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Journal of Pharmaceutical Sciences 110 (2021) 952-958

Contents lists available at ScienceDirect

Journal of Pharmaceutical Sciences

journal homepage: www.jpharmsci.org

Note

Immunological Evaluation *In Vitro* of Nanoparticulate Impurities Isolated From Pharmaceutical-Grade Sucrose

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ARTICLE INFO

Article history:
Received 2 September 2020
Revised 12 November 2020
Accepted 12 November 2020
Available online 19 November 2020

Keywords:
Immunogenicity
In vitro model
Monoclonal antibody
Nanoparticles
Sucrose

ABSTRACT

Sucrose is a commonly used stabilizing excipient in protein formulations. However, recent studies have indicated the presence of nanoparticulate impurities (NPIs) in the size range of 100–200 nm in pharmaceutical-grade sucrose. Furthermore, isolated NPIs have been shown to induce protein aggregation when added to monoclonal antibody formulations. Moreover, nanoparticles are popular vaccine delivery systems used to increase the immunogenicity of antigens. Therefore, we hypothesized that NPIs may have immunostimulatory properties. In this study, we evaluated the immunomodulatory effects of NPIs in presence and absence of trastuzumab *in vitro* with monocyte-derived dendritic cells (moDCs). Exposure of trastuzumab, the model IgG used in this study, to NPIs led to an increase in concentration of proteinaceous particles in the sub-micron range. When added to moDCs, the NPIs alone or in presence of trastuzumab did not affect cell viability or cytotoxicity. Moreover, no significant effect on the expression of surface markers, and cytokine and chemokine production was observed. Our findings showed, surprisingly, no evidence of any immunomodulatory activity of NPIs. As this study was limited to a single IgG formulation and to *in vitro* immunological read-outs, further work is required to fully understand the immunogenic potential of NPIs.

Introduction

Protein pharmaceuticals are prone to physical and chemical instability.[1] This may result in deterioration of protein drug product quality and pose potential safety concerns, such as unwanted immunogenicity.[2] To inhibit protein degradation, protein pharmaceuticals typically contain a combination of stabilizing excipients.[3] Sucrose is a commonly used excipient in both liquid and lyophilized protein formulations because of its excellent stabilizing properties.[4,5]

Recently, it has been found that pharmaceutical-grade sucrose contains nanoparticulate impurities (NPIs) in the size range of 100–200 nm.[6] Based on analytical characterization, the nanoparticles were suggested to be agglomerates of various compounds, including dextran (with presence of β-glucans), inorganic metal salts and fluorescent compounds, potentially originating from raw materials and production processes.[6] Furthermore, NPIs have been shown to destablize several marketed monoclonal antibodies (mAbs).[7] Introduction of NPIs isolated from pharmaceutical-grade sucrose to the mAbs resulted in immediate or delayed formation of protein aggregates and particles. Therefore, it is recommended to use sucrose low in NPI content to reduce instability risks for biopharmaceutical drug products.

Immunogenicity of protein biopharmaceuticals is an ongoing concern and may be linked to multiple sources related to the medicinal product, the patient and the treatment regimen.[8] Impurities in drug products, such as proteinaceous particles, have been shown to evoke immune responses in *in vitro* and *in vivo* models.[9] The presence of metal ions can result in the formation of immunogenic protein-metal complexes or metal ion-catalyzed oxidized protein species, including aggregates.[10–12] Furthermore, polysaccharides such as glucans have been explored as adjuvants to improve vaccine immunogenicity.[13] Various types of β-glucans have been shown to increase the immunogenicity of antigens.[14,15] Although glucan-related contaminants can been found in therapeutic mAb

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https://doi.org/10.1016/j.xphs.2020.11.011

products at a much lower concentrations compared to concentrations tested in vaccine formulations with glucan-based adjuvants (100–1000 μg/mL), immune-related adverse reactions upon administration might still occur.[16,17]

Based on the above, we hypothesized that NPIs in absence or presence of protein, or protein aggregates generated from the presence of NPIs, may act as a danger signal and induce the activation of innate immune cells such as dendritic cells. To test this hypothesis, we evaluated the effect of NPIs in presence and absence of trastuzumab (humanized mAb, isotype IgG1) on human dendritic cells, which are pivotal in the establishment of a $CD4^+$ T-cell dependent immune response leading to the production of high affinity anti-drug antibodies detected in patients' sera.[18]

Materials and Methods

Materials

The humanized mAb, isotype IgG1, trastuzumab (Herzuma®) was purchased from Leiden University Medical Centre (Leiden, The Netherlands). Pharmaceutical-grade surcose was purchased from VWR (Bruchsal, Germany) and RPMI 1640 medium was supplied by ThermoFisher Scientific (Waltham, USA). Polyvinylidene fluoride (PDVF) syringe filters with a pore size of 0.22 μm were purchased from Merck (Darmstadt, Germany). Labeled monoclonal antibodies used for flow cytometry were purchased from BD Biosciences (New Jersey, USA). A cytokine and chemokine multiplex assay (U-PLEX) was purchased from MSD (Rockville, USA). Highly purified water (conductivity: 18.2 mUΩ*cm) obtained from a Milli-Q® Advantage A10 system (Merck, Newark, USA) was used throughout the study.

