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Aggregates associated with instability of antibodies during aerosolization induce adverse immunological effects

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Abstract:

Background: Immunogenicity refers to the inherent ability of a molecule to stimulate an immune response. Aggregates are one of the major risk factors for undesired immunogenicity of therapeutic antibodies (Ab) and may ultimately result in immune-mediated adverse effects. For Ab delivered by inhalation, it is necessary to consider the interaction between aggregates resulting from the instability of the Ab during aerosolization and the lung mucosa. The aim of this study was to determine the impact of aggregates produced during aerosolization of therapeutic Ab on the immune system.

Methods: Human and murine immunoglobulin G (IgG) were aerosolized using a clinically-relevant nebulizer and their immunogenic potency was assessed both *in vitro* using a standard human monocyte-derived dendritic cell (MoDC) reporter assay and *in vivo* in immune cells in the airway compartment, lung parenchyma and spleen of healthy C57BL/6 mice after pulmonary administration.

Results: IgG aggregates, produced during nebulization, induced a dose-dependent activation of MoDC characterized by enhanced production of cytokines and expression of co-stimulatory markers. Interestingly, *in vivo* administration of high amounts of nebulization-mediated IgG aggregates resulted in a profound and sustained local and systemic depletion of immune cells, which was attributable to cell death. This cytotoxic effect was observed when nebulized IgG was administered locally in the airways as compared to a systemic administration but was mitigated by improving IgG stability during nebulization, through the addition of polysorbates to the formulation.

Conclusion: Although inhalation delivery represents an attractive alternative route for delivering Ab to treat respiratory infections, our findings indicate that it is critical to prevent IgG aggregation during the nebulization process to avoid pro-inflammatory and cytotoxic effects. Optimization of Ab formulation can mitigate adverse effects induced by nebulization.

Keywords: therapeutic antibody, aerosol, aggregates, immunogenicity

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1. Introduction

Therapeutic antibodies (Ab), which mainly consist of monoclonal IgG, represent the fastest growing class of protein therapeutics, accounting for 90% of proteins on the market [1]. As recently highlighted during the COVID-19 pandemic, Ab have a tremendous potential to provide a rapid neutralizing response to emerging viral respiratory pathogens, augmenting vaccines for control of respiratory infections. Interestingly, both pulmonary and nasal delivery of therapeutic proteins are receiving increasing interest, as the airways are a relevant non-invasive entry portal to the respiratory tract for local-acting protein therapeutics [2-4]. For the treatment of respiratory diseases, several preclinical studies demonstrated that inhaled protein therapeutics, including Ab, are efficacious and display a better pharmacokinetic profile, as compared to other routes [5-7]. Despite recent clinical development of oral anti-infective Ab, in the context of the global response to SARS-CoV2 pandemic, the inhalation route remains unexploited for Ab. One of the unknowns is the biological consequence of Ab instability during aerosolization. Inhalation requires transformation of a (bulk) protein formulation in an aerosol, i.e., dispersion of a solution/suspension or a dry-powder dispersed into micron-sized particles suspended in a gaseous medium. As with 75% of the inhaled protein therapeutics in clinical development, nebulization of liquid formulations is often the primary technique used for inhalation of proteins [5]. Nebulization generates a huge air-liquid interface which, combined with the potential for nebulization-induced temperature increase and/or shear forces, can be deleterious for proteins. In response to such stresses, proteins are prone to unfolding, aggregating and, in some cases, being partly inactivated [8,9]. Aggregation is a key marker of instability of full-length Ab during nebulization [10,11] and its extent mainly depends on the type of Ab, the aerosol generator type and the formulation characteristics.

Aggregates are associated with Ab-related adverse immunogenicity [12-14]. Immunogenicity refers to the inherent properties of a molecule to stimulate an immune response. Adverse immunogenicity is due to uncontrolled and protracted immune responses and has major consequences on product safety and pharmacology [15-17]. For instance, adverse immunogenicity is associated with patient's immunization and production of anti-drug antibodies (ADAs) and affects protein therapeutics pharmacokinetics (PK), pharmacodynamics, efficacy, and safety, sometimes resulting in extremely harmful side effects [18,19]. To date, many factors have been implicated in adverse immunogenicity [20-23] and they include the content of aggregates and the route of administration, which are important parameters of Ab inhalation.

After inhalation, aggregates from aerosolized Ab will encounter the airways immune system, which has evolved over time to recognize, and prevent foreign particles, including aggregates, from penetrating the body. The airway mucosa is sentinelled by a high density of antigen-presenting cells (APC) that quickly and efficiently orchestrate local immune responses against inhaled antigens [24]. In the present study, we analyzed, both *in vitro* and *in vivo*, the immunological consequences of Ab aggregates generated during aerosolization and delivered through the airways. First, we screened the potency of IgG aggregates generated by nebulization to activate APC *in vitro*, using a standard human monocyte-derived dendritic cell (MoDC) assay. To take into consideration the complexity of the immune system and the lung mucosal environment, we evaluated the impact of nebulization-mediated IgG aggregates on immune cells *in vivo* after pulmonary administration. Our findings show that aggregation attributable to Ab nebulization induced immune cell activation, in a dose-dependent manner and delivering high-level of aggregates through the pulmonary route had a dramatic effect on immune cell homeostasis but also that this was avoidable through appropriate formulation approaches.

2. Materials and Methods

Mice

Adult male C57BL/6jrj (B6) mice (5 to 7 weeks old) were obtained from Janvier (France). All mice were housed under specific-pathogen-free conditions at the PST Animaleries animal facility (France) and had access to food and water ad libitum. All animal experiments complied with the current European legislative, regulatory and ethical requirements and were approved by the local animal care and use committee (reference: APAFIS#10200-2017061311352787).

Antibodies

Abs 1, 2 and 3 are full-length IgG1 (named hIgG1-1, hIgG1-2, hIgG1-3) used in the clinics, supplied in their commercial formulation with endotoxin levels meeting acceptance thresholds (according to their certificate of analysis). To avoid any interference on the aggregation propensity of each Ab, excipients were removed by hydroxyapatite chromatography and subsequent dialysis against phosphate-buffered saline (PBS). Protein concentration was then evaluated for each Ab: hIgG1-1 [1.7mg/mL], hIgG1-2 [1.9mg/mL], hIgG1-3 [2.38mg/mL]

mAb166 (mIgG2b-1) is a murine monoclonal IgG2b,κ Ab against pcrV, a component of a type three secretion system (T3SS) of P. aeruginosa [25]. MPC11 (mIgG2b-2) is the control isotype of mAb166. Both were supplied as sterile, pyrogen-free solution in phosphate-buffered saline (PBS), in accordance with good manufacturing practice, by BioXcell (USA) with endotoxin level < 1 EU/ml (according to their certificate of analysis). They were supplied as follows: mIgG2b-1 [2.5mg/mL], mIgG2b-2 [8.5mg/mL]. In some experiments, Ab formulations were supplemented with Polysorbate 80 (Sigma-Aldrich, France) at 0.01 or 0.05% (final volume) prior to nebulization.

