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### ► To cite this version:

Maurizio Luisetti, Philipp Kroneberg, Takuji Suzuki, Zamir Kadija, Bernhard Muellinger, et al.. Physical Properties, Lung Deposition Modeling, And Bioactivity Of Recombinant Gm-Csf Aerosolised With A Highly Efficient Nebulizer. *Pulmonary Pharmacology & Therapeutics*, 2011, 24 (1), pp.123. <10.1016/j.pupt.2010.08.004>. <hal-00721578>

**HAL Id: hal-00721578**

**<https://hal.science/hal-00721578v1>**

Submitted on 28 Jul 2012

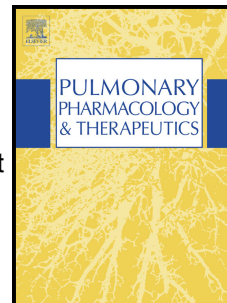
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# Accepted Manuscript



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PII: S1094-5539(10)00098-2

DOI: [10.1016/j.pupt.2010.08.004](https://doi.org/10.1016/j.pupt.2010.08.004)

Reference: YPUPT 1028

To appear in: *Pulmonary Pharmacology & Therapeutics*

Received Date: 24 May 2010

Revised Date: 21 July 2010

Accepted Date: 12 August 2010

Please cite this article as: Luisetti M, Kroneberg P, Suzuki T, Kadija Z, Muellinger B, Campo I, Gleske J, Rodi G, Zimlich WC, Mariani F, Ferrari F, Frey M, Trapnell BC. Physical Properties, Lung Deposition Modeling, And Bioactivity Of Recombinant Gm-Csf Aerosolised With A Highly Efficient Nebulizer, *Pulmonary Pharmacology & Therapeutics* (2010), doi: 10.1016/j.pupt.2010.08.004

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**PHYSICAL PROPERTIES, LUNG DEPOSITION MODELING, AND  
BIOACTIVITY OF RECOMBINANT GM-CSF AEROSOLISED WITH A  
HIGHLY EFFICIENT NEBULIZER**

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## Abstract

Pulmonary alveolar proteinosis (PAP) is a rare condition characterized by the accumulation of lipoproteinaceous material within air spaces. Although whole lung lavage is the current standard of care, recent advances in our understanding of PAP pathophysiology suggest that the disorder may benefit from inhalation of recombinant granulocyte-macrophage colony-stimulating factor (rGM-CSF). The aim of this study was to determine the physical properties and bioactivity of rGM-CSF aerosolized by the highly efficient AKITA<sup>2</sup> APIXNEB® nebulizer system. The physical properties of aerosolised rGM-CSF were investigated in terms of droplet size, output and output rate by laser diffraction and gravimetric analysis. Lung deposition was assessed using deposition modeling (ICRP). Molecular mass before and after aerosolisation was determined by SDS-PAGE, while the bioactivity of rGM-CSF was evaluated by measuring the GM-CSF-stimulated increase in pSTAT-5 using mAM-hGM-R cells. Ninety-six % of the rGM-CSF filling dose was aerosolized with the Akita<sup>2</sup> Apixneb® nebulizer system. Particle size was highly reproducible, and the amount deposited within the lung was 80.35% of the delivered dose. The aerosolisation did not alter the molecular structure of rGM-CSF, nor its ability to stimulate the pSTAT-5, which increased by 99.5%, similar to values for rGM-CSF prior to aerosolisation. We conclude that the highly efficient AKITA<sup>2</sup> APIXNEB® nebulizer system is likely to efficaciously deliver rGM-CSF to the airways of patients with autoimmune PAP.

## **Introduction**

Pulmonary alveolar proteinosis (PAP) is a rare condition characterized by the accumulation of lipoproteinaceous material within air spaces, and occurs in different clinical forms [1]. The presence of autoantibodies (Abs) neutralizing granulocyte macrophage colony-stimulating factor (GM-CSF) [2] and the reproduction of the pathologic disease manifestations by passive transfer of human Abs to primates [3] has permitted the reclassification of this condition to “autoimmune PAP”, the most frequent clinical form of PAP; previously it was referred to as “idiopathic” or “acquired” PAP [1]. These findings have opened the way to novel therapeutic scenarios.

