Cytokine 123 (2019) 154768

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.elsevier.com/locate/cytokine

Variability of cytokine concentration in whole blood serum and bronchoalveolar lavage over time

Alan Valaperti[a,1], Pascal Bezel[b,1], Maya Vonow-Eisenring[a], Daniel Franzen[b], Urs C. Steiner[a,*]

[a] Department of Clinical Immunology, University Hospital Zurich, Gloriastrasse 23, 8091 Zurich, Switzerland
[b] Division of Pulmonology, University Hospital Zurich, Raemistrasse 100, 8091 Zurich, Switzerland

ARTICLE INFO

Keywords:
Cytokines
Pre-analytics
Interleukin-8
Serum
Bronchoalveolar lavage fluid (BALF)

ABSTRACT

Measurement of cytokines in peripheral blood and bronchoalveolar lavage fluid (BALF) is a useful method to assess human immune responses in a large range of pulmonary diseases. One of the major pre-analytical challenges of cytokine analysis is the quality and stability of cytokines in the timeframe between sample collection and the separation of supernatant from cells.

To evaluate if the method of storage may affect cytokine quantification, whole blood and BALF were collected, aliquoted, and left at room temperature (RT) to be processed at different time points. In addition, sera and BALF were left either at RT or at 4 °C for 24 h after cell separation to test cytokine variations in the absence of cells. Samples were analysed by a multiple array containing ten cytokines.

Most of the cytokines analysed (interleukin (IL)-4, IL-5, IL-6, IL-12p70, IL-13, IL-17A, IL-23, interferon (IFN)-γ, and tumour necrosis factor (TNF)-α) did not show significant variations in whole blood and BALF. Levels of IL-8 however, increased after storage of whole blood and BALF for 24 h at RT. *Ex vivo* IL-8 production seems to correlate with higher numbers of macrophages in collected BALF.

These data demonstrate that many cytokines are stable for a brief time after sample collection. For IL-8, freshly collected whole blood and BALF should be quickly processed and frozen to avoid false positive results.

1. Introduction

Cytokines are immunomodulatory proteins produced by immune cells, endothelial cells, or stromal cells. They regulate cell interaction as well as cell growth and maturation in health and disease [1,2]. Cytokines are involved in the effector phase of all inflammatory diseases and can exert their biological function already at low concentrations [3–6]. Imbalance in the cytokine regulatory system can lead to chronic inflammation as seen in auto-immune and auto-inflammatory diseases or in cancer [7–10].

Cytokines can be specific for certain disease entities, and they also can serve both as biomarkers for disease severity and as targets for therapy [3,11–18]. Cytokines measured in whole blood often do not reflect the degree and type of inflammation in a specific organ and thus the extent of local inflammation can be underestimated or misinterpreted. The local sample collection of an affected organ represents the best option to characterise inflammation [19–24]. Bronchoalveolar lavage (BAL) is a common method used to collect BALF, which is then used to locally measure cytokines in the lungs of patients with various lung diseases [11,12,15,25,26]. However, proper pre-analytical conditions are crucial, and thus it is important to figure out if there could be potential pre-analytical issues that would reduce the reliability of cytokine analysis.

The method of sample collection, the anticoagulant used, storage conditions, transportation, and the time between collection and measurement are relevant pre-analytical aspects that determine the validity of the final results. The major issue raised is the variability of cytokine concentrations over time, from sample collection to analysis [27–37]. It is mandatory to preserve and handle samples using procedures that minimise cytokine variations [29]. Therefore it is necessary to have standardised protocols for all various types of samples used for cytokine analysis including plasma, serum, urine, saliva, synovial fluid, cerebrospinal fluid, bronchoalveolar fluid, eye fluid, intestinal fluid, exhaled breath condensates, middle ear effusion or lysed biopsies of a diseased organ.

In this paper, we analysed the variability of cytokine concentrations over time in whole blood and in BALF before and after cell separation with the aim of establishing a protocol that reflects the best conditions

* Corresponding author.
E-mail address: urs.steiner@usz.ch (U.C. Steiner).
[1] Shared first authorship.

https://doi.org/10.1016/j.cyto.2019.154768
Received 17 December 2018; Received in revised form 28 May 2019; Accepted 25 June 2019
Available online 02 July 2019

for reliable cytokine measurement. To our knowledge, there are no studies on cytokine stability in BALF after different storage conditions.

2. Materials and methods

2.1. Patient selection

We included a total of twelve patients with different lung diseases, including pulmonary sarcoidosis, interstitial lung disease (ILD), or adenocarcinoma, who underwent routinely performed flexible bronchoscopy (FB) and BALF at the University Hospital Zurich. Exclusion criteria included patient vulnerability, such as pregnancy and emergency conditions, denied informed consent, and time delay between BALF collection and the first sample handing in the laboratory. The local ethical review committee approved this study (numbers 2017-02307 and 2018-01724).

2.2. Blood specimens

Blood samples were drawn by a nurse with considerable experience in phlebology as part of the routinely performed pre-interventional peripheral vein access right before BALF collection. Whole blood was collected into 10-ml BD Vacutainer Clot Activator Tube (CAT, Plus Blood Collection Tubes, Becton Dickinson, Plymouth, UK) to obtain serum after spinning them down, while for whole blood count and differentiation, blood was collected in 10-ml BD Vacutainer K2E (EDTA) tubes (Plus Blood Collection Tubes, Becton Dickinson, Plymouth, UK), which were never spun down.

