Endotoxin levels were quantified on three independent samples and were always

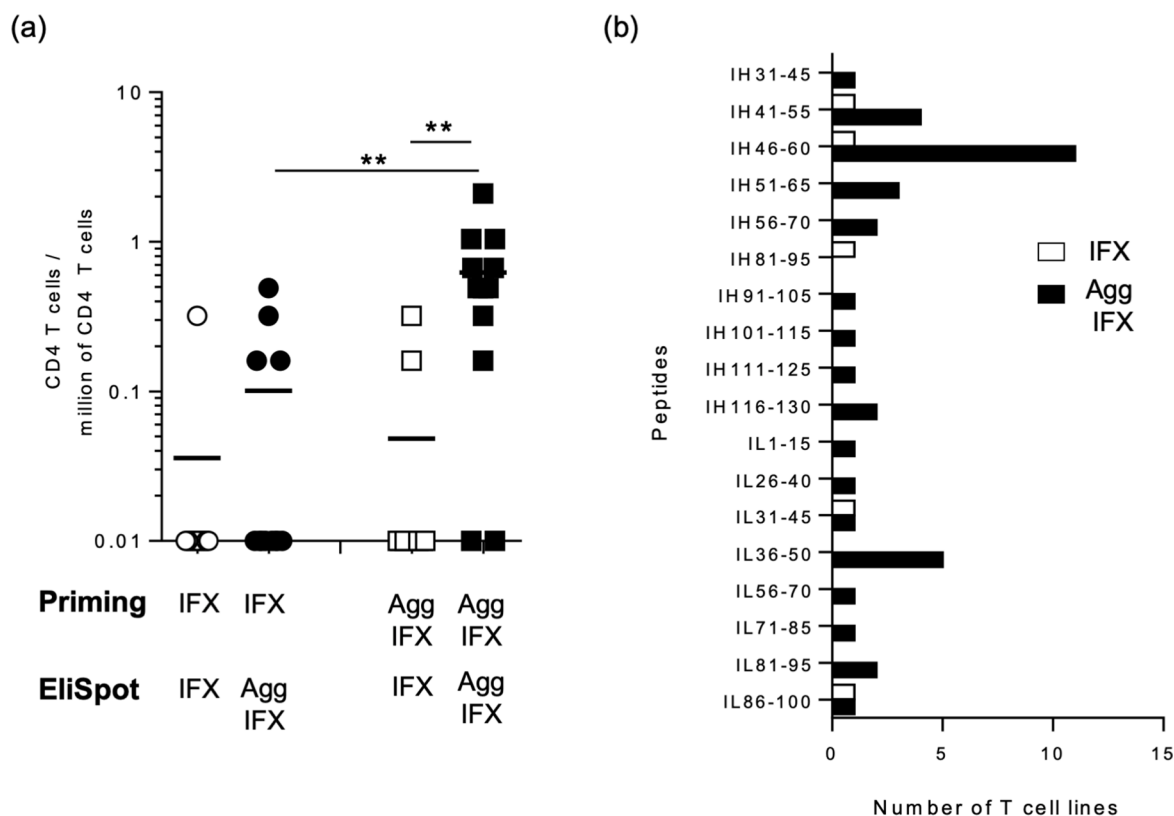


Fig. 1. Aggregation increases CD4 T-cell response to infliximab peptides.

CD4 T cells from healthy donors were repeatedly stimulated *in vitro* with IFX or aggregated IFX. After 3 weeks of culture, CD4 T cell response was analyzed by IFN- γ ELISpot assays. (a) The T cell lines raised against IFX (circle) or its aggregated form (square) were tested in the ELISpot assay with the therapeutic antibody (empty) or the aggregated form (filled). Frequencies of specific CD4 T-cells were estimated for 12 healthy blood donors and were calculated using the Poisson's distribution as described in the M&M section. The statistical significance was assessed using the Wilcoxon matched-pairs signed rank test (**: $p < 0.01$). (b) The T cell lines raised against the therapeutic antibody (empty) or the aggregated form (filled) were tested in the ELISpot assay using overlapping peptides covering the VH and VL domains of each Mab.

under the acceptable limit of 0.25 EU/mL (data not shown). Aggregates were first characterized by two complementary methods and on three independent samples (with three measures for each sample) to verify reproducibility.

Native and aggregated proteins were first characterized by size exclusion chromatography. A single peak of monomer was observed for native IFX (data not shown). For aggregated IFX, a stress time-dependent decrease in the area of the chromatogram peak of the monomer compared to the native solution was observed (data not shown). Concentrations of monomeric antibody present after aggregation were calculated according to the area of the chromatogram peaks (Fig. 2a). For instance, after 1 hour of incubation at 55 °C (S1), 90.3 % of monomeric antibody (0.903 ± 0.028 mg/mL) was left in the aggregated solution and no aggregates of intermediate size were detected (Fig. 2a), meaning that S1 induced the formation of around 9.7 % of insoluble aggregates that were not eluted from the column. However, after 24 h of stress (S24), less than 1 % of monomeric antibody (0.005 ± 0.001 mg/mL) was left in the aggregated solution and no aggregates of intermediate size were detected (Fig. 2a), indicating that S24 induced the formation of around 99.5 % of insoluble aggregates. These results indicated that formation of insoluble aggregates upon incubation at an elevated temperature is increased in a time-dependent manner.