The current standard of care for PAP is whole lung lavage (WLL), which results in the complete and durable resolution of PAP lung abnormalities in a percentage ranging from 20 to 70 of lavaged patients [4-5]. In addition, WLL is a highly invasive procedure, and even though complications are infrequent it requires prolonged anesthesia. The evidence that autoimmune PAP is caused by a disruption in GM-CSF signaling, has prompted investigations with recombinant GM-CSF (rGM-CSF) supplementation. A small series of PAP patients have been treated with rGM-CSF either subcutaneous (s.c.) or by inhalation, the overall response rate was slightly better by the latter route [6]. To date, no side effects, even for prolonged treatments, have been reported. Clearly, this therapeutic option, although very promising, requires further verification, in larger randomized trials

A clinical trial, aimed at maximizing the therapeutic outcome in PAP patients, has been recently approved [7]. The objective of the trial is to demonstrate the superiority of WLL followed by inhaled rGM-CSF vs WLL alone to obtain sustained resolution of lung abnormalities in autoimmune PAP patients. To optimize drug delivery, we decided to utilize the AKITA<sup>2</sup> APIXNEB® apparatus, a vibrating mesh nebulizer with controlled breathing and aerosol pulse technology. A series of experiments, preliminary to the trial, were therefore planned to investigate the particle size distribution and output. In addition, an important variable to be determined was the retention of rGM-CSF biological activity after aerosolisation. A series of experiments were therefore planned to investigate this aim. In this study we demonstrate the highly effective performance of the Akita<sup>2</sup> Apixneb® nebulizer system with the rGM-CSF formulation. In addition, aerosolisation does not alter the molecular mass or the biological activity of rGM-CSF.

## Methods

The rGM-CSF (Sargramostim, Leukine®) used in all experiments was a lyophilized preparation (250 µg/vial preservative-free powder) purchased from Bayer HC (Seattle, WA), and kept at 4 °C until needed, and then dissolved in 1 ml saline. In all experiments, the AKITA<sup>2</sup> APIXNEB® nebulizer handset (Activaero, Gemünden, Germany) was equipped with a Smart Card with the following configuration : inhalation volumes : 1500 ml; bolus width : 1250 ml; bolus depth : 1500 ml; flow rate : 15 L/min).

### Characterization studies

Unless specified otherwise, all experiments were conducted in triplicate.

- **Particle size distribution.** This method was used to characterize the droplet size distribution of the nebulized aerosol for different nebulizer systems. For the measurements, a laser diffraction particle sizer (Sympatec) was used. The aerosol was sent into a closed conditioned channel, where a laser beam illuminated the aerosol. When the laser light interacts with the aerosol particles the light is diffracted, reflected and refracted and a characteristic light spectrum is generated (fig.1). A multi-element sensor detected the light spectrum at different angles and the droplet size distribution was calculated by using Mie-theory. For each experiment, the nebulizer was filled with 250 µg rGM-CSF dissolved in 1 ml sterile saline. The measurements were conducted at room temperature, but the temperature of the measurement chamber was cooled to 18 °C. The droplet size distribution [x50 (VMD): *volume median diameter* (µm; the geometric particle diameter of an aerosol); GSD: *geometric standard deviation* (value describing the width of a lognormal particle size distribution); C<sub>opt</sub>: *Optical concentration* (%)] was evaluated by the Sympatec Windox4-Software by using the Mie-Theory.
- **Output and output rate.** With this method, total output for each nebulizer was measured. Delivered dose was determined by gravimetric analysis. Therefore, the nebulizer weight was measured with a precision balance before and after filling with a pipette. The continuous output

rate was calculated from the cumulative nebulization time per treatment which was recorded and noted, as well as the total amount of nebulized material.

- **Lung deposition modeling.** Lung dose was calculated using the International Commission for Radiological Protection (ICRP) Deposition Model [8-10] . The inhalation flow rate was 15 L/min for the AKITA<sup>2</sup> APIXNEB® nebulizer. For the deposition calculations, laser diffraction results were used.