Antibody nebulization

For sterility purposes, this procedure was performed under a cell-culture hood. All antibodies, in suspension were filtered on a 0.22µm syringe filter (Millipore, France). For *in vitro* assay, all antibodies were diluted in PBS1X to a final concentration of ~1.7 mg/mL before nebulization. For each Ab, 1 mL were nebulized using a clinically used Aeroneb Pro™ vibrating-mesh nebulizer (Aerogen, Ireland), connected with a 13 mL polypropylene tube (Dutscher, France) or the VITROCELL Cloud 12 system (VITROCELL Systems, Germany), which is the commercial version of the Air–Liquid Interface Cell Exposure–Cloud (ALICE-cloud) system, described by Lenz and colleagues [26]. Nebulization duration was measured and lasted ~5min for each sample (i.e., 0.4 ml/min liquid output rate). For *in vitro* aerosol-cell exposures, using the VITROCELL Cloud 12 system, the aerosol cloud was allowed to settle for 20min before removing cells. When necessary, nebulized Ab solutions were filtered on a 0.45µm syringe filter (Millipore, France) prior to further analysis. Nebulizers were washed extensively between each nebulization session using 0.22µm autoclaved water and PBS. Subsequently, nebulized PBS was analyzed by flow cell microscopy and the nebulizer was considered cleaned and operating for Ab nebulization when the nebulized PBS samples contained less than 300 particles/mL. To rule out that cell activation may be attributable to contamination released during nebulization, the activation potency of nebulized PBS (vehicle solution) was also investigated *in vitro*. Our analysis revealed that nebulized PBS had no impact on MoDC activation (data not shown).

Dynamic Light Scattering (DLS) analysis of antibodies

Native and nebulized Ab were analyzed by dynamic light scattering with a Dynapro Nanostar® (Wyatt Technology, USA) after appropriate dilution to 100 µg/ml in PBS1X1X in UVette (Eppendorf, France). For each sample, the acquisition was performed 10 times for 7 seconds each, at a temperature of 25°C with a detection angle of 90°. Particle diameter –(nm) and polydispersion index (pdi) were analyzed using DynamicsTM software (Wyatt Technology, USA). Samples with less than 70% of successful analyses were con-

sidered multimodal and non-analyzable as recommended by the manufacturer (depicted as n/a).

Flow cell microscopy (FCM) of antibodies

Native and nebulized Ab were analyzed by flow cell microscopy with an Occhio® FC200S+ (Occhio, Belgium) after appropriate dilution to 100 µg/ml in medium in the flowcell. Briefly, 250 µL of each antibody solution passed continuously in the flow cell, where particles were automatically detected, sized and counted by a camera. Particle counting, size assessment and distribution were analyzed using CallistoTM software (Occhio, France).

Monocyte-derived dendritic cells (MoDC) preparation

Human peripheral mononuclear cells (PBMC) were purified from cytapheresis, obtained from naïve donors (Etablissement Français du Sang, Hôpital Bretonneau, France) by centrifugation on a Ficoll density gradient (Eurobio, France). MoDC were prepared from PMBC as previously described [27]. Briefly, CD14+ monocytes were isolated using magnetic cell sorting (Miltenyi Biotec, Germany) and cultured during 6 days, at 1x106cells/mL in RPMI1640-GlutamaxTM (Gibco, France) supplemented with 10% of FCS (Dutscher, France), 1X Penicillin/Streptomycin (Gibco, France) in the presence of 25ng/mL of IL-4 (Miltenyi Biotec, Germany) and 100 ng/mL of GM-CSF. On day 6, homogeneity and viability of MoDC population was checked by flow cytometry based on their physical characteristics (forward scatter (FSC) and side scatter (SSC)) and incorporation of vital dye (LiveDeadTM, Invitrogen, France). The immature phenotype of MoDC was checked based on their DC-SIGN+, CD80low, CD83low and HLA-DR low phenotype.

MoDC stimulation with antibody preparation

On day 6, immature MoDC were harvested and washed in complete medium (RPMI1640-Glutamax™ supplemented with 10% of FCS (Dutscher, France) and 1X Penicillin/Streptomycin). For experiments using Ab aerosols collected in a 13 mL polypropylene tube, $1x10^5$cells/well were plated in 75µL in a U-bottom 96-well plate (Falcon, BectonDickinson, France) for 4 hours. 75µL of nebulized or native Ab were then added to a final concentration of 1, 10, 100 and 200 µg/mL depending on the experiments. For experiments using the VITROCELL Cloud 12 system, $1x10^5$cells/well were plated in 150µL on a 24-well plate permeable insert (Corning, France) for 18 hours. 200µL of complete was added in the basolateral compartment to prevent cell drying. The insert was then placed in the VITROCELL Cloud 12 system with 200µL in the basolateral compartment and cells were exposed to Ab aerosol as describe above. Inserts were put back on a companion 24-well plate.

Cells were incubated for 18 hours at 37°C with 5% CO_2. Lipopolysaccharide (LPS, from Escherichia coli O111:B5, Sigma-Aldrich, France) was used at 1µg/mL as a positive control. Untreated and nebulized PBS1X (Gibco, France) were used as vehicle controls. Each condition was tested in 3-9 replicates.

Analysis of MoDC activation by flow cytometry

After 18 hours of stimulation, MoDC were harvested and saturated in PBS1X supplemented with 2% of FCS, 2 mM EDTA and 1X of human Fc-Block (Becton Dickinson, France) for 15 min at 4°C. Cells were washed and stained in FACS buffer (PBS1X supplemented with 2% FCS and 2 mM EDTA) with antibodies described in Supplementary table 2, for 20min at 4°C in the dark. Negative FMO controls were generated for each parameter analyzed. All antibodies were from Biolegend (UK). Data acquisition was made on the 8-colors MACSQuant flow cytometer (Miltenyi Biotec, Germany) on a minimum of 10,000 living cells. MoDC analysis was performed using VenturiOne software (Applied Cytometry, USA). Analysis was made on singlet (FSC-A / FSC-H gating)

and CD45+ live cells (negative for LiveDead staining). Costimulatory protein expression was expressed as a ratio of the median fluorescence intensity (MFI) of cells treated with nebulized Ab / MFI of cells treated with native Ab.

Cytokine, chemokine and protein assays

All assays were perfomed on Immulon™ 96-well plates (ThermoFischer, France). Concentrations of CXCL8 (IL-8), human IL-6 in cell-free MoDC supernatants and TNF, IL6, IL1b and CXCL1 (KC) were measured using specific ELISAs (Biolegend, UK; limit of detection: at 15.6 pg/mL) according to the manufacturer's instructions and were normalized based on the mean concentration of total protein. Total protein concentration was determined using a BCA assay (ThermoFischer Scientific, France), according to the manufacturer's instructions (limit of detection: 15 µg/mL).

Animal experiments

Animal experiments have been performed 2-3 times. For each experiments, 5 mice per groups were used. Nebulized or native mIgG2b-1 and mIgG2b-2 (100 µg/animal, in 40µL) were administered orotracheally or through intravenous injection using a restraining tube at days 0, 7, 14, 21 and 28. For orotracheal administration, mice were anesthetized with isofluorane 4% and an operating otoscope fit with intubation specula was introduced to both to maintain tongue retraction and to visualize the glottis. A fiber optic wire threaded through a 20G catheter and connected to torch stylet (Harvard Apparatus, France) was inserted into the mouse trachea. Correct intubation was confirmed using lung inflation bulb test and 40µL of the bacterial solution was applied using an ultrafine pipette tip. For the acute treatment assessment, animals were euthanized using a lethal dose of ketamine/xylazine at 4 hours, 1 day and 14 days after a single administration. In the chronic treatment groups, animals were euthanized at day 29.