To characterize the insoluble aggregates, flow imaging microscopy was used to analyze the shape and size distribution of particles present in the aggregated solutions. A number-weighted length distribution is illustrated for IFX S1 (Fig. 2b), and for S2, S3, S4, S6 and S24 each (Supplementary Fig. 1). The aggregated samples showed a monomodal population of polydisperse particles and the number-weighted lengths,

d_{10} , d_{50} , d_{90} , corresponding to the first, the fifth, and the ninth deciles, were 2.82 ± 0.14 , 6.62 ± 0.71 and 17.49 ± 1.34 , respectively. The relative span reflecting the width of the distribution was rather stable, around 2.18 ± 0.13 for IFX S1. Results for the different aggregate preparations (Table 1) showed a comparable size distribution for aggregates generated under 1 to 6 h of stress with relatively similar number-weighted lengths, d_{10} , d_{50} , d_{90} . Moreover, 75 % of the aggregates contained in these preparations were smaller than 11.5 μm in length. However, aggregate size distribution was altered after 24 h of stress (S24) with the formation of larger aggregates. Thus, aggregate characterization showed a stress time-dependent increase of the size and quantity of IFX insoluble aggregates.

3.3. Infliximab aggregates modulate moDC phenotype in a size and quantity-dependent manner

To investigate the effect of these preparations with increasing quantity and size of IFX aggregates on moDC phenotype, we analyzed the expression of surface markers CD83 and CD86 as well as the secretion of cytokines IL-6, IL-8, IL-10, IL-12p40 and TNF- α and chemokines CCL3 and CCL4 after 24 h of stimulation with native or aggregated IFX. These markers were chosen for their implication in moDC activation in response to antibody aggregates (Gallais et al., 2017; Morgan et al., 2019; Nabhan et al., 2020a).

We first studied the expression of CD83 and CD86 on moDCs after stimulation with native or aggregated IFX (S1, S2, S3, S4, S6 or S24) at 100 $\mu\text{g}/\text{mL}$, or LPS at 25 ng/mL as positive control. The cytotoxicity of native and aggregated antibodies was first analyzed using propidium

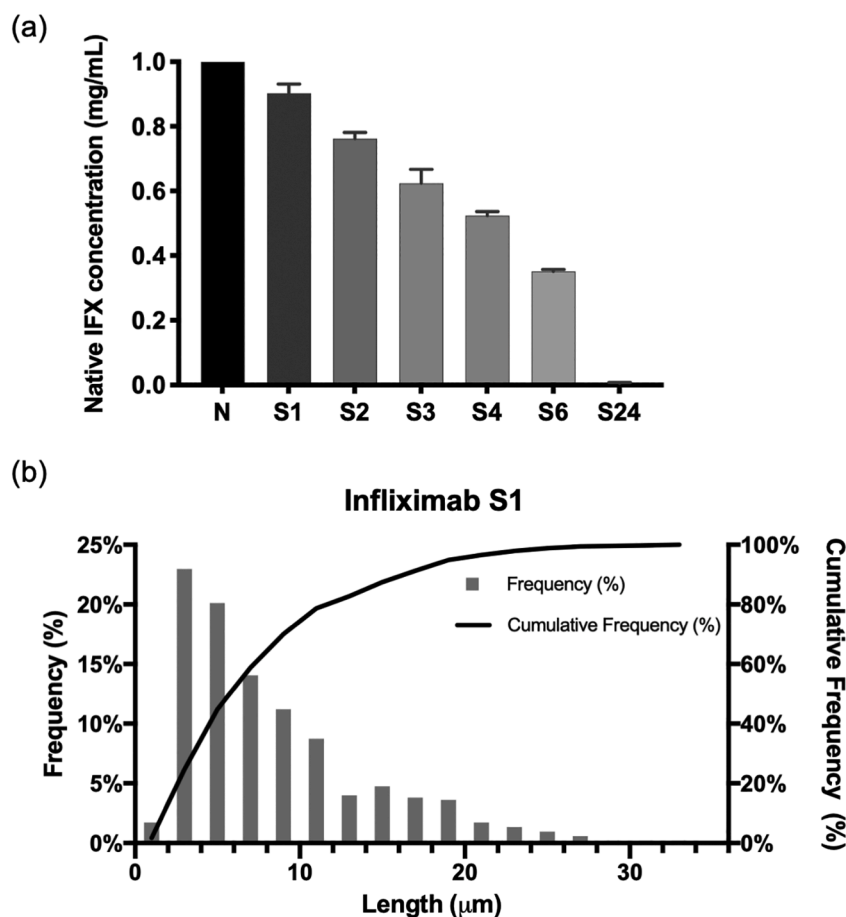


Fig. 2. Elevated temperature stress induces a time-dependent formation of micrometer-size infliximab aggregates.

Native and aggregated infliximab (1 mg/mL) were characterized by physico-chemical methods: (a) Native infliximab concentrations were determined by Size Exclusion Chromatography-Fluorescence detection (SEC-FLD), according to the stress duration (from 1 h, S1 to 24 h, S24). N: native; (b) Particle size distribution for S1 stress: histogram represents the number of particles per size range, with the lower limit of quantitation of the instrument set to 1 μm and the upper limit being the size of the 100 μm-chosen cell with a minimal luminance set at 10%; the curve represents the cumulative frequency of particles.