### **Biological Activity**

For this set of experiments, rGM-CSF was aerosolized with the AKITA<sup>2</sup> APIXNEB® nebulizer, collected in a sterile glass bottle, kept on ice where it condensed; it was then harvested, divided into aliquots and kept frozen at -20°C until needed. A dissolved rGM-CSF sample, which was not aerosolized served as control.

- **Determination of the molecular mass.** The molecular mass of samples before and after aerosolisation was evaluated by submitting aliquots of rGM-CSF to SDS-polyacrilamide gel electrophoresis (SDS-PAGE).
- **Concentration of rGM-CSF pre- and post-aerosolization.** The rGM-CSF concentration in samples before and after aerosolisation was determined by a commercially available ELISA kit (R&D Systems)
- **Biological activity of rGM-CSF pre- and post-aerosolization.** The biological activity of rGM-CSF before and after aerosolisation was determined by measuring the GM-CSF-stimulated increase in phosphorylated signal transducer and activator of transcription 5 (pSTAT-5). This indicator of GM-CSF signaling utilizes mAM-hGM-R cells, which is a GM-CSF-deficient murine cell line (mAM cells) [11] retrovirally expressing functional human GM-CSF receptors. This novel genetically modified cell line exhibits cytokine-specific, dose-and time-dependent GM-CSF signaling responses. Briefly, aliquots of pre- and post- aerosolized rGM-CSF samples were diluted 1:25,000 and incubated with mAM-hGM-R cells for 15 min, after which cells were

lysed and evaluated by western blotting using the antibodies of pSTAT5 (Milipore) and STAT5 (SantaCruz) as described previously [12]. Phosphorylation of STAT5 was quantified by PathScan-STAT5(Y694) Sandwich ELISA Kit (Cell Signaling Technology). Phosphorylation of STAT5 by post-aerosolised samples was calculated as % phosphorylation by pre-aerosolised samples. GM-CSF samples of a known concentration were used as calibration standards [12].

## Results

**Particle size distribution, output and output rate.** Particle size distribution, analyzed by laser diffraction, showed a volume median diameter (VMD) of  $4.31 \mu\text{m} \pm 0.11$  (mean  $\pm$  SD; range 4.28 - 4.43  $\mu\text{m}$ ). The geometric standard deviation (GSD) of all measurements showed the mean value  $1.58 \pm 0.03$ . The output dose, determined by gravimetric analysis, was estimated as  $95.71\% \pm 0.018$  for a filling dose of 1 mL. Finally, the continuous output rate observed was 1.043 mL/min. These data are reported in detail in tables 1-3.

**Lung deposition modeling.** For the detected particle size and the specified breathing pattern, the estimated overall lung deposition in percentage of delivered dose was 80.35%. Interestingly, approximately 50% of the delivered dose was calculated to reach the alveolar spaces (table 4). Since, according to the dose calculation, the estimated lung deposition, from a starting volume of 250  $\mu\text{g}$ , was 192.3 $\mu\text{g}$ . We estimated that approximately 96  $\mu\text{g}$  of rGM-CSF would come into contact with the GM-CSF neutralizing Abs present in the alveolar spaces of the PAP subject.

**Biological activity.** In contrast with the output method, the collection system we used to harvest the post-aerosolization samples, yielded a mean recovery, in two different experiments, of  $0.625 \pm 0.020 \mu\text{l}$  from the initial 1 ml volume. Nevertheless, the concentration of rGM-CSF determined by ELISA in the post-aerosolisation samples was respectively 94.1 % and 95%, of the pre-aerosolisation samples. This would suggest that this method yielded a significant concentration in the aerosol volume, likely due to evaporation, with no rGM-CSF loss. The ELISA determination