At the time of necropsy, blood was recovered by intracardiac puncture. Bronchoalveolar lavage fluid (BALF) was then collected by cannulating the trachea and washing the lung twice with 1.2 mL of PBS1X at room temperature. The lavage fluid was centrifuged at 400 g for 10 min at 4°C, and the supernatant was stored at -20°C until analysis. The cell pellet was resuspended in FACS buffer and counted in a hemocytometer chamber. Peripheral blood was washed out by intracardiac perfusion with 10 mL of PBS1X. Lung and spleen homogenates were then prepared in 2mL of RPMI1640 containing 125 µg/mL of Liberase (Sigma-Aldrich, France) and 100 µg/mL of DnaseI (Sigma-Aldrich, France) using a GentleMACS tissue homogenizer (Miltenyi Biotec, Germany). Cells were isolated through a 100µm cell strainer and purified with a 20% Percoll (GE Healthcare, France) density gradient. Cell preparation was centrifuged at 400 g for 10 min at 4°C, and the pellet was resuspended in FACS buffer, and counted in a hemocytometer chamber.

Immune cell phenotyping by flow cytometry

BALF, lung and spleen cells isolated as described above were saturated in PBS1X supplemented with 2% of FCS, 2 mM EDTA and 1X of human Fc-Block (BectonDickinson, France) for 15 min at 4°C. Cells were washed and stained with antibodies described in Supplementary table 2 for 20min at 4°C in the dark. Data acquisition was made on the 8-colors MACSQuant flow cytometer (Miltenyi Biotec, Germany) on a minimum of 100,000 living cells. Analysis was performed using VenturiOne software (Applied Cytometry, USA). Analysis was made on singlet (FSC-A / FSC-H gating) and CD45+ live cells (negative for LiveDead staining). Immune cell phenotypes were determined as described in the Supplementary table 2. Data were normalized based on the mean concentration of respective native Ab conditions or expressed as a number of cell calculated as follow: % of the total CD45+ immune cell population x total number of cell. For Annexin-V/PI staining, data were normalized as follows: % of the positive population for nebulized Ab / % of the positive population for native Ab.

Histology

Lungs were fixed in 10% buffered formalin (Shandon), dehydrated in ethanol and embedded in paraffin. Serial sections (3 mm) were stained with haematoxylin and eosin (HE) or Congo Red. Ten HE sections per mouse were randomly evaluated to count cell nucleus using a machine-learning pixel classification plugin for Image J (https://imagej.net/plugins/tws/), as described previously [28].

Statistical analysis

Differences between experimental groups were determined using Kruskal-Wallis or t-test comparing two groups, using one or two-way analysis of variance (ANOVA) followed by Newman-Keuls or Bonferroni post-test (for comparison between more than two groups), after confirmation that the data were normally distributed. All statistical tests were performed with GraphPad Prism software (version 4.03 for Windows, GraphPad Software Inc., USA). All data are presented as mean ± standard error of the mean (SEM). The threshold for statistical significance was set to $p<0.05$.

3. Results

3.1. Aggregation of antibodies is heterogeneous during nebulization

Nebulization promotes Ab aggregation [10,11]. In this study, mesh-nebulization was used since it is less deleterious on Ab and often considered for aerosolization of proteins [5]. Full-length Ab commercially available or under preclinical development were reformulated in PBS and nebulized with the Aeroneb Pro™ vibrating-mesh nebulizer.

Ab aggregation results in a broad range of particles, from dimers (several nanometers) to micron-sized, and even visible particles in some cases [29], requiring the combination of different complementary analytical techniques. The aggregation profiles of Ab were assessed using DLS and FCM to report particles at the submicrometric and micrometric scales, respectively (Figure 1).

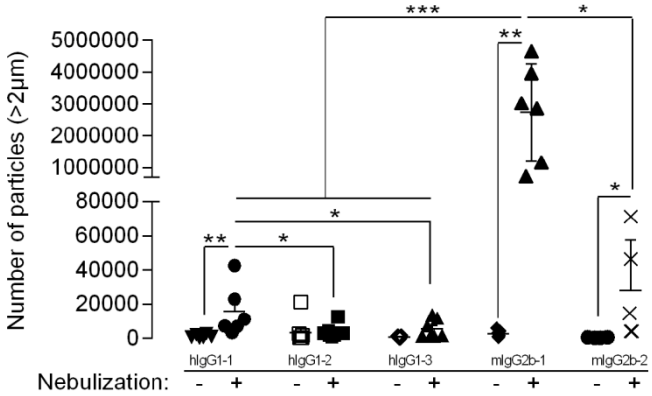


Figure 1. Antibodies were either nebulized using an Aeroneb Pro™ vibrating-mesh nebulizer and collected (Nebulization +) or left untreated (Nebulization -). The total number of particles (with diameter >2µm) was quantified using a flow microscope. The data are quoted as the mean ± SEM. *, ** : $p<0.05$, and $p<0.01$ respectively, in a one-way ANOVA with Newman-Keuls's correction for multiple comparisons. The results represent three to eight independent nebulizations.

Nebulization led to an increase in the concentration of particles > 2 µm for hIgG1-1, mIgG2b-1 and mIgG2b-2, as compared to their native counterparts. Conversely, no significant increase in particles was observed for hIgG1-2, hIgG1-3 (Figure 1). Furthermore, comparing nebulized Ab, we observed that hIgG1-1 and mIgG2b-1 had significantly more particles than the other human and murine Ab, respectively. In addition to differences in total particle concentration, there were also differences in particle size distribu-

tion. Nebulized hIgG1-1 and -2 had more than 78% of their particles smaller than 5 µm, while hIgG1-3, mIgG2b-1 and mIgG2b-2 comprised more than 33% of their particles above 5 µm (Table 1). For murine mIgG2b-1 and mIgG2b-2, aggregation was also observed at the submicron scale, most notably for nebulized mIgG2b-1, which could not even be analyzed by DLS due to the large heterogeneity of the particle size distribution (most likely all-sized Ab aggregates), which cannot be clearly structured in defined size modes – a prerequisite for DLS measurements. Nebulized mIgG2b-2 exhibited a significant reduction (7%) of monomeric Ab amount (Supplementary Table 1). Overall, our results highlight the huge heterogeneity of Ab aggregation during mesh nebulization, most likely depending on Ab sequence/structure.

Table 1. Particle size distribution (%) of nebulized antibodies.

Antibody	2-5µm	5-25µm	>25µm
hIgG1-1	84,3	14,7	1,1
hIgG1-2	78,4	21,1	0,4
hIgG1-3	53,8	45,8	0,4
mIgG2b-1	67,0	32,3	0,7
mIgG2b-2	57,8	40,7	1,6

3.2. Ab aggregates, produced during mesh-nebulization, activate antigen-presenting cells

The dramatic consequences of adverse immunogenicity have prompted regulatory authorities to establish a guidance to test the immunogenicity of therapeutic protein products and industry to propose screening approaches [30,31]. They include investigating the ability of protein aggregates to activate immune cells, *in vitro*.