Table 1
Infliximab aggregate characterization by flow imaging microscopy.

	S1	S2	S3	S4	S6	S24
d10	2.82 ± 0.14	3.01 ± 0.20	3.02 ± 0.08	3.16 ± 0.13	3.17 ± 0.10	3.29 ± 0.21
d50	6.62 ± 0.71	7.30 ± 0.65	7.14 ± 0.52	7.15 ± 0.35	7.23 ± 0.46	10.29 ± 0.71
d90	17.49 ± 1.34	15.86 ± 2.28	16.30 ± 2.09	15.89 ± 1.58	16.27 ± 1.01	26.44 ± 1.70
Relative span	2.18 ± 0.13	1.86 ± 0.19	1.83 ± 0.16	1.80 ± 0.15	1.86 ± 0.12	2.33 ± 0.17

The number-weighted lengths for IFX S2, S3, S4, S6 and S24, d10, d50, d90, are presented and correspond to the first, the fifth, and the ninth deciles. The relative span reflects the width of the distribution.

iodide staining, and we observed that cell viability was never less than 80% (data not shown). Results obtained from 4 donors showed that native IFX had little to no effect on the expression of CD83 and CD86. IFX aggregates generated under 1 hour (S1) and 2 h (S2) of incubation at an elevated temperature did not increase expression of these activation markers compared to native IFX. However, a trend to increase in CD83 and CD86 expression was detected in response to IFX aggregates generated upon 3 and 4 h of stress (S3, S4) and a significant increase was observed with IFX aggregates S6 and S24 compared to native IFX (Fig. 3a).

We then measured cytokine and chemokine secretion in moDC

culture supernatants in response to 100 μg/mL of native or aggregated IFX (S1 and S24, since these two stress conditions have the lowest and highest effect on DC surface marker expression) respectively. Native IFX did not induce an increase in cytokine and chemokine production compared to untreated cells (Fig. 3b). IFX S1 aggregates induced a slight but not statistically significant increase in IL-6, IL-8, IL-10, IL-12p40, CCL3 and CCL4 secretion compared to native IFX (Fig. 3b). As for IFX S24 aggregates, secretion of IL-6, IL-8, IL-10 and CCL3 was significantly increased in response to S24 aggregates compared to the native protein whereas IL-12p40, CCL4 and TNF-α showed a trend to increase (Fig. 3b). These results showed that IFX aggregates induced moDC maturation in a stress time and quantity-dependent manner.

3.4. S1-aggregated infliximab increases response of CD4 T cells

We then wanted to assess the CD4+ T-cell response to S1 aggregates that were denied of any capacities of modifying DC phenotype as assessed previously. CD4 T cells collected from 6 healthy donors were cultured with autologous moDCs loaded with either native IFX or S1-aggregated IFX for three weeks and their specificity after this culture phase was tested by IFN-γ ELISPOT. KLH induced a strong T-cell response in all tested donors as we determined a mean frequency of 14.26 KLH-specific CD4 T cells per million circulating CD4 T cells (data not shown). On one hand, we detected CD4 T cells recognizing native IFX in the 6 tested donors, with a mean calculated frequency of 1.11 cells per million circulating CD4 naïve T cells (Fig. 4a). On another hand, CD4