substantially confirmed the output dose determination, described above. In addition, the SDS-PAGE experiments showed that the aerosolisation did not alter the structure of rGM-CSF. In fact, the three major bands corresponding to the rGM-CSF were identical in aspect and molecular mass before and after aerosolisation (figure 2). The three major rGM-CSF bands are consistent with the description provided by the manufacturer that Leukine® is “..characterised by 3 primary molecular species having molecular masses of 19,500, 16,800, and 15,500 daltons..” [13] . To evaluate GM-CSF bioactivity, mAM-hGM-R cells were incubated with Pre- or Post- aerosolized rGM-CSF samples after dilution 25,000 and were evaluated by detecting the phosphorylation of STAT5 (pSTAT5), which is one of the GM-CSF receptor signaling molecules. Western blotting of pSTAT5 showed the similar bioactivity in Pre- and Post- aerosolized rGM-CSF (Figure 3A). Quantification of pSTAT5 by ELISA demonstrated that the mean ( $\pm$  S.E.M.) increase in pSTAT5 in post-aerosolization aliquots was  $99.5\pm 4.1\%$  of pre-aerosolization ( $n=5$ ;  $P = 0.872$ ) (Figure 3B), indicating that aerosolization did not significantly alter GM-CSF bioactivity

## Discussion

The experiments described in this paper provide important information on nebulized GM-CSF. These data are essential for the design of a trial to provide local delivery of GM-CSF in patients with autoimmune PAP. In fact, GM-CSF supplementation in patients with PAP characterized by the presence of anti-GM-CSF Abs was first delivered s.c. with variable results. Over the last 14 years, approximately 50 PAP patients have been treated with rGM-CSF by this route [14-17] , with the following results: 1) improvement was observed in about 50% of patients; 2) a variable response among patients; 3) response depended on dose and duration of therapy, and 4) a lag of about eight weeks was necessary before a response was detected [6] . Since it is conceivable that the alveolar space is the site of GM-CSF signal disruption leading to the impairment of surfactant catabolism in

autoimmune PAP, then it is reasonable to propose that local GM-CSF supplementation would result in better treatment outcome. After a preliminary, but interesting report showing that aerosol delivery of GM-CSF in three PAP patients resulted in a decrease in GM-CSF neutralizing capacity in the lungs [18], a paper including 12 PAP patients treated with 250 mg *b.i.d.* aerosolized GM-CSF for varying time periods, showed a positive response in 91% of the subjects [19]. In a more recent, well designed Japanese study, including a run-in period, followed by an induction dose, and finally a maintenance dose, the response rate in 35 PAP patients was 68% [20]. Taken together, the above reported data seem to suggest the superiority of the inhalation route vs the subcutaneous one.

Interestingly, these results were achieved with an aerosol apparatus (LC Plus – PARI, Midlothian, VA), whose performance differs from that of the AKITA<sup>2</sup> APIXNEB® herein utilized. We therefore considered it a priority to perform a preliminary study on the characteristics of rGM-CSF aerosolized with the apparatus intended for use in the future clinical trial. This apparatus combines a vibrating mesh nebulizer with a computerized compressor to control both inhalation flow rate and inhaled volume [21]. In recent studies, the apparatus has been used to investigate the deposition of radiolabelled alpha1-antitrypsin (AAT) in healthy controls, and patients with either alpha1-antitrypsin deficiency or cystic fibrosis (CF) [22], and the effect of inhaled AAT on the inflammatory process in the airways of patients with CF [23]. Taken together, these studies suggest that the total lung deposition of AAT is approximately 70% of the filling dose, irrespective of the degree of lung function impairment, and that this resulted, in short term treatment, and a clear reduction in signs of airway inflammation in CF patients. The AKITA<sup>2</sup> APIXNEB® equipment has also been used to aerosolize all-trans retinoic acid in a single case of severe emphysema, with improvement in lung function and inflammatory parameters [24].