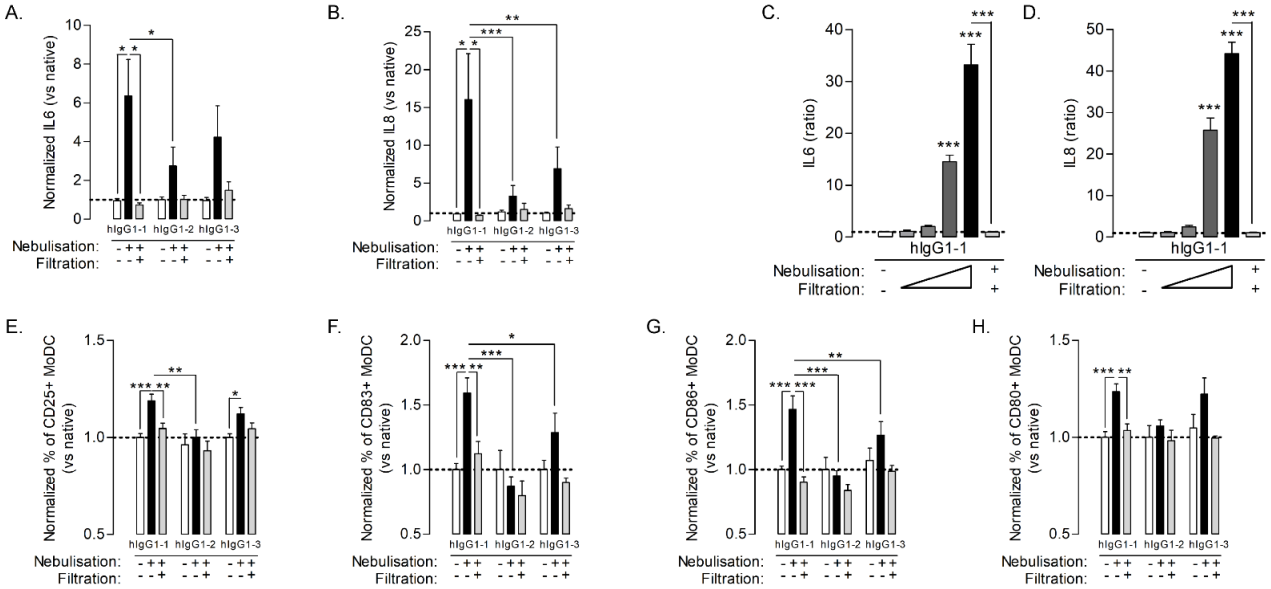
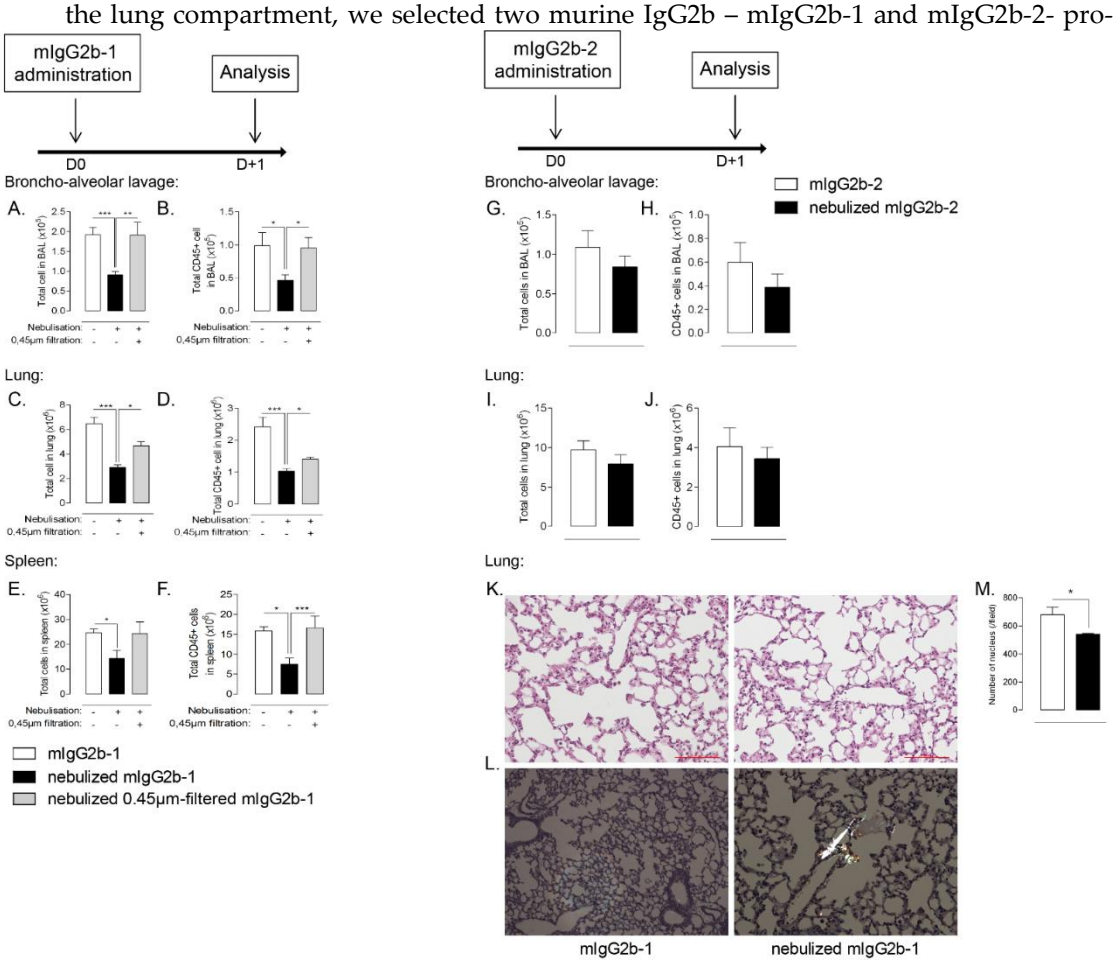


Figure 2. MoDC were stimulated using equal final concentration of Ab at 100 µg/mL either native (white bars), nebulized (black bars) or nebulized and 0.45µm-filtered (gray bars) for 18h. IL6 (A) and IL8 (B) were quantified in cell-free supernatant. MoDC were stimulated with 1, 10, 100 or 200µg/mL (gray to black bars) of nebulized hIgG1 or 100µg/mL of nebulized and 0.45µm-filtered hIgG1 (last bar) or left untreated (white bars) for 18h. IL6 (C) and IL8 (D) were quantified in cell-free supernatant. MoDC were stimulated using equal final concentration of Ab at 100 µg/mL either native (white bars), nebulized (black bars) or nebulized and 0.45µm-filtered (gray bars) for 18h.CD25 (E), CD83 (F), CD86 (G), CD80 (H) expression were measured using flow cytometry. The data are quoted as the mean ± SEM. *, **, *** : $p<0.05$, $p<0.01$ and $p<0.001$ respectively, in a one-way ANOVA with Newman-Keuls's correction for multiple comparisons. The results are representative of six independent experiments (n=6-9 technical replicates/experiment).