Preparation of NPIs

Preparation of NPIs was performed as described before.[7] Briefly, a 50% w/v of sucrose solution in water was prepared and submitted to an ultra-/dia-filtration process by using a Minimate II Tangential Flow Filtration (TFF) system (Pall, Crailsheim, Germany) equipped with a 30-kDa TFF capsule (Pall). Diafiltration of the sucrose solution was performed against 20 vol of water. Further, the sucrose-free retentate (confirmed by DLS, data not shown) was upconcentrated 1000-fold and filtered by using a 0.22-μm PDVF filter. Upconcentrated stock retentate containing NPIs at a concentration 1×10^{11} particles/mL (as determined by NTA) was stored at −80 °C until needed.

Water without addition of sucrose was submitted to the same process described above and served as a control sample (Ctl) throughout the study.

Sample Preparation

Stock suspensions of NPIs (two-fold higher in concentration compared to the target concentration) were prepared in RPMI medium and spiked into trastuzumab samples to target concentrations stated in the results section. Trastuzumab was diluted with RPMI medium to 2 mg/mL. Further addition of NPIs and RPMI medium was performed to reach a final protein concentration of 1 mg/mL. Trastuzumab was incubated in presence of NPIs or control (water) for 1 h at room temperature. The formulations were added to moDC suspensions to reach a target IgG concentration of 0.1 mg/mL.[19]

Nanoparticle Tracking Analysis (NTA)

NanoSight (Model LM20, Malvern Instrument, Malvern, UK) instrument equipped with a 405-nm laser (blue) was used to quantify and determine the size of nanometer sized particles (size range 50–1000 nm). Samples were injected into the chamber by using a 1-mL silicone-free syringe and the purging volume was 0.3 mL. A video capture was initiated immediately after injection and a triplicate measurement of 60-s replicates was performed. All measurements were collected at room temperature with camera levels set to optimal values until at least 100 valid tracks were recorded.

Flow Imaging Microscopy (FIM)

MFI 5200 (ProteinSimple, USA) was used for characterization and quantification of particles $\geq$ 1 μm in equivalent circular diameter. The system was equipped with a silane-coated high-resolution 100-μm flow cell. RPMI medium was used to perform optimization of illumination after a 0.17-mL purge volume and 0.28 mL of sample was analyzed within each measurement, resulting in a ca. 2.89 min measurement time. The number of image frames stored per measurement was between 138 and 1133, dependent on the particle concentration within each sample. Data were processed by using MVAS V2.3 software and each sample was measured once.

Generation of Human Monocyte-Derived Dendritic Cells (moDCs)

Human peripheral blood mononuclear cells were purified from buffy coats obtained from Etablissement Français du Sang (EFS Rennes, France) by density centrifugation on a Ficoll gradient (lymphocyte separation medium; GE Healthcare, Buc, France). Healthy donors gave their written consent for the use of blood donation for research purposes. MoDC preparation was performed as described before.[20] Briefly, 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, Bergish Gladbach, Germany). Monocytes were cultured at 1×10^6 cells/mL in the presence of GM-CSF (550 U/mL) and IL-4 (550 U/mL) (Miltenyi Biotec) in RPMI-1640 25 mM HEPES GlutaMAX™ supplemented with 10% fetal calf serum, antibiotics and 1% pyruvate at 37 °C in humidified air containing 5% CO_2. Within 4 days, monocytes had differentiated into moDCs with an immature phenotype.

Treatment of moDCs

After differentiation, immature moDCs were treated with water (negative control, water treated the same way as NPIs) or NPIs (10^8, 10^9 or 10^{10} NPIs/mL) in 12-well plates. Additionally, moDCs were treated with trastuzumab (100 μg/mL) alone or in presence of NPIs (10^{10} NPIs/mL). Lipopolysaccharide (LPS from Escherichia coli 055:B5 strain, at a final concentration of 25 ng/mL; Sigma-Aldrich, St. Louis, USA) served as a positive maturation control. All treated moDC samples were incubated at a final concentration of 10^6 cells/mL in 1 mL at 37 °C in humidified air containing 5% CO_2 for 24 h prior to analysis.

Flow Cytometric Analysis of moDC Maturation

MoDC viability and phenotype were analyzed by using flow cytometry. Cell viability was assessed in preliminary experiments by staining with propidium iodide (PI) (Invitrogen, California, USA), used at a final concentration of 625 ng mL^{-1}, on a small fraction of moDC cultures. For the phenotypic analysis, the surface labeling procedure was as follows: 2×10^5 cells/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