Data herein presented seem to support the concept that the AKITA<sup>2</sup> APIXNEB® is an apparatus capable of providing optimal delivery of rGM-CSF to the air spaces of PAP patients. In fact, the particle sizing experiments showed highly reproducible particle sizes for rGM-CSF, with a 0.2 µm maximum difference within three measurements. Such a low variation in particle size will not have a significant effect on drug delivery. The effective range of emitted dose was in the typical range for the nebulizer system used. The available dose at the mouthpiece was about 96% of the filling dose, thus suggesting that aerosol generation by the device is highly effective for the rGM-CSF formulation. Interestingly, the emitted dose, evaluated by gravitational analysis, was confirmed at the same rate by an ELISA test, in a different laboratory. Lung deposition modeling was performed to calculate the amount that will deposit within the patient's lungs. For deposition modeling the ICRP deposition model was selected, which is a semi empirical deposition model [8-10] . Calculated lung deposition values in percentage of delivered dose in this study showed results similar to those reported in the literature [10] . In particular the results achieved showed a highly effective lung deposition for the rGM-CSF formulation : assuming an exhaled rate of 7.63%, and an extrathoracic deposition of 12.02%, the lung deposition of the particles would account for 80.35% of the delivered dose, of which 30.48% is at the tracheo-bronchial level and 49.94 % at the alveolar level. If we assume that the delivered dose, from a filling dose of 250 µg, is 239 µg, then the dose of rGM-CSF directly delivered to the alveolar spaces would be 96 µg (table 4).

However, in spite of the above described encouraging results from physical properties and deposition modeling investigation, it must bear in mind that the presence *in vivo* of lipoproteinaceous material in the airspaces of patients with PAP might alter the GM-CSF deposition, thus making the expected performance less efficient.

A second important achievement of this study was the demonstration that not only the delivery pattern, but also the biological activity of rGM-CSF was maintained with the AKITA<sup>2</sup>

APIXNEBb® apparatus. We evidenced that, after aerosolisation, the rGM-CSF showed the same electrophoretic migration pattern as before aerosolisation (figure 2). The three major rGM-CSF bands, detected at 20,600, 17,800, and 16,500 daltons are consistent with the description provided by the manufacturer [13] .

More importantly, aerosolized rGM-CSF fully retained its biological activity after aerosolisation. In fact, the aerosolisation of the drug through AKITA<sup>2</sup> APIXNEB® did not alter either its molecular mass or its biological activity, which is almost fully retained, as suggested by the ability to activate the signal transduction in the cell line expressing the human GM-CSF receptor.

No comparative studies have been performed, to the best of our knowledge, on the performance of both the AKITA<sup>2</sup> APIXNEB® device and the Pari LC-plus nebulizer, the latter having been used to aerosolize rGM-CSF to PAP patients in the above discussed papers [18-20] . The only available paper compared the effects of inhaled tobramycin in cystic fibrosis patients using an older version of AKITA [25] . In this paper, it was shown that the AKITA significantly reduced the amount of drug required, the inhalation time, and the variability of dosage. Thus we speculate that the performance of AKITA<sup>2</sup> APIXNEB® should be at least not inferior.

We conclude that the highly efficacious AKITA<sup>2</sup> APIXNEB® nebulizer system, based on the effective *in vitro* performance and the complete preservation of the structure and bioactivity of the aerosolized drug, is likely to produce positive results when applied to the *in vivo* delivery of rGM-CSF to the airways of autoimmune PAP patients.

### **Authors contribution**

ML and BCT designed the study and drafted the paper; WCZ, BM, JG, PK and MF designed, performed, and collected data on the aerosolized rGM-CSF physical characterization (particle sizing, output rate and lung deposition modeling); TS, ZK, IC, FM, FF and GR designed, performed, and collected data on the bioactivity experiments.

### **Acknowledgements**

This study was supported by the AIFA (Italian Agency for Medicines) project FARM7MCPK4.

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## FIGURE LEGENDS

Figure 1 - Schematic laser diffraction setup

Figure 2 - 16 % SDS-PAGE (1 mm). Lane 1: Molecular markers. Lanes 2 and 4 : Pre-aerosolization sample. Lanes 3 and 5: Post-aerosolization sample. Denaturing Loading Buffer (LB) with SDS, not reducing. Lanes 2 and 3: two part of sample and one part of LB. Lanes 4 and 5: one part of sample and one part of LB. A total of 30  $\mu$ l was loaded in each well. Staining with Coomassie solution.

Figure 3 - A- Functional assay of rGM-CSF. mAMhGMR cells were incubated with different concentrations of native rGM-CSF (0,1; 1; 10 ng/ml, 15 min) and with rGM-CSF before (1~5) and after (6~10) aerosolization through AKITA<sup>2</sup> APIXNEB<sup>®</sup>. Western analysis was then performed to detect phosphorylated STAT5 (pSTAT5) and total STAT5 (STAT5).