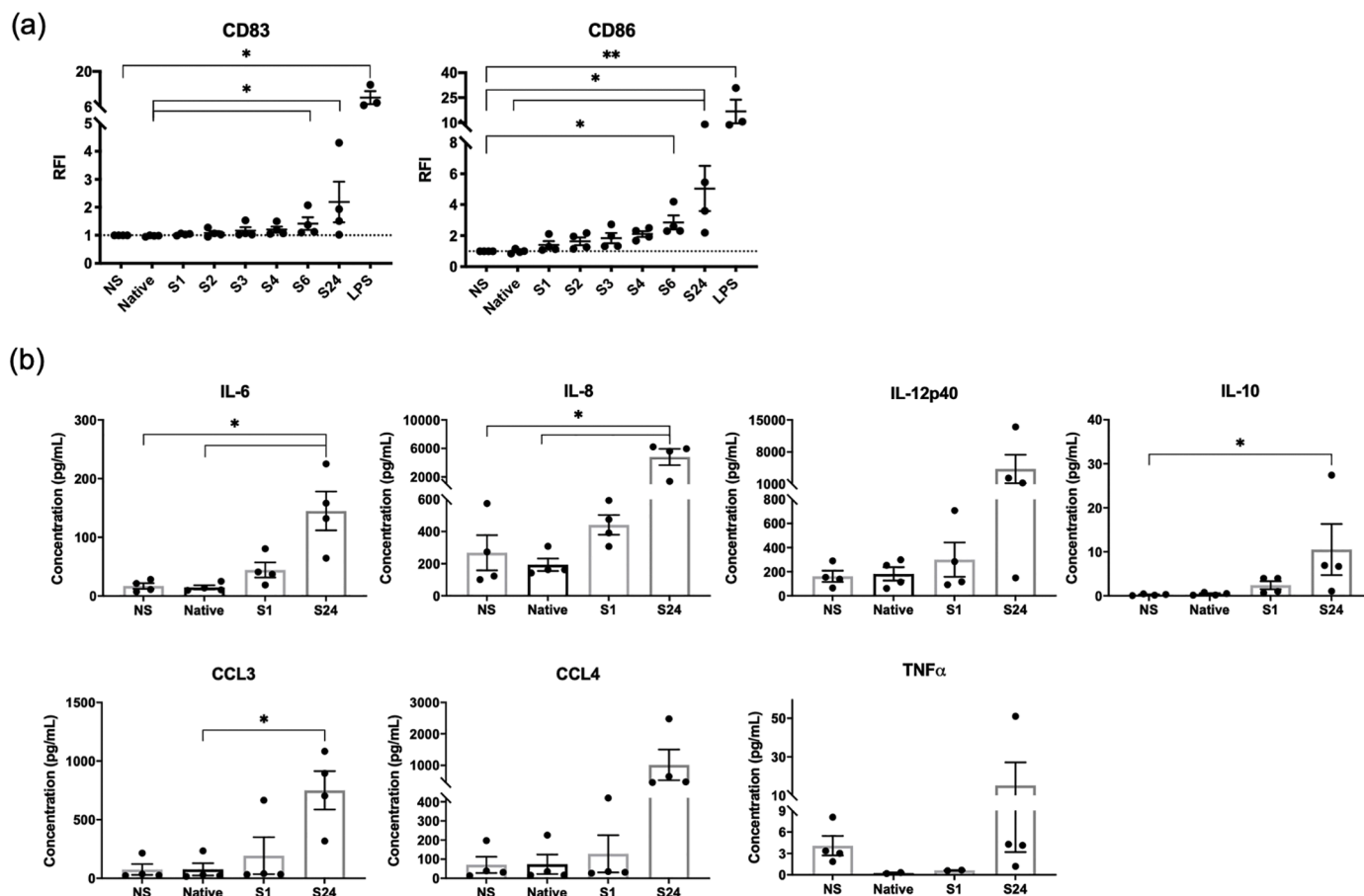


Fig. 3. Infliximab aggregates modulate moDC phenotype in a size and quantity-dependent manner.

Immature moDCs were treated for 24 h with native (IFX N) or aggregated (S IFX) infliximab (S1, S2, S3, S4, S6, S24; 100 μ g/mL) or lipopolysaccharide (LPS; 25 ng/mL). Cells were then collected, washed and analyzed by flow cytometry for surface markers expression. (a) Results are expressed as relative fluorescence intensity (RFI) compared to non-stimulated cells and represented the mean \pm SEM of 4 independent experiments. (b) Cytokine and chemokine concentrations in culture supernatants were measured in duplicate using an electroluminescent multiplex assay. Detection limits are indicated in the Methods section. Results of 4 independent experiments are represented as dot plot. *: $p < 0.05$; **: $p < 0.01$ Kruskal-Wallis test followed by Dunn's multiple comparison. NS: non-stimulated cells.

naïve T cells recognizing S1-aggregated IFX in all tested donors were identified with a mean calculated frequency of 2.05 cells per million circulating CD4 naïve T cells (Fig. 4a). These results showed that the frequency of CD4 T cells recognizing IFX S1 aggregates was significantly higher than the one of T cells recognizing native IFX.

To confirm the involvement of MHC class II molecules in the response of CD4 T cells, inhibition experiments were conducted for one donor, using anti-HLA-DP, -DQ and -DR antibodies. Results for donor 6 are presented in Fig. 4. Results showed that T-cell activation appeared to be mainly dependent on HLA-DR molecules that are preponderantly expressed, whether in the presence of native IFX (Fig. 4b and d) or aggregated IFX (Fig. 4c and e).

3.5. Some aggregate-responding CD4 T cells don't recognize native infliximab

To study the specificity of the T-cell response to IFX aggregates vs native IFX, we tested the cross-reactivity of the identified aggregate-specific CD4 T-cell lines to native IFX. A total of 27 S1 IFX-recognizing naïve T cells identified by a first ELISpot assay from the 6 donors were tested in a second ELISpot assay for their cross-reactivity to native IFX. Our results showed that for all the donors combined, 14 T-cell lines were positive only for IFX S1 aggregates (S1+N-; Fig. 5a), whereas 13 CD4 T-cell lines were able to recognize both native and S1 IFX (S1+N+; Fig. 5a). For each donor, some T-cell lines only recognized the aggregated form of IFX while the rest recognized both native and aggregated

forms. A representative result is shown for one donor in Fig. 5b. For donor 3 (D3), 7 CD4 T-cell lines were found specific for IFX aggregates out of the 50 tested T-cell lines in the first ELISpot assay. The second ELISpot assay showed that 4 (W14, W15, W31, W32) out of the 7 positive cell-lines were also positive for native IFX as the IFN- γ spot number with native IFX-treated moDCs was 2-fold higher than the spot number with unloaded moDCs. However, the 3 other wells (W3, W19, W21) were only positive for aggregated IFX as the IFN- γ spot number with S1 IFX-treated moDCs was 2-fold higher than the spot number with unloaded moDCs while it was lower with native IFX-treated moDCs (Fig. 5b). Results for the other five donors are shown in Fig. 5c and spot-counts are shown in Supplementary Fig. 2.