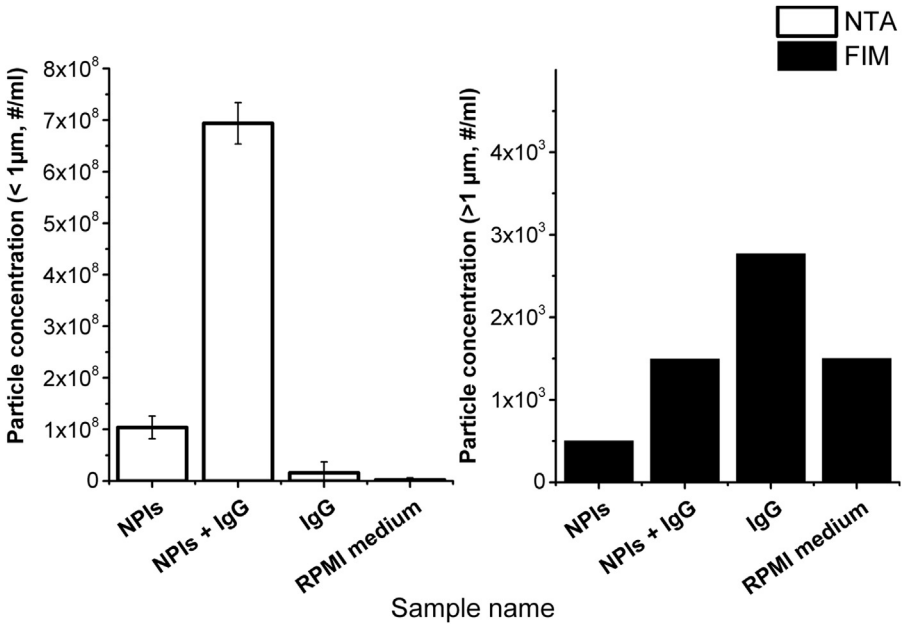

Fig. 1. Nanometer sized and micron sized particle concentrations determined by using a) NTA and b) FIM, respectively. Samples measured with NTA were diluted 100-fold in RPMI medium prior to measurement and stated concentrations are not corrected for dilution. Error bars represent standard deviation of triplicate measurements. FIM analysis was performed as single measurements of undiluted samples. NPIs: nanoparticulate impurities, IgG: trastuzumab.

used: allophycocyanin (APC)-labeled mouse anti-human CD86 (555660, BD Biosciences, New Jersey, USA) and anti-human CXCR4 (555976, 12G5, BD Biosciences); fluorescein isothiocyanate (FITC)-labeled mouse anti-human CD80 (5557226, L307.4, BD Biosciences) and anti-human PD-L1 (558065, MIH1 BD Biosciences); phycoerythrin (PE)-labeled mouse anti-human CD83 (556855, HB15e, BD Biosciences). Appropriate isotype controls (mouse IgG1κ or IgG2aκ, BD Biosciences) were used at the same concentration to determine non-specific staining. Cells were analyzed on an Attune Nxt (Invitrogen) by using FlowJo software (version 10; FlowJo LLC, Oregon, USA), 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 are expressed as the relative fluorescence intensity (RFI), by using the corrected mean fluorescence intensity (cMFI), as follows: cMFI = MFI−MFI of isotype control; RFI = cMFI of treated cells/cMFI of untreated cells.

Quantification of Cytokines and Chemokines in Cell Culture Supernatants

Culture supernatants were analyzed by using Meso Scale Discovery (MSD, Rockville, MA, USA) multiplex assay, according to the manufacturer's instructions, to assess the concentrations of the following cytokines and chemokines: IL-1β, IL-6, IL-8, IL-10, IL-12/23p40, CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), IFN-β, TNFα. The quantification ranges were: IL-1β: 0.91−3730 pg/mL, IL-6: 0.51−2080 pg/mL, IL-8: 0.50−2050 pg/mL, IL-10: 0.90−3700 pg/mL, IL-12/23p40: 5.57−22,800 pg/mL, CCL2: 1.45−5920 pg/mL, CCL3: 1.46−6000 pg/mL, CCL4: 0.45−1860 pg/mL, IFN-β: 21.97−90,000 pg/mL, TNFα: 0.89−3650 pg/mL. Each plate was imaged on a Meso Quickplex SQ120 (MSD) according to manufacturer's instructions. The analysis software (Discovery Workbench, MSD) provided with the platform contains the

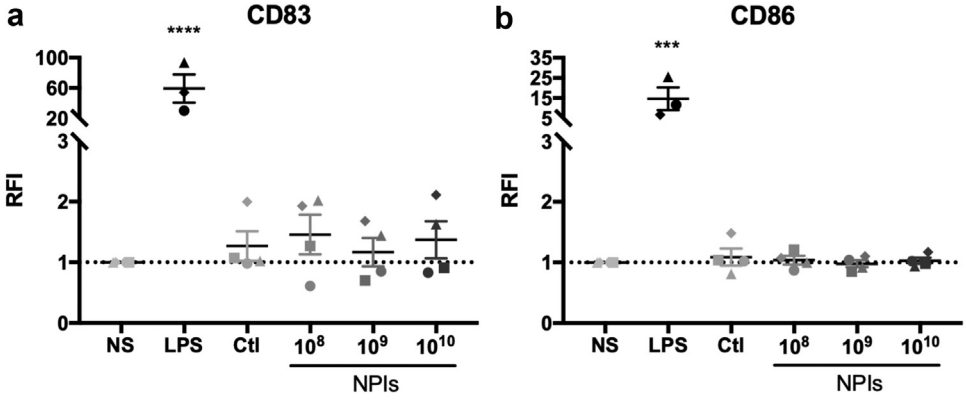

Fig. 2. Effect of NPIs on the expression of moDC surface markers. Immature moDCs were treated for 24 h with NPIs (10^8, 10^9 and 10^{10} particles/mL), water or LPS (25 ng/mL). Cells were then collected, washed and analyzed by flow cytometry for expression of surface maturation markers a) CD83 and b) CD86. Results are expressed as relative fluorescence intensity (RFI) compared to non-stimulated cells and represent the mean ± SEM of 4 independent experiments. ***$p < 0.001$, ****$p < 0.0001$; one-way ANOVA followed by Tukey's analysis. NS: non-stimulated cells; LPS: lipopolysaccharide; Ctl: control water treated as NPIs; NPIs: nanoparticulate impurities.

quantification, detection ranges and quality criteria for the standard curve, which were applied automatically during analysis.