B- rGM-CSF signaling measured by pSTAT5 ELISA, before (N=5) and after (N=5) aerosolization through AKITA<sup>2</sup> APIXNEB<sup>®</sup>.

**Table 1- Results of particle sizing experiments**

Measurement No.	Nebulizer No.	Filling Volume [mL]	VMD [ $\mu\text{m}$ ]	GSD	$C_{opt}$ [%]	VMD Mean Value [ $\mu\text{m}$ ]	VMD SD	GSD Mean Value	GSD SD
1.1	GMP-01	1	4.4	1.6	14.				
1.2	GMP-01	1	4.2	1.5	17.	4.31	0.1	1.58	0.0
1.3	GMP-01	1	4.28	1.57	17.13				

**Table 2- Output measurement results**

Measurement No.	Nebulizer No.	Filling Vol [mL]	Emitted dose [mL]	Mean Emitted dose [mL]	SD	Emitted dose [% of filling]	Mean Emitted dose	SD
2.1	GMP-01	1	0.95			97.7%		
2.2	GMP-01	1	0.95	0.956	0.073	95.4%	95.71%	0.018
2.5	GMP-01	1	0.96			94.1%		

**Table 3 - Calculated continuous output rate**

Measurement No.	Nebulizer No.	Filling Vol [mL]	Emitted dose [mL]	No. of breaths	Nebulization time per breath [s]	Calculated output rate [mL/min]	Mean output rate [mL/min]	SD
2.1	GMP-01	1	0.95	11	5	1.041	1.043	0.008
2.2	GMP-01	1	0.95	11	5	1.037		
2.3	GMP-01	1	0.96	11	5	1.052		

**Table 4- Results from lung deposition modeling in % of delivered dose and deposited drug amount**

<b>AKITA<sup>2</sup> APIXNEB</b>		
<b>Regional Deposition of Particles</b>		
Extrathoracic Deposition	%	12.02
Tracheobronchial Deposition	%	11.09
Bronchial Deposition	%	19.32
Alveolar Deposition	%	49.94
Exhaled	%	7.63
<b>Lung Deposition</b>		
(=Tracheal + Bronchial + Alveolar)	%	<b>80.35</b>
<b>Dose Calculation</b>		
Filled Dose	$\mu\text{g}$	250 $\mu\text{g}$
Delivered Dose	$\mu\text{g}$	239 $\mu\text{g}$
<b>Lung Deposition</b>	<b><math>\mu\text{g}</math></b>	
<b>(no evaporation assumed)</b>		<b>192.3 <math>\mu\text{g}</math></b>