4. Discussion

Patient immunization to therapeutic proteins is problematic for the treatment of chronic diseases and the presence of aggregates in clinical batches has been suggested to increase the risk of immunization (Moussa et al., 2016; Wang et al., 2012). For this reason, pharmaceutical companies pay extremely close attention to minimize the content of aggregates in the formulated batches of therapeutic antibodies (U.S. Food and Drug Administration, 2014). However, mishandling of the vials through brief exposure to elevated temperatures, or even after mild conditions of elevated temperature stress might unfortunately occur and can promote aggregate formation (Vieland et al., 2018; Wang et al., 2012). To date, *in vitro* studies highlighted the role of aggregates in

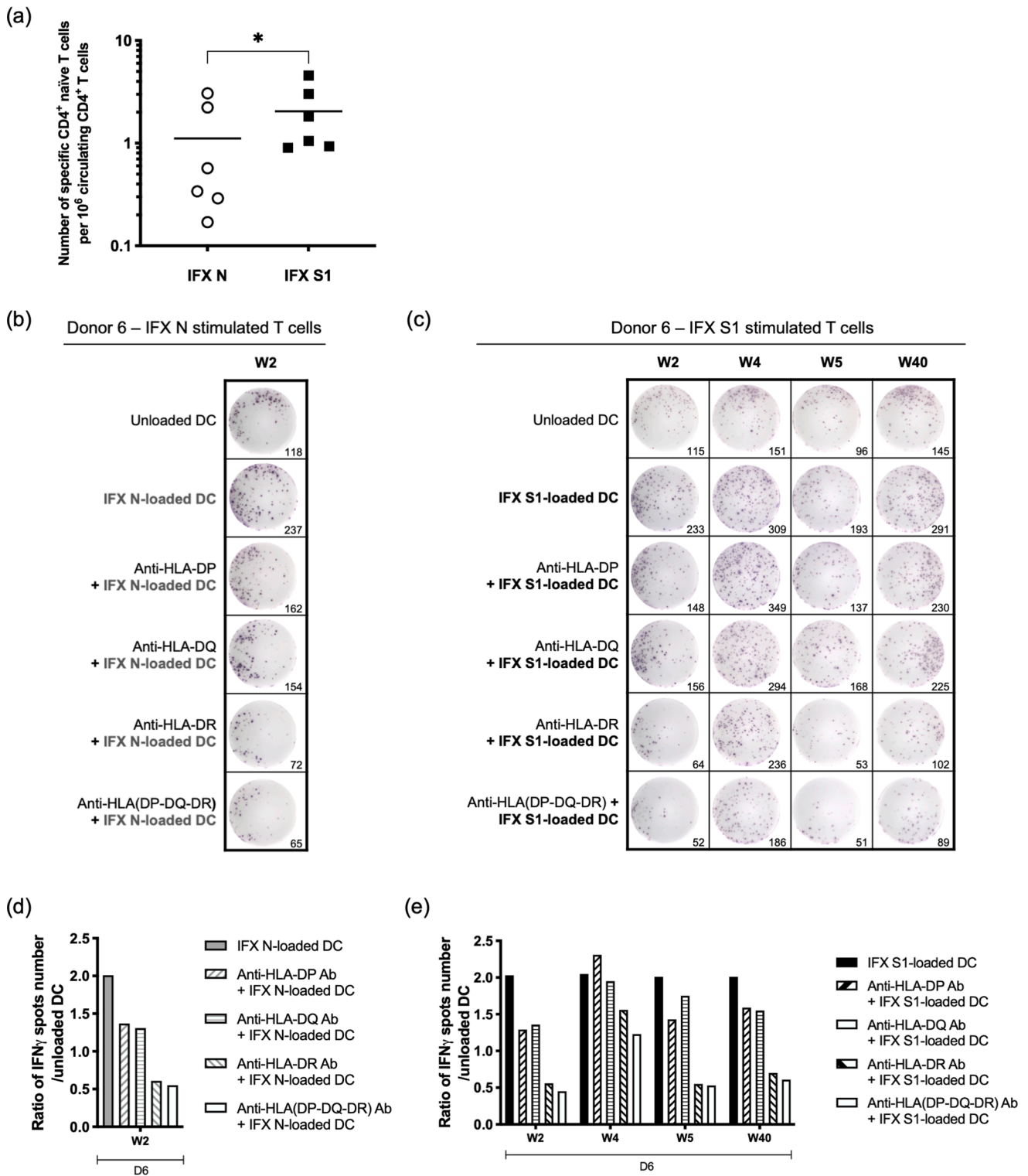


Fig. 4. S1-aggregated infliximab increases CD4 T-cell response.

(a) Frequencies of naïve CD4 T cells recognizing native or S1-aggregated infliximab. The y-axis represents the frequency of CD4⁺ T cells calculated for each donor using the Poisson distribution. Naïve CD4 T cells primed with native infliximab were tested for their specific response in an ELISpot assay towards native infliximab (white circles). Naïve CD4 T cells primed with S1-aggregated infliximab were tested for their specific response in an ELISpot assay towards S1-aggregated infliximab (black squares). Results of 6 independent experiments are represented as dot plot. *: $p < 0.05$, Wilcoxon signed-rank test.