Here, we incubated human monocyte-derived dendritic cells (MoDC) overnight with native or nebulized Abs (hIgG1-1 to -3) and analyzed both the release of pro-inflammatory cytokines and expression of co-stimulatory proteins involved in the DC-T synapse (CD25, CD83, CD86 and CD80). Neither the native Ab, nor the nebulized buffer without Ab (data not shown) promoted IL-6 or IL-8 production by MoDC (Figures 2A and 2B) and modulated cell markers as compared to untreated MoDC (Figures 2E-H). Nebulized and aggregated hIgG1-1 induced a significant and dose-dependent increase of cytokine production (Figures 2A-D) whereas the other nebulized Ab solutions had only a minor and inconsistent impact on cytokine level. The nebulized and aggregated hIgG1-1 induced a slight but significant increase in all cell markers, whereas the effect of other nebulized Ab was limited (Figures 2E-H). Interestingly, the filtration of Ab solutions after nebulization, removing micrometric particles (data not shown), resulted in the abrogation of both cytokine release and expression of co-stimulatory markers by MoDC (Figures 2A-H, gray bars). In addition, when comparing nebulized antibodies, our analysis revealed that activation potency of hIgG1-1 was higher than for hIgG1-2 and hIgG1-3. Altogether, our results suggest that activation of APC was attributable to the presence of aggregates and that the extent of this activation was correlated to the number of particles (Figure 1). Since aerosol collection system may modulate the amount and the size distribution of Ab aggregates after nebulization [11], we determined whether collection might induce a bias in APC activation. We exposed directly MoDC to hIgG1-1 aerosols using the VITROCELL Cloud 12 system [26]. As observed in Supplementary Figure 1, we obtained similar results on MoDC activation independent of whether the cells were directly exposed to hIgG1-1 aerosols (through use of the VITROCELL Cloud 12 system) or exposed to them after collection as a bulk solution supporting our decision of adopting the latter approach. Taken together, our results showed that nebulized Ab induced MoDC maturation and activation as compared to native Ab, and confirmed the involvement of Ab aggregates in this response.

3.3. High-level of nebulization-mediated antibody aggregates impair lung cell homeostasis after lung delivery

Historically, *in vitro* assays have been widely used to describe the potential immunogenicity of biotherapeutic aggregates [13]. However, they display several limitations: (i) the amount of aggregates inducing a response *in vitro* may not directly translate into *in vivo* response and (ii) they may not predict the impact of the pulmonary delivery route. To gain insight into the broad effects of Ab aggregates produced by aerosolization within the lung compartment, we selected two murine IgG2b – mIgG2b-1 and mIgG2b-2- pro-



ducing different amounts of aggregates during mesh-nebulization (Figure 1) which differentially activate MoDC (Supplementary figures 2). We administered them through the airways in naïve mice. Remarkably, the nebulized and aggregated mIgG2b-1 resulted in a dramatic reduction in the total cell number in the airway compartment of mice (BAL), as compared to native antibodies administered by the same route (Figure 3A). Moreover, nebulized mIgG2b-2, producing 10-fold less aggregates after mesh-nebulization than mIgG2b-1 (Figure 1), showed no statistically significant reduction in BAL cell number (Figures 3G). Of note, the animals that received the native Ab through the airways had similar cell count as sham animals (data not shown), which implies that the oro-tracheal application itself did not obfuscate our results.

Figure 3. B6 mice received a 40 µL orotracheal instillation of mIgG2b-1 at 100 µg/mL either native (white bars), nebulized (black bars) or nebulized and 0.45µm-filtered (gray bars). The total number of cells (A, C and E) and CD45+ cells (B, D and F) were quantified in BAL (A and B), in the lungs (C and D) and the spleen (E and F) using flow cytometry, 18h after the administration. B6 mice received a 40 µL orotracheal instillation of mIgG2b-2 at 100 µg/mL either native (white bars) or nebulized (black bars). The total number of cells (G and I) and CD45+ cells (H and J) were quantified in BAL (G and H), in the lungs (I and J) using flow cytometry, 18h after the administration. Lung tissues of mice treated with either native (white bars) or nebulized (black bars) mIgG2b-1 were histologically examined 18h after the administration. (K) Hematoxylin-eosin sections were used to

quantify cell nucleus (M) by machine-learning (see material and methods section). (L) Congo-Red sections were observed under polarized light. Aggregates are identified as apple-green birefringence artifacts.. 10 sections / mouse were observed at x20 magnification and used for machine-learning quantification. The data are quoted as the mean ± SEM. *, **, *** : $p<0.05$, $p<0.01$ and $p<0.001$ respectively, in a one-way ANOVA with Newman-Keuls's correction for multiple comparisons. The results are representative of three independent experiments (n=5 mice/experiment).

Analysis of the cellular phenotype of the BAL cells revealed an analogous result for immune (CD45+ leukocytes) cells (Figures 3B and 3H). Further analysis did not reveal any impact on specific lineage as almost all immune cell lineages were affected after the administration of aggregated Ab (Supplementary Figures 3A-E). In the lung tissue, nebulized mIgG2b-1 caused a 2-fold reduction in total cell and leukocyte counts relative to controls (Figures 3C-D), while nebulization of mIgG2b-2 did not modify leukocyte counts (Figures 3I and J). This decrease affected both myeloid (neutrophil, monocyte, dendritic cell) (Supplementary Figures 3F-I) and lymphoid lineages (B, T CD4+/CD8+ cells) (Supplementary Figures 3J-L). The modification of cell homeostasis in the airway compartment was not associated with an alteration of the lung epithelial barrier (Supplementary figure 4), but was mostly attributable to micron-sized particles as 0.45μm-filtration of nebulized mIgG2b-1 prevented these adverse effects (Figures 3A-F, gray bars). The filtration step did not significantly modify Ab particle size distribution (Supplementary Tables 4 and 5) or concentration (Supplementary Table 6), as compared to native Ab.

Interestingly, we observed protein aggregates (appearing as apple-green birefringence structures under polarized light) on Congo-red stained lung sections from animals treated with nebulized mIgG2b-1 (Figure 3L). Unsupervised machine learning, which was used to quantify cell nucleus on HE-stained lung section, showed that local administration of nebulized mIgG2b-1 was associated with a significant reduction of lung cells after 18 hours (Figures 3K and 3M). Unexpectedly, the number of total and immune cells was also diminished, even though to a lesser extent, in the spleen (Figures 3E-F), indicating that the impairment of cellular homeostasis reached the systemic compartment. Contraction of cell number 18h after a single airway administration of nebulized Ab primarily occurred in the airways, and then probably extended through the lung tissue and systemically as it was restricted to the airway compartment after 4 hours (Figures 4A-4F). Moreover, this effect was sustained for at least up to 14 days after Ab administration (Figures 4G and 4H), or after repeated administrations (Figures 4I-4N). For either single or repeated administrations of nebulized mIg2b-1, we did not observe any sign of general toxicity, including body-weight loss (data not shown). Overall, our results suggest that airway administration of aggregated IgG (>0.45 μm) profoundly affected cellular homeostasis, in a time-dependent manner, both locally and systemically.