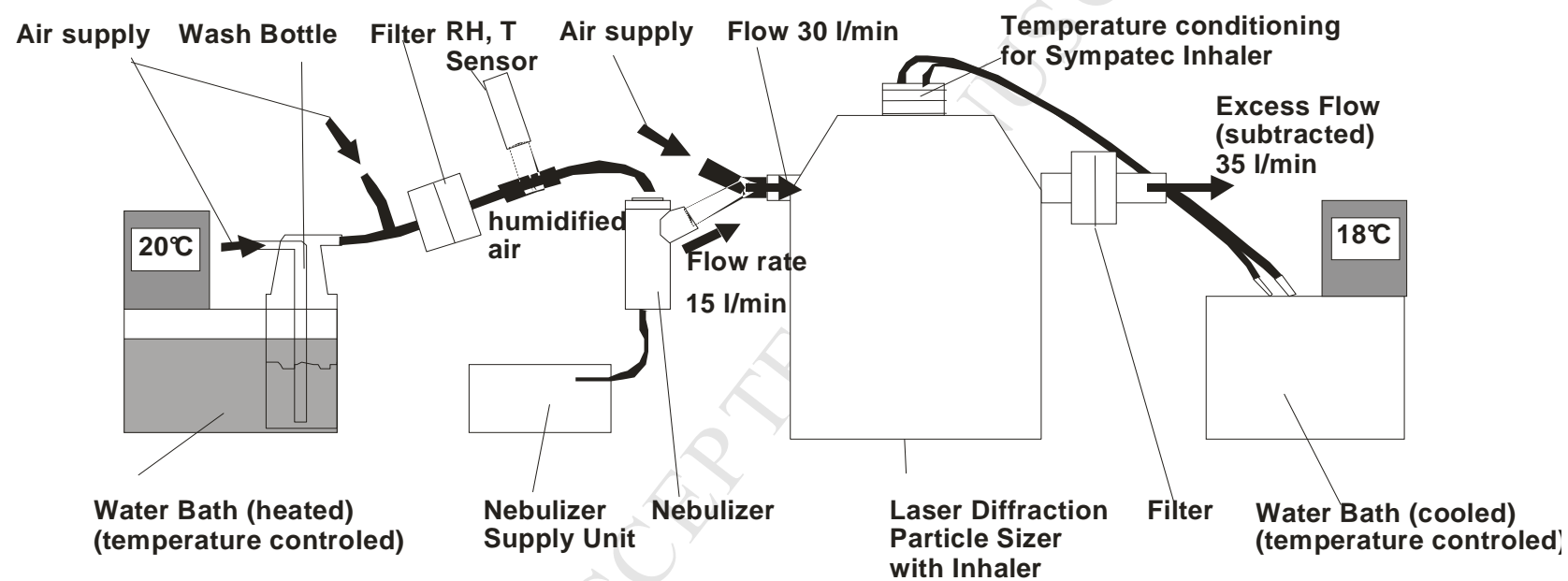
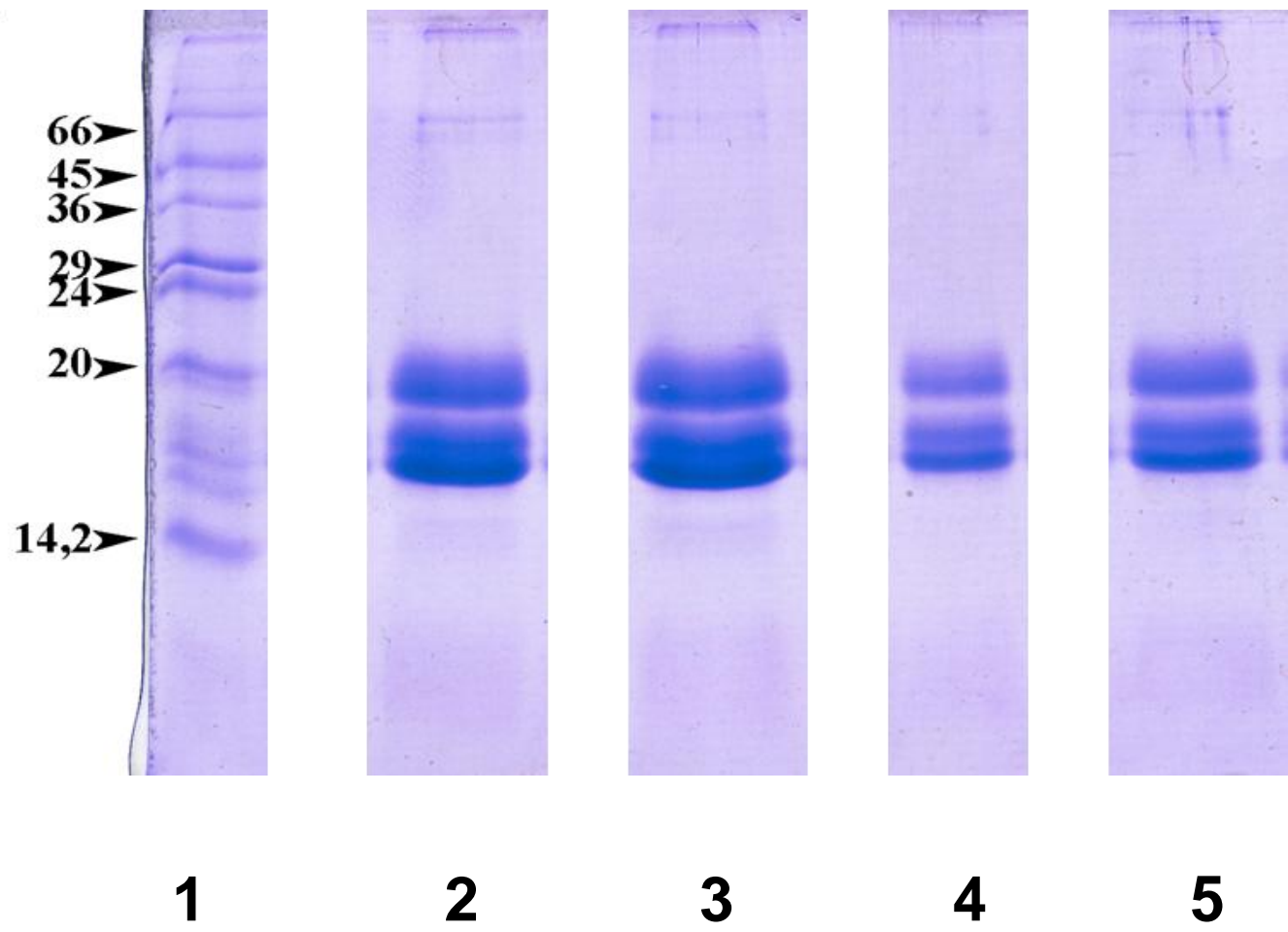