Statistical Analysis

ANOVA with post hoc Tukey's analysis was used to assess statistical significance, with $P < 0.05$ considered significant. Data were analyzed on Graphpad Prism software (La Jolla, CA, USA).

Results and Discussion

Particle Characterization

Isolated NPIs from sucrose were added to trastuzumab to a target concentration of 1×10^{10} NPIs/mL (based on dilution). This concentration was chosen as a worst-case scenario, as it is slightly above the maximum concentration of NPIs that might be present in an isosmotic sucrose formulation.[6] The resulting mixture was incubated for 1 h at room temperature prior to analysis. A noticeable higher concentration of nanometer sized particles was detected by NTA for the trastuzumab formulation spiked with NPIs as compared to NPIs alone and the non-treated IgG sample (Fig. 1), indicating the formation of nanometer sized trastuzumab aggregates. Nanometer sized IgG aggregates previously were shown to be highly immunogenic *in vivo*[21] and an assessment of risks imposed by such particles with respect to the clinical performance of drug products is recommended by regulatory agencies.[22] On the other hand, no increase in micron sized particles in MFI was measured upon addition of NPIs to the trastuzumab formulation. These results vary from the results of a previous study, where the originator drug product (Herceptin®) was used, and the addition of NPIs induced the formation of micron sized particles.[7] Even though