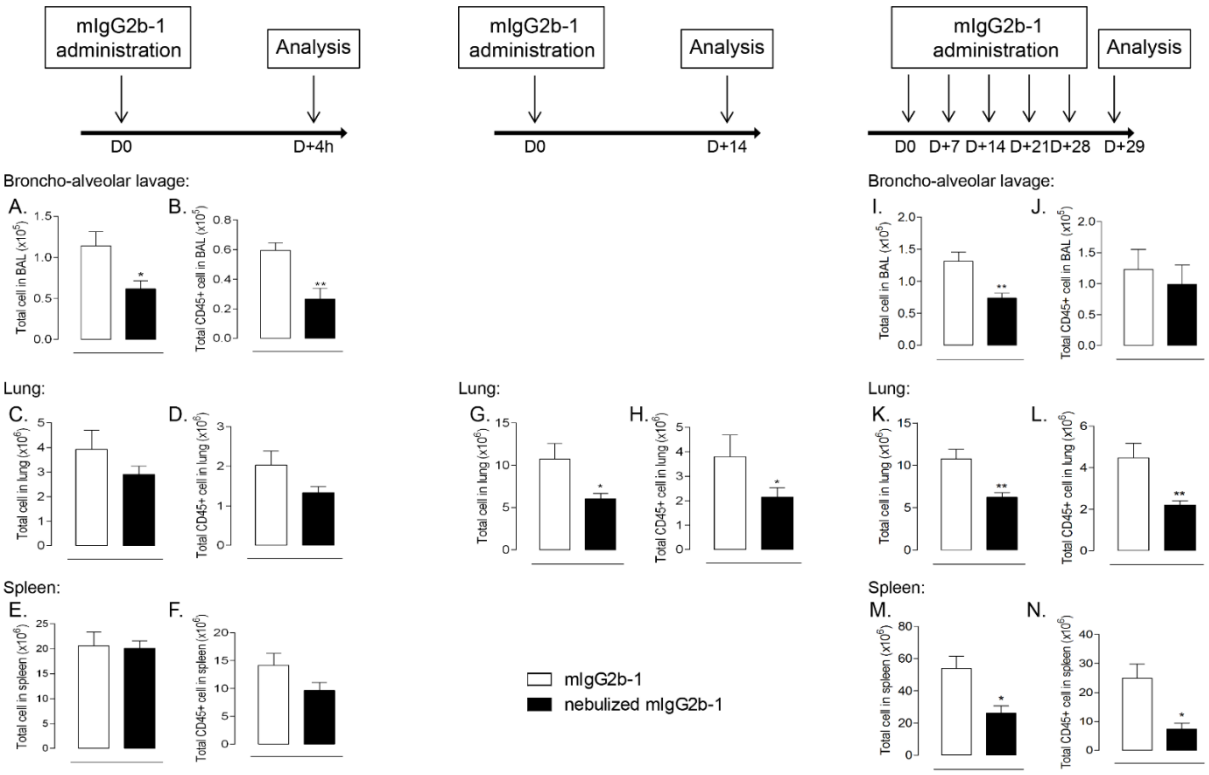


Figure 4. B6 mice received a 40 µL orotracheal instillation of mIg2b-1 at 100 µg/mL either native (white bars) or nebulized (black bars) through the airways at D0, or D+0, D+7, D+14, D+21 and D+28. The total number of cells (A , C, E, G, I, K and M) and CD45+ cells (B, D, F, H, J, L and N) were quantified in BAL (A, B, I and J), in the lungs (C, D, G, H, K and L) and the spleen (E, F, M and N) using flow cytometry, 4h, 14 days or 29 days after the first administration. The data are quoted as the mean ± SEM. *, ** : $p<0.05$ and $p<0.01$ respectively, in a t-test. The results are representative of two independent experiments (n=5 mice /experiment).

3.4. Nebulized aggregated antibody induced immunologically silent cell death after lung administration

Next, we investigated the mechanisms accounting for host cell contraction and hypothesized that it was associated with cell death. Cell death occurs in multiple forms and can be divided in accidental cell death (ACD; necrosis) or regulated cell death (RCD; apoptosis) [32]. ACD is characterized by a dramatic and instantaneous collapse of cells and can be triggered in response to different stresses, including chemical, physical or mechanical insults, whereas RCD relies on committed molecular machinery [33]. Using Annexin-V/propidium iodide (PI) staining, which allows the discrimination of early apoptotic cells (Annexin-V+/PI-), late apoptotic cells (Annexin-V+/PI+) and necrotic cells (Annexin-V-/PI+) [34], we quantified the proportion of each cell death phenotypes in both total cells and CD45+ leukocytes population, 18 hours after a single administration of mIgG2b-1. We observed that administration of nebulized mIgG2b-1 provoked a significant increase of both late apoptotic and necrotic spleen cells while lung and airway cells were suffering from necrosis.as compared to mice treated with native Ab (Figures 5A-5F) or with nebulized mIgG2b-2 (Supplementary Figure 5). The type of cell death could also be determined by the analysis of mediators released in the environment. When comparing BALs from animals treated with native and nebulized mIgG2b-1, we did not observe any significant difference in the production of TNF, IL-6, IL-1b or CXCL1 (KC) (Figures 5G-J). These data suggested that the cell contraction occurring after single or multiple airway administration of nebulized and aggregated IgG was associated with an inflammatory silent cell death process.

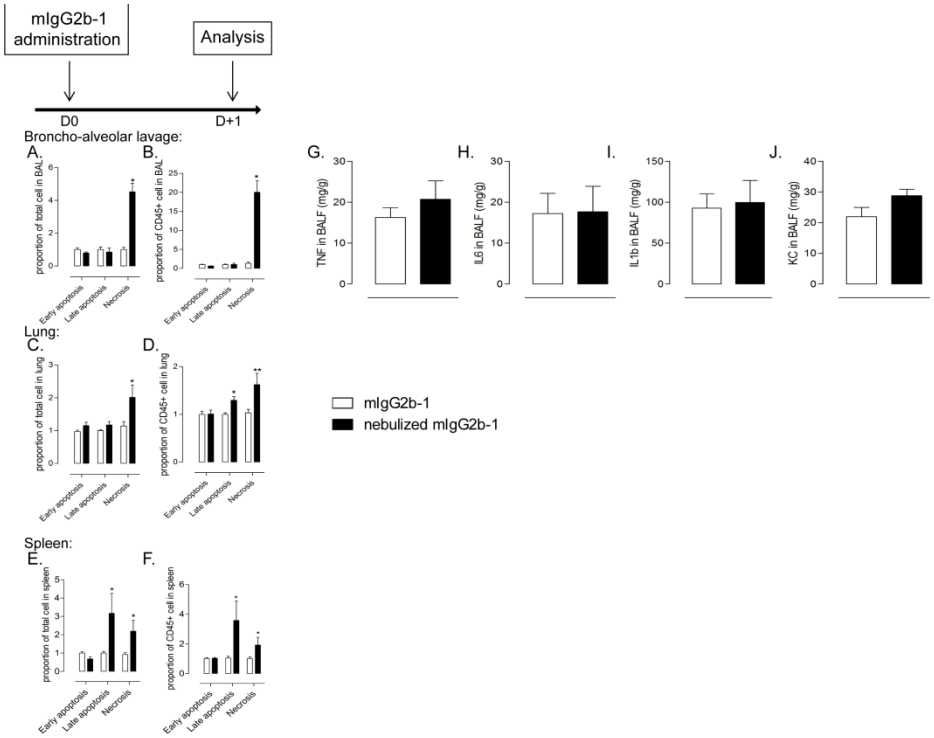


Figure 5. B6 mice received a single 40 µL orotracheal instillation of mIg2b-1 at 100 µg/mL either native (white bars) or nebulized (black bars). The proportion of early apoptotic cells (Annexin-V+/PI-), late apoptotic cells (Annexin-V+/PI+) and necrotic cells (Annexin-V-/PI+) were quantified in total cell (A, C and E) or CD45+ cell (B, D and F), 18h after the administration in BAL (A and B), lungs (C and D) and spleen (E and F) relative to mice treated with native Ab using flow cytometry. The concentrations of TNF (G), IL6 (H), IL1b (I) and KC (J) in BALF were determined 18h after the administration. The data are quoted as the mean ± SEM. *, **: $p<0.05$ and $p<0.01$ respectively, in a t-test. The results are representative of three independent experiments (n=5 mice/experiment).