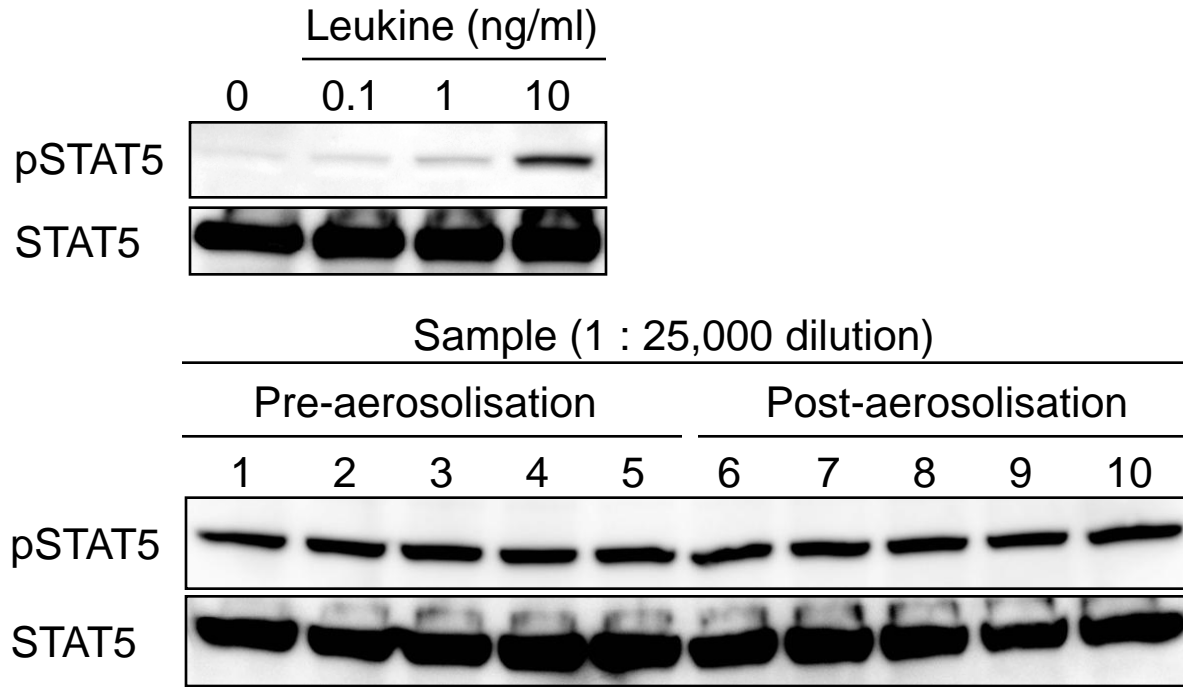


Figure 1B



# Functions of GM-CSF samples

A



B