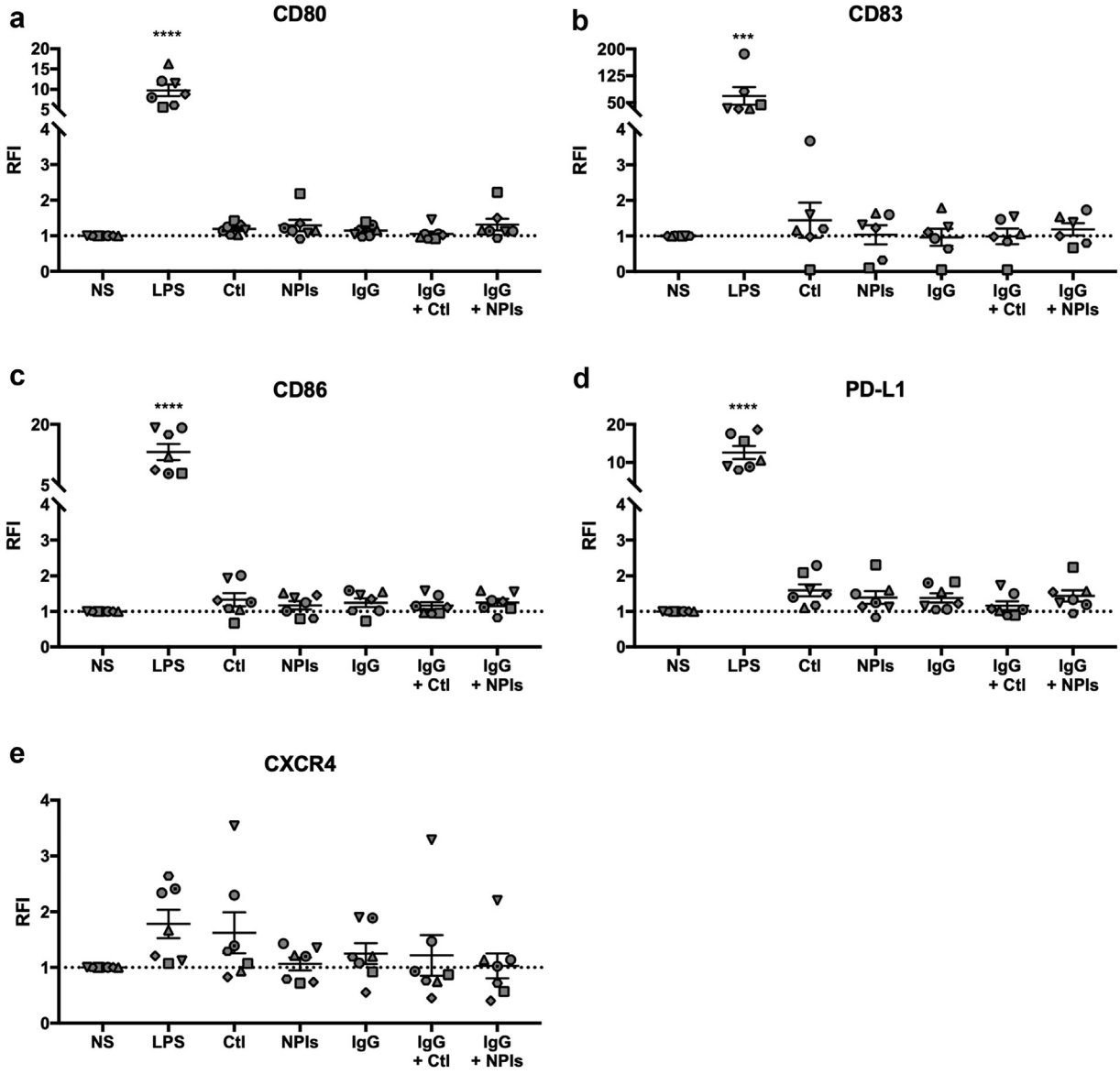

Fig. 3. Effect of NPIs and trastuzumab on the expression of moDC surface markers. Immature moDC were treated for 24 h with a mixture of NPIs (1010 NPIs/mL) and trastuzumab (100 μg/mL) or corresponding controls or LPS (25 ng/mL). Cells were then collected, washed and analyzed by flow cytometry for expression of surface maturation markers. Results are expressed as relative fluorescence intensity (RFI) compared to nonstimulated cells and represent the mean ± SEM of 6 or 7 independent experiments with values for each donor represented by one given symbol. ***p < 0.001, ****p < 0.0001; one-way ANOVA followed by Tukey's analysis. NS: non-stimulated cells; LPS: lipopolysaccharide; Ctl: control water treated as NPIs; NPIs: nanoparticulate impurities, IgG: trastuzumab.

the concentration of NPIs was adjusted based on a NTA method used in both studies, the composition of NPIs used in our study could have differed from the NPIs used in the study of Weinbuch et al., because we used a different batch. Additionally, the different IgG behavior in presence of NPIs could be explained by the different formulation composition used in this study and/or the slightly different glycosylation profiles between Herceptin® and Herzuma®.[23,24]