(b-e) MHC class II molecules required for the activation of naïve CD4 T cells recognizing native and aggregated infliximab. (b-c) Images and spot-count for naïve CD4 T cells recognizing (b) native infliximab or (c) S1-aggregated infliximab detected for donor 6 (D6) stimulated with unloaded moDCs or moDCs loaded with native infliximab (IFX N-loaded DC) or moDCs loaded with native infliximab and treated with MHC class II blocking antibodies (HLA-DP, HLA-DQ, HLA-DR or HLA (DP-DQ-DR)). (d-e) Histograms represent ratio of IFN- γ spots detected in response to (d) native infliximab or (e) S1-aggregated infliximab loaded moDCs with or without MHC class II blocking antibodies compared to IFN- γ spots detected in response to unloaded moDCs calculated for donor 6.

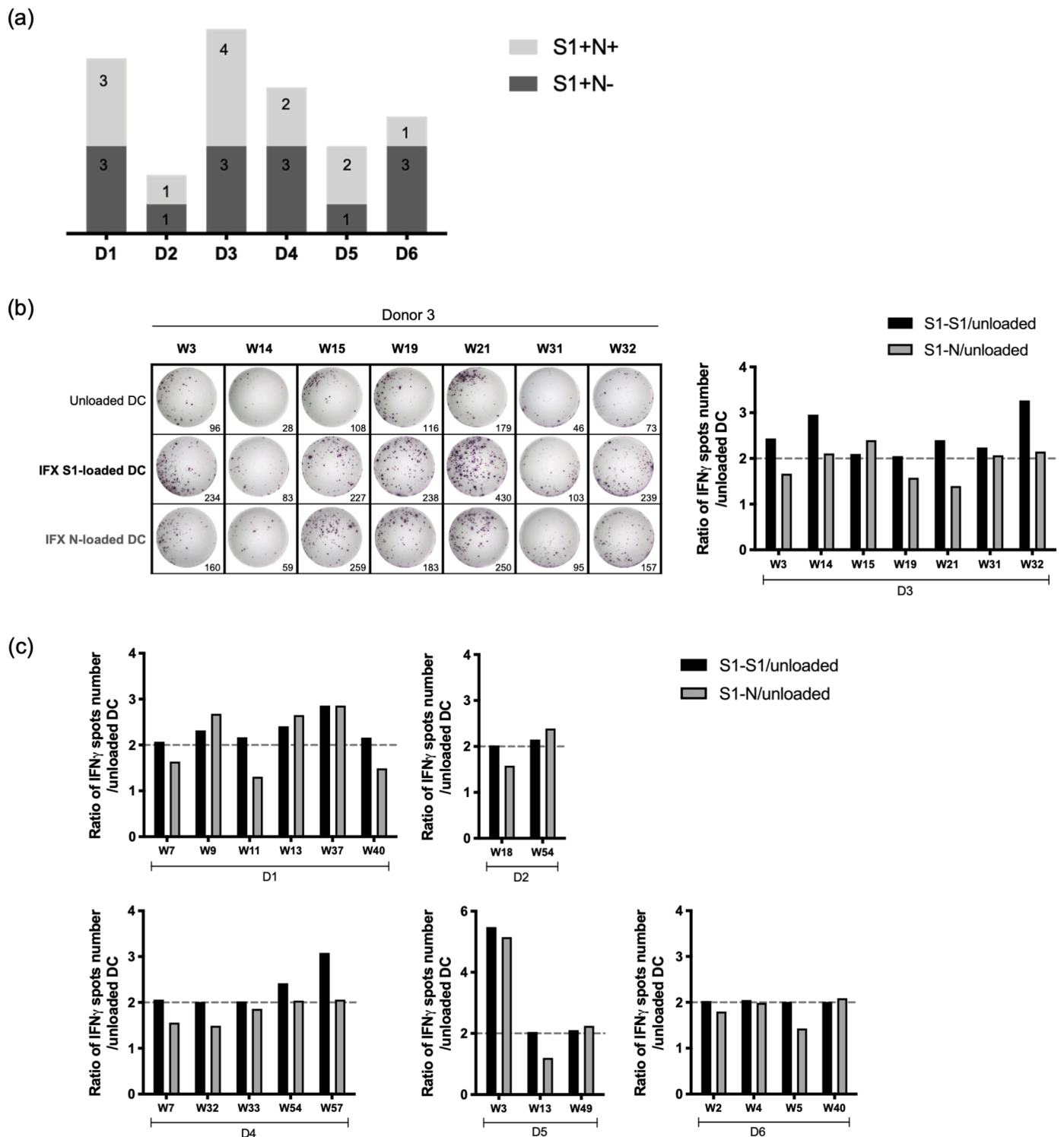


Fig. 5. Cross-reactivity of S1-aggregated infliximab responding-naïve CD4 T cells with native infliximab. CD4 naïve T cells identified in the first ELISpot assay as specific of S1-aggregated infliximab were tested in a second ELISpot assay for their response towards native infliximab. These T cells were stimulated with unloaded moDCs, or moDCs loaded with S1-aggregated infliximab, or moDCs loaded with native infliximab; (a) The number of aggregate specific T-cell positive wells detected in the second round of the ELISpot assay is presented. The number of T-cell wells positive for both S1-aggregated and native infliximab (S1+N+) is presented in the light grey bars and the number of T-cell wells only positive for S1-aggregated infliximab (S1+N-) is presented in the dark grey bars (refer to ELISpot positivity criteria in Methods section); (b) Representative results shown for donor 3: images and spot-count for naïve CD4 T cells recognizing S1-aggregated infliximab detected for donor 3. In an ELISpot assay, aggregate-specific T cells detected in the first ELISpot assay were stimulated with unloaded moDCs or moDCs loaded with S1-aggregated infliximab (IFX S1-loaded DC) or moDCs loaded with native infliximab (IFX N-loaded DC); histogram represents ratio of IFN- γ spots detected in response to S1-aggregated infliximab (black bars) and native infliximab (grey bars) compared to IFN- γ spots detected in response to unloaded moDCs calculated for donor 3; (c) Histograms showing results for donors 1, 2, 4, 5 and 6: ratio of IFN- γ spots detected in response to S1-aggregated infliximab (black bars) and native infliximab (grey bars) compared to IFN- γ spots detected in response to unloaded moDCs were calculated for each of the six tested donors. D: donor, N: native, S1: 1 h-elevated temperature stress.

inducing maturation of innate immune cells such as dendritic cells (DCs) and in activating CD4 T cells (Gallais et al., 2017; Joubert et al., 2012; E. M. Moussa et al., 2016). However, these studies mainly focused on aggregates produced under severe stress conditions and have yet to address the specificity of the CD4 T-cell response to aggregates, especially those that cannot be detected and eliminated prior to administration to patients. We therefore aim to explore the role of antibody aggregates produced in mild and severe conditions in the initiation of a CD4 T-cell response that could contribute to the development of anti-drug antibodies.