3.5. The effect of nebulization-mediated antibody aggregates on immune cell homeostasis is specific of the pulmonary route

Immunogenicity of Ab is also dependent of their route of administration [20-22]. Thus, we investigated the effect of native or nebulized-mIgG2b-1 after intravenous injection. In contrast to what was observed after airway administration, there were no significant differences in the BAL or lung cell counts in the animals who received native or nebulized mIgG2b-1 intravenously (Figures 6A-6D). Interestingly, these results were substantiated when analyzing cell number after repeated administration of nebulized mIgG2b-1 (Supplementary Figure 6), where no differences were noticed in the airways or lungs of animals treated by repeated intravenous injections as compared to animals, which received the IgG in the lungs. Our data suggested that the route of administration played an important role on the adverse effect of IgG aggregates produced during nebulization on cell homeostasis.

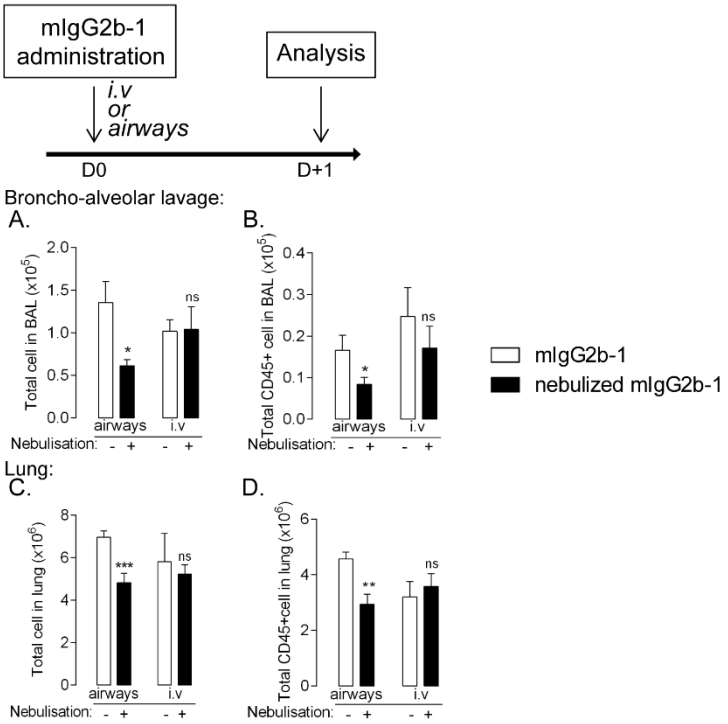


Figure 6. B6 mice received a 40 µL orotracheal instillation or 100µL intravenous injection of mIgG2b-1 at 100 µg/mL either native (white bars) or nebulized (black bars). The total number of cells (A and C) and CD45+ cells (B and D) were quantified in BAL (A and B) and in the lungs (C and D) using flow cytometry, 18h after the administration. The data are quoted as the mean ± SEM. *, **, ***: $p<0.05$, $p<0.01$ and $p<0.001$ respectively, in a t-test. The results are representative of two independent experiments (n=5 mice/experiment).

3.6. Reducing aggregation limits pulmonary cytotoxicity associated to lung administration of nebulized Ab

Pharmaceutical development aims to design a high-quality product ensuring an efficacious and safe treatment along the life of the product. Hence, formulation and protein engineering are often adapted to limit Ab aggregation, especially considering chronic-based therapies. Different parameters, including addition of surfactant, have a protective effect, limiting Ab aggregation during nebulization [10]. Here, we added polysorbate80 (PS80) in mIgG2b-1 formulation to significantly reduce its aggregation during nebulization (Supplementary Figure 7). The addition of surfactant did not significantly modify Ab particle size distribution (Supplementary Tables 4 and 5) or concentration (Supplementary Table 6) as compared to unformulated Ab. Single administration of nebulized mIgG2b-1 supplemented with 0,05% of PS80 abrogated the reduction of lung cell number (Figures 7C and D) and to a lesser extent of airway cell (Figures 7A and B) as compared to non-formulated mIgG2b-1. This was likely attributable with a reduction of cell death in the same compartment (Supplementary Figure 8). These results suggest that optimizing IgG formulation improved its molecular stability and might limit adverse effects on cell homeostasis.

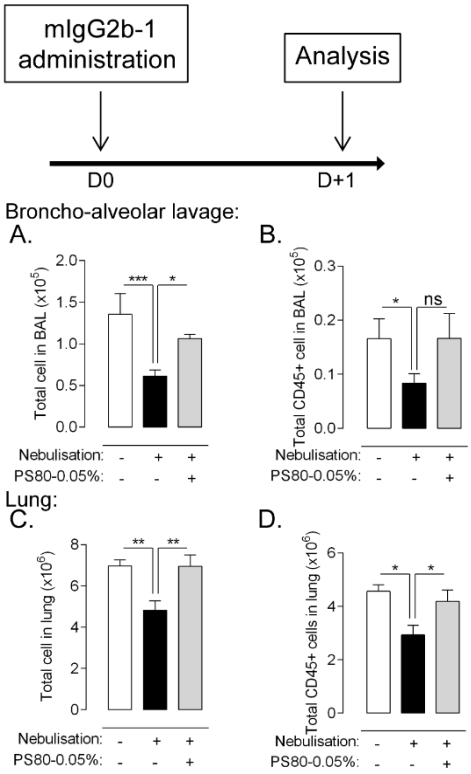


Figure 7. B6 mice received a 40 µL orotracheal instillation of mIgG2b-1 at 100 µg/mL either native (white bars), nebulized (black bars) or nebulized + Polysorbate80 0.05% (PS80 0.05%, gray bars). The total number of cells (A and C) and CD45+ cells (B and D) were quantified in BAL (A and B) and in the lungs (C and D) using flow cytometry, 18h after the administration. The data are quoted as the mean ± SEM. *, **, ***: $p<0.05$, $p<0.01$ and $p<0.001$ respectively, in a t-test. The results are representative of two independent experiments (n=5 mice/experiment).

4. Discussion

Ab are highly sensitive to stresses encountered during their product development, manufacturing, storage or clinical use, and they often require high therapeutic doses, necessitating high concentration drug products with a higher risk of aggregation. Tracking aggregates prior and after Ab bioprocessing is essential to avoid risks for patients, treatment failure and ultimately termination of drug development/commercialization.

Protein aggregation has been widely demonstrated as an influential factor in the induction of adverse immunogenicity of biotherapeutics [35,36]. However, some of the stress conditions used in the literature are not representative of those experienced during product development, manufacturing, storage or clinical use, making the aggregates generated far different from those found in marketed products. This may hamper conclusions about the exact potency of protein aggregation in the occurrence of drug immunogenicity. Several intrinsic or extrinsic attributes of aggregates might work synergistically to induce immunogenicity. Among them, parameters related to the delivery route can affect the immune response associated with the administration of drugs. For example, subcutaneous delivery has been associated with higher immunogenicity than the intravenous route, for several biotherapeutics [37,38]. To our knowledge, there is not any study conducted so far to assess the immunogenicity associated with lung administration of an aerosolized and aggregated Ab. We set out a dual experimental approach to investigate the quality and the extent of the immune responses induced by a nebulized and aggregated Ab using both *in vitro* and *in vivo* models. Here, we showed for the first time that the aggregates resulting from IgG nebulization induced immune cell

over-activation and that their delivery to the lung markedly and durably impaired cell homeostasis.