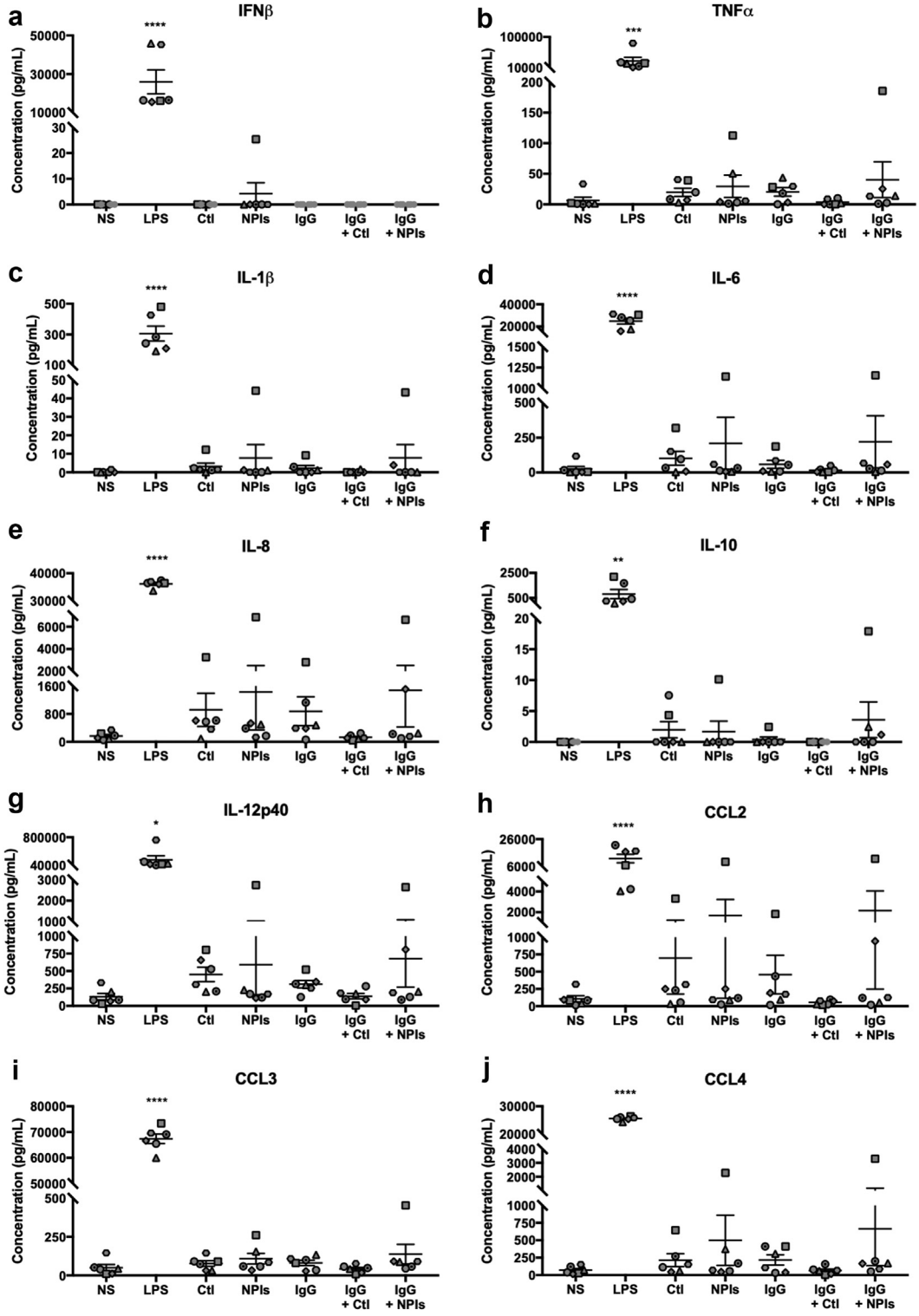

Fig. 4. Effect of NPIs and trastuzumab on moDC cytokine and chemokine secretion. Immature moDC were treated for 24 h with a mixture of NPIs (1010 NPIs/mL) and trastuzumab (100 μg/mL) or corresponding controls or LPS (25 ng/mL). Cytokine and chemokine concentrations in culture supernatants were analyzed in duplicate by using an electroluminescent multiplex assay. Detection limits are indicated in the Methods section. Results of 6 independent experiments are presented with values for each donor represented by one given symbol. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; one-way ANOVA followed by Tukey's analysis. NS: non-stimulated cells; LPS: lipopolysaccharide; Ctl: control water treated as NPIs; NPIs: nanoparticulate impurities, IgG: trastuzumab.

Previously performed Glucatell assays with the isolated NPIs indicated a prominent presence of (1 → 3)-β-D-glucans (10 ng/mL per 1×10^{10} NPIs/mL),[25] which are well-known immunomodulatory agents.[26] β-glucans are believed to trigger the innate immunity via dectin-1, Toll-like receptors 2 and 6, and complement receptor 3, which are transmembrane receptors abundantly found on myeloid cells, such as dendritic cells.[27] However, opposed to endotoxin levels, glucans are not regulated in terms of maximum permitted concentrations in pharmaceutical products and their levels in IgG formulations is rarely tested.[28,29] Whereas the immunostimulatory effect of β-glucans is considered relatively low, they can act as adjuvants in presence of other immunogenic species, such as proteinaceous particles.[30,31]

Effect of NPIs Alone on moDC Phenotype

To investigate the effect of NPIs on moDC maturation, we analyzed the expression of moDC surface markers after 24 h of stimulation with different concentrations of NPIs (10^8, 10^9 and 10^{10} NPIs/mL). The toxicity of NPIs was first analyzed by using PI staining and we observed that cell viability was unaffected at all tested concentrations and remained above 75% (data not shown). Surface markers CD83 and CD86 were highly up-regulated after incubation with LPS for 24 h, indicating that moDCs could undergo maturation. However, the NPIs at the three tested concentrations did not modulate CD83 and CD86 expression compared to water, processed as NPIs and used as control, and untreated cells (Fig. 2). On the basis of these observations, the following experiments were carried out with 10^{10} NPIs/mL (maximal concentration which could be prepared).

Effect of NPIs and Trastuzumab Aggregates on moDC Maturation

To evaluate if nanometer sized particles generated after adding NPIs to a trastuzumab solution modulate the phenotype of moDCs, we first analyzed the expression of surface markers. NPIs and trastuzumab aggregates had no significant effect on the expression of CD80, CD83, CD86, PD-L1 and CXCR4 compared to the corresponding controls: control water alone, NPIs alone, trastuzumab alone and trastuzumab mixed with water after 24 h of incubation (Fig. 3 and Supplementary Fig. 1). NPIs in absence and presence of trastuzumab did not increase inflammatory cytokine and chemokine production (Fig. 4). These results indicate that NPI-induced aggregates of trastuzumab have little impact on antigen-presenting cells.

The immunomodulatory properties of aggregated antibodies have often been studied following application of extreme stress conditions to protein formulations, such as heat- or stir-stress, resulting in formation of very high numbers of proteinaceous particles.[21,32–34] In one of the studies, the high abundance of particles was determined to be a dominant factor in evoking an immune response *in vitro*.[32] Furthermore, forced degradation studies result in a mixture of variable aggregate sizes, which induced a strong moDC maturation. In fact, results from various studies comparing different size range-aggregates are controversial. Oligomeric, nanometer or micron sized antibody aggregates were found to have variable immunogenic potency *in vitro*[32–34] and *in vivo*.[8,21,35] In our case, a relatively low concentration of proteinaceous particles in the nanometer size range was formed within trastuzumab formulations in presence of NPIs, which were insufficient to activate moDCs in the experimental setting used in the current study.

The inherent immunogenicity of the monomeric protein and the physicochemical properties of protein aggregates are other important influencing factors in unwanted immunogenicity.[32,34] Generation of immunogenicity via moDCs depends on the interactions of IgG aggregates and innate receptors such as immunoglobulin receptors (FcγRs), danger signal receptors (e.g., TLRs) or complement receptors.[19] Morgan et al. showed infliximab aggregates formed upon heat stress to induce full moDC maturation, as opposed to infliximab aggregates induced by mechanical stress.[19] Other protocols attempted to refine aggregate formation in order to expose moDCs to more homogeneous preparations and to induce low levels of aggregation of the total protein. For instance, applying stir stress to a trastuzumab preparation induced the formation of aggregates within the sub-micron and micron size range, while heat stress mainly induced the formation of oligomers. Both preparations induced moDC maturation, but aggregates generated via stir stress induced an increased maturation state.[33] Therefore, both the protein and the stress applied to induce aggregates will have a considerable effect on the immunogenic potential of protein therapeutics.