Although CD4 T-cell epitopes of immunogenic antibodies such as IFX, rituximab and adalimumab have been previously identified in healthy donors (Hamze et al., 2017; Meunier et al., 2019), their impact on aggregate immunogenicity has not been yet explored. We have previously shown that IFX has a high susceptibility to aggregate after 24 h at 55 °C and that IFX aggregates produced in these severe conditions can act as an immune danger signals by increasing DC maturation and CD4 T-cell proliferation (Morgan et al., 2019; Nabhan et al., 2020a). To investigate if these conditions of IFX aggregation can influence the nature and the number of T-cell epitopes, we isolated T-cell lines from CD4 T cells following multiple rounds of *in vitro* stimulation with native or aggregated IFX. To note, moDC stimulation was done in the presence of LPS to induce efficient peptide presentation. For each tested healthy donor, the number of T-cell lines responding to IFX aggregates was always higher than the one responding to native IFX, with a significantly increased frequency of preexisting aggregate-specific T cells. Identified T-cell lines were then evaluated for their reactivity to IFX-derived peptides encompassing the VH and VL sequences. An increased response was observed with aggregate-specific T cells, with 17 T-cell epitopes found with aggregated IFX *versus* only 5 with the native antibody. These results suggested that aggregation of IFX in severe conditions can induce an increased peptide presentation in terms of number and variety. This is consistent with results of a MAPPs assay showing that aggregation of monoclonal IgGs increased the frequency of presented peptides as well as the presentation of new IgG peptide clusters compared to unstressed IgGs (Rombach-Riegraf et al., 2014). These combined studies show that antibody aggregates can participate to DC activation by increasing MHC-peptide presentation, and by increasing the expression of surface co-stimulation molecules. Both signals are known to allow a proper activation of specific CD4 T cells (Chen and Flies, 2013; Fooksman et al., 2010), that could in turn lead to efficient B cell recruitment.

The presence of submicron and micron-sized aggregates in IV solutions has been recently observed for different preparations, including Remicade® (IFX) i.v. solutions (Pardeshi et al., 2021, 2017). Even though filtration greatly reduces their quantity, these particles can still be found in clinical batches administered to patients (Pardeshi et al., 2021, 2017; Werner and Winter, 2018) or might appear due to mishandling errors (Dill et al., 2020; Siska et al., 2020; Vlieland et al., 2018). In most studies exploring aggregation-related immunogenicity, aggregates are generated under an accelerated stress condition that favors the formation of uncontrolled quantities of heterogenic submicron and micron-sized aggregates. In our study, we first submitted a concentrated IFX solution to elevated temperature stress for 24 h, so the formed aggregate size ranged from 1 µm to more than 100 µm and these aggregates activate moDCs (Nabhan et al., 2020a) as well specific CD4 T cells as mentioned above. We then generated more homogeneous aggregate preparations, produced in mild conditions that could be more relevant to those possibly endured by commercialized vials. To study the role of aggregate size and quantity on the immune response, we incubated a diluted IFX solution at an elevated temperature for various durations. This stress induced the formation of micron-sized aggregates of a relatively homogeneous size range, 75 % of which ranged between 0.5 and 11.5 µm when stress duration varied between 1 and 6 h. Moreover, length of stress also increased the quantity of aggregates, starting with 10 % of aggregated antibody following 1 hour of stress, to a fully aggregated preparation after 24 h. Assessing moDC maturation in