Protein aggregation is a process of non-specific association of monomers through multiple physical and chemical pathways which are well documented [39]. The characteristics of protein aggregates are variable in terms of particle size, number, morphology, chemical modifications, reversibility, conformation or hydrophobicity [40]. This phenotypic heterogeneity results from the various stresses applied to proteins and require specific analytical methods [41]. Aerosol generation involves the dispersion of liquid droplets into a gas. This process may be associated with physical stresses including temperature variations and the generation of a massive air-liquid interface, which ultimately induce changes in protein conformation and lead to its aggregation [10]. We measured the aggregation of several human IgG submitted to aerosolization using a vibrating-mesh nebulizer, which is expected to be less deleterious than other nebulizers [42,43]. Flow cell microscopy and dynamic light scattering revealed that the number and size of aggregates were Ab dependent, confirming the results in the literature on the necessity of a case-by-case approach. Besides, drastic differences were observed between commercial Abs (hIgG1-1 to 3) and murine Abs (mIgG2b-1 and -2), where the latter displayed higher aggregation upon nebulization. This may be explained by the fact that commercial Abs underwent advanced development programs and were thus selected for their limited aggregation potency. We next determined whether the aggregates found in the therapeutic product after nebulization may induce immunogenicity using a MoDC-based assay, which has been widely used to describe the potential immunogenicity of biotherapeutics or unwanted products [44]. Our analysis revealed that nebulized and aggregated hIgG1 – in particular hIgG1-1, were able to induce MoDC activation and maturation, as evidenced by the enhanced secretion of cytokines and increased expression of co-stimulatory proteins on MoDC. This response correlated with the fraction of micron-sized aggregates in the Ab aerosol. There are still discrepancies regarding the size and type of aggregates involved in the generation of immunogenic responses [45]. This depends on the type and strength of the stress applied and the multiple experimental protocols which have been described [21,46]. Here, hIgG1-1 aerosol is mainly composed of small-sized particles (2-10μm), which have been shown to enhance the immune response and be the most immunogenic [45,47].

One potential bias regarding Ab aggregation could be attributed to the aerosol collection step, which uses a polypropylene tube to re-pool aerosol droplets into a bulk liquid. Indeed, it has been evidenced that the aerosol collection device could influence protein stability, generating different aggregation profiles [11,48]. In this context, we used the VITROCELL technology, which was developed to improve reliability of toxicological studies of aerosolized compounds on air-liquid interface cell cultures [49,50]. This system, which allows direct deposition of aerosols on cells, avoids the collection step [51]. Our results showed that direct hIgG1-1 deposition on MoDCs resulted in cell activation, as evidenced by the similar increase in both cytokine production and expression of costimulatory proteins than those obtained with the nebulized-collected Ab. Thus, the collection system used here was considered relevant for these assays and was kept for the *in vivo* experiments.

To take into account the complexity of the lung mucosal-associated immune system, it was necessary to use an animal model to predict the immunogenicity of nebulized Ab *in vivo* [52,53]. We chose murine antibodies to avoid a high immunogenic background response due to the non-specific activation of the mouse immune system by foreign proteins. In our study, we observed that single or repeated administration of a nebulized antibody in the lungs induced a significant contraction of the total and immune cell number in the airways starting quickly after the administration as compared to native Ab or saline controls. This was dependent on the number of aggregates as low aggregated Ab, filtered or PS80 preparation of nebulized Ab did not have any impact on cell number. Interestingly, at later time-points this contraction reached the lung parenchyma and the

spleen, reducing significantly the number of both myeloid and lymphoid cells. We wondered whether this cell number reduction induced by the administration of nebulized Ab was associated with cell death. We observed that apoptosis were significantly increased in lung total cells and in leukocytes after airway administration of nebulized Ab. Interestingly, these cellular injuries were dependent on the presence of aggregates, as Ab formulated with surfactant, known to limit aggregation [10], and even filtered nebulized Ab preparation did not promote cell death. These adverse effects were also dependent on the route of administration as nebulized Ab administered through the intravenous route was not associated with the same cell number reduction. The complete understanding of the molecular and cellular mechanism associated with the massive cytotoxicity of nebulized and aggregated antibodies requires further investigations.

Protein aggregation underlies many chronic diseases where aggregates are thought to elicit injury including cell apoptosis [54,55]. To the best of our knowledge, this is the first study reporting that extracellular therapeutic protein aggregates may sensitize the host to cytotoxicity. This cellular injury occurred in the absence of a pro-inflammatory response which is contradictory with the current paradigm regarding the induction of innate immune responses by protein aggregates [13]. This discrepancy may come from the attributes of Ab aggregates associated with nebulization, as compared to the ones associated with other aggregation stresses, including the formation of neoepitopes, the immunomodulatory properties of the aggregates, the exposure of post-translational modifications or the generation of danger signals [56]. A complete understanding of the physical mechanisms accounting for the immunogenic properties of nebulized Ab aggregates is beyond the scope of this study. Immunogenicity may also be associated with the breakdown of self-tolerance rather than an active immune response [56]. It is particularly concordant with repeated exposure which may occur during dosing regimen for chronic disease [36].

In conclusion, we demonstrated that aerosolization using a clinically relevant nebulizer induced Ab aggregation and resulted in immune cell activation and immunocytotoxicity *in vivo*. Although there are still many questions to address to understand better the relationship between Ab aggregates and immunogenicity, our findings point a significant role of the route of administration in the immunogenic/biological response associated to Ab aggregates. Further investigations will be required to determine the types and the amount of aggregates and the role of the Fc domain in the immunocytotoxic response of Ab aggregates produced during nebulization. Our findings also highlight the importance to explore further the different methods (protein engineering, aerosolization process, formulations) to stabilize further Ab during aerosolization to minimize risks for the patients.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: *in vitro* aerosol-MoDC exposure using Vitrocell; Figure S2: MoDC activation by nebulized mIgG2b-1; Figure S3: Lung cell immunophenotyping after administration of nebulized mIgG2b-1; Figure S4: Protein content in BALF after administration of nebulized mIgG2b-1; Figure S5: Apoptosis of lung and airway cells after administration of nebulized mIgG2b-2; Figure S6: Lung and airway cell count after i.v administration of nebulized mIgG2b-1; Figure S7: Effect of PS80 on the generation of aggregate after mIgG2b-1 nebulization; Figure S8: Lung cell count after administration of nebulized mIgG2b-1 supplemented with PS80-0.05%; Table S1: Dynamic light scattering analysis of nebulized antibodies; Table S2: List of antibodies used for flow cytometry; Table S3: Phenotype of analyzed cells; Table S4: Particle size distribution (%) of nebulized antibodies; Table S5: Dynamic light scattering analysis of nebulized antibodies; Table S6: Nanodrop analysis of antibody concentration.

Author Contributions: TS and NHV conceived the study. All authors substantially contributed to the acquisition, analysis or interpretation of data. GI performed machine-learning analysis of histological sections. OS loaned the VITROCELL Cloud 12 system and provided expertise regarding the ALICE–cloud system. TS and NHV contributed to manuscript drafting, revising and critically reviewing. All authors approved the final version of this manuscript to be published.

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Data Availability Statement: The data supporting this article will be shared on reasonable request to the corresponding author.

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Conflicts of Interest: NHV is co-founder and scientific expert for Cynbiose Respiratory. In the past two years, she received consultancy fees from Eli Lilly, Argenx, Novartis and research support from Sanofi and Aerogen Ltd. RML is Head of R&D, Science and Emerging Technologies in Aerogen. All other authors have no competing interests to declare.

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