The weak immunostimulatory effect of NPIs in presence of trastuzumab aggregates could be rationalized by the minimal impact of NPIs on the monomeric IgG three-dimensional structure.[7] Harsh artificial stress, such as heat or stir stress, generates aggregates consisting of altered protein structures which may increase the presentation of peptide sequences on the aggregate's surface that are recognized by moDC receptors. For instance, heat-stressed infliximab activated moDCs by cross-linking of FcγRIIa receptors.[36] Overall, the studies cited above focused on the direct impact of the pure protein aggregates on immune cells, while in our work the potential effect of NPIs in presence of monoclonal antibodies was investigated.

Another explanation for the lack of moDC maturation seen in our study could be the relatively small size of the NPIs (100−200 nm). For example, for particulate vaccines an increase in mean particle diameter resulted in a stronger immune response.[37,38] Similarly, many investigational applications of β-glucans involve preparations consisting of particulates in the lower micron-size range. However, further studies are required to evaluate the impact of particle size and other physico-chemical attributes of protein aggregates on their immunogenicity.[39]

Conclusion

Surprisingly, NPIs derived from pharmaceutical-grade sucrose, when added to moDCs *in vitro* at a level of 1×10^{10} NPIs/mL, did not show immunomodulatory effects in absence or in presence of trastuzumab, even though the NPIs induced the formation of nanometer sized protein aggregates. It is worth noting, however, that the observations made in this study relate solely to *in vitro* models using one dendritic cell type, and are unlikely to fully predict the immunological potential of NPIs *in vivo*. Examination of the immunogenicity of the NPIs with other IgGs and protein formulations in both *in vitro* and *in vivo* models would be required to obtain a broader picture of the immunogenic risk of NPIs. However, with respect to pharmaceutical quality and stability the induction of aggregation and particles by NPIs remains critical and should be considered during drug product development.

Appendix A. Supplementary Data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.xphs.2020.11.011.