response to these different preparations allowed to show an effect dependent on the quantity of aggregates. Indeed, preparations with the lowest abundance of aggregates (S1 and S2) did not appear to modulate moDC phenotype compared to native IFX, whereas a progressive activation of moDCs was observed with preparations with increasing quantities of aggregates (S3 to S24). This clearly indicates that moDC maturation is dependent on the quantity of aggregates in the preparations. These results are consistent with previous findings that have demonstrated that applying increased concentrations of an aggregated-protein preparation to moDC led to a subsequent increase in surface marker expression and moDC maturation. Indeed, this was the case for heat-stressed and shear-stressed IFX (Morgan et al., 2019), stir-stressed rituximab, growth hormone and commercial human IgG1 (Gallais et al., 2017). However, it remains to be confirmed whether this activation is due to an increase in the amount of aggregates of a specific size or in the overall aggregates observed with increasing duration of stress. In fact, results from studies exploring the immunogenicity of aggregates of a specific size range or quantity are controversial. Fathallah et al. showed that soluble native-like oligomers (< 100 nm) were the most immunogenic (Fathallah et al., 2015), whereas Kijanka et al. showed that submicron antibody aggregates (100–1000 nm) were more immunogenic than soluble oligomers and micron aggregates (Kijanka et al., 2018) when injected in mice. Similarly others *in vivo* studies showed an increased immune response to aggregated proteins compared to administered native proteins, however the highest induced ADA production differed depending on the aggregated protein: preparations containing insoluble and denatured murine anti-TNF-α antibody aggregates (Freitag et al., 2015) and aggregated recombinant human interferon alpha2b with native-like conformation (Hermeling et al., 2006) were the most immunogenic. Moreover, aggregated murine antibodies were more immunogenic at low concentrations compared to higher doses that could induce tolerance (Shomali et al., 2015). One *in vitro* study comparing submicron and micron aggregates showed that antibody aggregates in a 5–10 µm size range induce cytokine secretion by peripheral blood mononuclear cells (PBMC) compared to 1–2 µm and 2–5 µm-sized aggregates (Telikepalli et al., 2015). Another study by Ahmadi et al. evaluating the immune response to antibody preparations with low quantities of aggregates showed that preparations with less than 3 % of rituximab aggregates did not induce changes in surface marker expression, whereas trastuzumab aggregates induced a slight increase in CD86 and HLA-DR (Ahmadi et al., 2015).

Focusing on preparations with small amounts of aggregates (IFX S1) that do not appear to affect moDC phenotype, we then evaluated these aggregates for their capacity to induce a CD4 T-cell response in comparison to native IFX. For this, we used an autologous co-culture model of moDCs and naïve CD4 T lymphocytes from healthy donors to assess the frequency of T cells responding to IFX aggregates and to compare it to native IFX. Overall results obtained in 6 healthy donors indicated a significant increase in the number of T-cell lines responding to IFX S1 aggregates compared to native IFX. This increased frequency of aggregate responding-T cells was similar to what we observed with aggregates that can also induce moDC maturation. Furthermore, HLA-DR molecules seemed to play a primary role in the T-cell response, both in the presence of native and aggregated IFX. Indeed, aggregates have been shown to be uptaken by moDCs and to co-localize with HLA-DR molecules after incubation of moDCs with preparations with low levels of micron-sized antibody aggregates (Ahmadi et al., 2015). Additionally, it has been suggested that internalization of a micron-sized antibody aggregate could induce an increase in protein concentration in endolysosomes, which would promote increased peptide formation and presentation (Rombach-Riegraf et al., 2014). Hence, our results confirm that aggregation can increase CD4 T-cell recruitment by interfering with moDC MHC II-peptide presentation.

To test whether some of the IFX S1-activated T cells were specific of aggregates only, the identified T-cell lines were then evaluated for their cross-reactivity with native IFX for each tested donor. While some T cells

responded to both native and aggregated IFX thus recognizing native IFX epitopes, other CD4 T cells were activated only by aggregate-loaded moDCs suggesting that they solely responded to peptides present due to IFX aggregation. On one hand, as we found with high quantities of aggregates, this could be explained by an increased presentation of native IFX peptides allowing the recruitment of rare specific T cells with a lower T cell receptor avidity. Moreover, aggregation can lead to antibody denaturation and unfolding and the possible exposure of new immunodominant epitopes. The increase in presentation of peptides could be explained by differences in the interaction of aggregates with DCs compared to the native form (*i.e.* different receptors engaged or different internalization pathways), that could lead to an increased uptake of aggregates. Another possible hypothesis could be the generation of neopeptides following stress-induced conformational changes. Thus, these results suggested once again that aggregation could favor an increase in the quantity and diversity of presented peptides leading to a stronger T-cell response.

In summary, immunization due to aggregation of therapeutic antibodies poses a significant challenge and our study highlights the importance of evaluating the immune effect of aggregates even at low abundance as they could increase the probability of recruiting aggregate-recognizing CD4 T cells.

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CRedit authorship contribution statement

Myriam Nabhan: Methodology, Investigation, Formal analysis, Writing – original draft. **Sylvain Meunier:** Methodology, Investigation. **Victor Le-Minh:** Methodology, Investigation. **Baptiste Robin:** Methodology, Investigation. **Marie de Bourayne:** Methodology, Investigation. **Claire Smadja:** Writing – review & editing. **Bernard Maillère:** Writing – review & editing. **Marc Pallardy:** Conceptualization, Writing – review & editing. **Isabelle Turbica:** Conceptualization, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors state no conflict of interest.

Data availability

Data will be made available on request.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.ejps.2023.106670](https://doi.org/10.1016/j.ejps.2023.106670).

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