References

1. Roberts CJ. Therapeutic protein aggregation: mechanisms, design, and control. *Trends Biotechnol.* 2014;32:372-380.

2. Jiskoot W, Randolph TW, Volkin DB, et al. Protein instability and immunogenicity: roadblocks to clinical application of injectable protein delivery systems for sustained release. *J Pharm Sci*. 2012;101:946-954.
3. Jameel F, Hershenson S. *Formulation and Process Development Strategies for Manufacturing Biopharmaceuticals*. Hoboken NJ: Wiley; 2010:955.
4. Timasheff SN. Protein hydration, thermodynamic binding, and preferential hydration. *Biochemistry*. 2002;41:13473-13482.
5. Timasheff SN, Xie G. Protein-solvent preferential interactions, protein hydration, and the modulation of biochemical reactions by solvent components. *Proc Natl Acad Sci U S A*. 2002;99:9721-9726.
6. Weinbuch D, Cheung JK, Ketelaars J, et al. Nanoparticulate impurities in pharmaceutical-grade sugars and their interference with light scattering-based analysis of protein formulations. *Pharm Res (N Y)*. 2015;32:2419-2427.
7. Weinbuch D, Ruigrok M, Jiskoot W, Hawe A. Nanoparticulate impurities isolated from pharmaceutical-grade sucrose are a potential threat to protein stability. *Pharm Res (N Y)*. 2017;34:2910-2921.
8. Jiskoot W, Kijanka G, Randolph TW, et al. Mouse models for assessing protein immunogenicity: lessons and challenges. *J Pharm Sci*. 2016;105:1567-1575.
9. Moussa EM, Panchal JP, Moorthy BS, et al. Immunogenicity of therapeutic protein aggregates. *J Pharm Sci*. 2016;105:417-430.
10. Filipe V, Jiskoot W, Basmeleh AH, et al. Immunogenicity of different stressed IgG monoclonal antibody formulations in immune tolerant transgenic mice. *mAbs*. 2012;4:740-752.
11. van Beers MMC, Sauerborn M, Gilli F, et al. Oxidized and aggregated recombinant human interferon beta is immunogenic in human interferon beta transgenic mice. *Pharm Res (N Y)*. 2011;28:2393-2402.
12. Torosantucci R, Mozziconacci O, Sharov V, et al. Chemical modifications in aggregates of recombinant human insulin induced by metal-catalyzed oxidation: covalent cross-linking via michael addition to tyrosine oxidation products. *Pharm Res (N Y)*. 2012;29:2276-2293.
13. Lukashevich I, Shirwan H. Novel. In: *Technologies for Vaccine Development*. Springer-Verlag Wien; 2014:3-32.
14. Irvine DJ, Hanson MC, Rakhra K, Tokatlian T. Synthetic nanoparticles for vaccines and immunotherapy. *Chem Rev*. 2015;115:11109-11146.
15. Gregory AE, Titball R, Williamson D. Vaccine delivery using nanoparticles. *Front Cell Infect Microbiol*. 2013;3:13.
16. Ferwerda G, Meyer-Wentrup F, Kullberg B-J, et al. Dectin-1 synergizes with TLR2 and TLR4 for cytokine production in human primary monocytes and macrophages. *Cell Microbiol*. 2008;10:2058-2066.
17. Wang M, Zhang L, Yang R, et al. Improvement of immune responses to influenza vaccine (H5N1) by sulfated yeast beta-glucan. *Int J Biol Macromol*. 2016;93:203-207.
18. Jawa V, Terry F, Gokemeijer J, et al. T-cell dependent immunogenicity of protein therapeutics pre-clinical assessment and mitigation-updated consensus and review 2020. *Front Immunol*. 2020;11:1301.
19. Morgan H, Tseng S-Y, Gallais Y, et al. Evaluation of in vitro assays to assess the modulation of dendritic cells functions by therapeutic antibodies and aggregates. *Front Immunol*. 2019;10:601.
20. Gallais Y, Szely N, Legrand F-X, et al. Effect of growth hormone and IgG aggregates on dendritic cells activation and T-cells polarization. *Immunol Cell Biol*. 2017;95:306-315.
21. Kijanka G, Bee JS, Korman SA, et al. Submicron size particles of a murine monoclonal antibody are more immunogenic than soluble oligomers or micron size particles upon subcutaneous administration in mice. *J Pharm Sci*. 2018;107:2847-2859.
22. U.S. Department of Health and Human Services FDA, Center for biologics evaluation and research. In: *Guidance for Industry: Immunogenicity Assessment for Therapeutic Protein Products*; 2014. Available at: http://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/default.htm. Accessed June 30, 2020.
23. Alsenaidy MA, Jain NK, Kim JH, et al. Protein comparability assessments and potential applicability of high throughput biophysical methods and data visualization tools to compare physical stability profiles. *Front Pharmacol*. 2014;5:39.
24. U.S. Department of Health and Human Services FDA, Center for drug evaluation and research. In: *Complete Response BLA 761091*. Trastuzumab-Pkrb Celltrion, Inc; 2017. Available at: https://www.accessdata.fda.gov/drugsatfda_docs/nda/2018/761091Orig1s000OtherR.pdf. Accessed June 30, 2020.
25. Weinbuch D. *Nanoparticulate Impurities in Pharmaceutical-Grade Sucrose Are a Potential Threat to Protein Stability*. Leiden: Doctorate thesis; 2016:24.
26. Goodridge HS, Wolf AJ, Underhill DM. Beta-glucan recognition by the innate immune system. *Immunol Rev*. 2009;230:38-50.
27. Novak M, Vetvicka V. Beta-glucans, history, and the present: immunomodulatory aspects and mechanisms of action. *J Immunotoxicol*. 2008;5:47-57.
28. Gefroh E, Hewig A, Vedantham G, et al. Multipronged approach to managing beta-glucan contaminants in the downstream process: control of raw materials and filtration with charge-modified nylon 6,6 membrane filters. *Biotechnol Prog*. 2013;29:672-680.
29. Barton C, Vigor K, Scott R, et al. Beta-glucan contamination of pharmaceutical products: How much should we accept? *Cancer Immunol Immunother*. 2016;65:1289-1301.
30. Vetvicka V, Vannucci L, Sima P. β-glucan as a new tool in vaccine development. *Scand J Immunol*. 2020;91:e12833.
31. Sonck E, Devriendt B, Goddeeris B, Cox E. Varying effects of different β-glucans on the maturation of porcine monocyte-derived dendritic cells. *Clin Vaccine Immunol*. 2011;18:1441-1446.
32. Joubert MK, Hokom M, Eakin C, et al. Highly aggregated antibody therapeutics can enhance the in vitro innate and late-stage T-cell immune responses. *J Biol Chem*. 2012;287:25266-25279.
33. Ahmadi M, Bryson CJ, Cloake EA, et al. Small amounts of sub-visible aggregates enhance the immunogenic potential of monoclonal antibody therapeutics. *Pharm Res (N Y)*. 2015;32:1383-1394.
34. Rombach-Riegraf V, Karle AC, Wolf B, et al. Aggregation of human recombinant monoclonal antibodies influences the capacity of dendritic cells to stimulate adaptive T-cell responses in vitro. *PLoS One*. 2014;9:e86322.
35. Fathallah AM, Chiang M, Mishra A, et al. The effect of small oligomeric protein aggregates on the immunogenicity of intravenous and subcutaneous administered antibodies. *J Pharm Sci*. 2015;104:3691-3702.
36. Nabhan M, Legrand F-X, Le-Minh V, et al. The FcγRIIa-syk Axis controls human dendritic cell activation and T cell response induced by infliximab aggregates. *J Immunol*. 2020;205:2351-2361.
37. Benne N, van Duijn J, Kuiper J, et al. Orchestrating immune responses: How size, shape and rigidity affect the immunogenicity of particulate vaccines. *J Control Release*. 2016;234:124-134.
38. Elder MJ, Webster SJ, Chee R, et al. β-Glucan size controls dectin-1-mediated immune responses in human dendritic cells by regulating IL-1β production. *Front Immunol*. 2017;8:791.
39. Han B, Baruah K, Cox E, et al. Structure-functional activity relationship of β-glucans from the perspective of immunomodulation: a mini-review. *Front Immunol*. 2020;11:658.