After collection, whole blood was separated into 5 aliquots of 1 ml each and left unprocessed at RT until later processing. The first aliquot was processed exactly one hour after collection (baseline), the following aliquots were processed after three hours, six hours, and 24 h. Whole blood was centrifuged at 3′500 rpm for 10 min at RT. Supernatants were collected and stored at −80 °C for later analysis. To check for stability of cytokines in the absence of cells, additional whole blood samples were stored at RT for one hour and then centrifuged as previously described. Supernatants were collected, left for 24 h at RT or at 4 °C, and then stored at −80 °C for later analysis. Cytokine measurement was always performed with a new aliquot. After thawing, aliquots were not frozen again for further analysis. Blood leukocyte differentiation in whole blood was measured and carried out automatically with the ADVIA 2110i (Siemens). The ADVIA 2120i differentiates white blood cells via peroxidase and nuclear density displays (cytogramm) based on their morphology.

2.3. Bronchoscopy-/BAL-technique and processing of BALF

All patients underwent FB using Olympus bronchoscopes under moderate sedation with propofol and alfentanil. BALF was performed according to the guidelines published by Rennard and colleagues [38] and standardised by following the procedure suggested by Baughman by injecting four portions of 50 ml (total of 200 ml) of isotonic saline solution into the wedged segmental bronchus, leading to the target lesion with the most prominent radiological finding [39]. Thereafter, BALF was recovered by gentle suctioning with the same 50 ml syringe and collected in a graduated plastic cylinder. Approximately 50 ml of BALF was used for routinely performed cytological and microbiological analyses, the remaining fluid was used for the purposes of this study. BALF was collected in regular plastic tubes without preservatives, anticoagulants, or clot activators and was separated into 5 aliquots of 1 ml each and left unprocessed at RT until later processing. The first aliquot was processed exactly one hour after collection (baseline), the following aliquots were processed after three hours, six hours, and 24 h. BALF was centrifuged at 1′000 rpm for 10 min at RT. Supernatants were collected and stored at −80 °C for later analysis. To check for stability of cytokines in the absence of cells, additional BALF samples were stored at RT for one hour and then centrifuged as previously described. Supernatants were collected, left for 24 h at RT or at 4 °C, and then stored at −80 °C for later analysis. Cytokine measurement was always performed with a new aliquot. After thawing, aliquots were not frozen again for further analysis.

ADVIA 2120i (Siemens) was used for the automatic leukocyte measurements in BALF. The ADVIA 2120i differentiates cells via peroxidase and nuclear density displays based on their morphology. It was able to recognize lymphocytes, macrophages, neutrophils, and eosinophils in BALF.

2.4. Cytokine analysis

Cytokines were analysed by a cytokine multiple array on a Luminex 200 platform (Luminex Corporation, Austin, TX) with a high sensitive Milliplex kit (HSTCMAG-28SK-10) customized by Merk Millipore, containing the following ten human cytokines: IL-4, IL-5, IL-6, IL-8, IL-12p70, IL-13, IL-17A, IL-23, IFN-γ, and TNF-α. This array includes cytokines that promote T helper (Th) cell differentiation or cytokines produced by differentiated Th cell subpopulations. IL-12p70 and IFN-γ are related to Th1 cells, IL-4, IL-5, and IL-13 to Th2 cells, and IL-6, IL-17A and IL-23 to Th17 cells [1]. Monocytes/macrophages and activated T cells are the major producers of TNF-α [40], while IL-8, also called CXCL8, is released by a wide variety of immune and non-immune cells to predominantly mobilise neutrophils [41]. With the Milliplex kit, each stock standard was serially diluted 1:4 to produce seven standards (Suppl. Table 1). The xPONENT software generated a five-parameter logistic curve-fit standard curve to measure unknown samples.

The high sensitive Milliplex kit was validated before measuring cytokines in serum and BALF. The acceptance criteria, which included the % coefficient of variation (CV) of the intra-assay, the %CV of the inter-assay, the low limit of detection, the low limit of quantification, the linearity, and the %CV of replicates of low concentrated BALF samples, were fixed before validation. All acceptance criteria were fulfilled, as shown in Suppl. Table 2 and Suppl. Fig. 1.

Since, so far, very few companies calibrate their cytokine kits against an internationally recognized standard, we used the National Institute for Biological Standards and Control (NIBSC; Potters Bar, Hertfordshire, UK; WHO International Laboratory for Biological Standards) standards to evaluate the accuracy of the Milliplex kit. Although the NIBSC guidelines provided an approximate cytokine amount contained in each ampoule, we used these values as starting concentration when spiking NIBSC standards into serum of healthy donors to perform serial dilutions and check for linearity and reproducibility of the Milliplex kit (Suppl. Fig. 1). A conversion rate relative to the NIBSC standard for each cytokine tested (except for IL-5 and IL-23) was calculated in the linear range of the assay and is shown in Suppl. Table 2.

2.5. Statistical analysis

Measured cytokine concentrations were plotted over time. Means were expressed as percentages of concentrations of the control samples, which corresponded to the sample centrifuged one hour after collection and immediately frozen at −80 °C. Concentrations in pg/ml were also shown in supplementary figures. Generalized estimating equations (GEE) were used to address the repeated measurements over varying storage times. The correlation structure was specified to be “exchangeable”. To reduce variability, the one hour measurement was set to 100 percent (baseline) and all subsequent measurements were calculated as percentage change from baseline. The baseline measurement was not included in the GEE analysis, because all values were 100%, without variability. The interpretation of the estimated coefficients in GEE is marginal in the sense that population averages are provided. All analyses were conducted with RStudio and plotted with Prism (GraphPad Software Inc., Version 5.0). Data are reported as

Table 1
Cytokine stability in serum and BALF processed at different time points and stored at 4 °C or at RT. Concentrations are represented in pg/ml.

Mean values in pg/ml	Cytokines processed at different time points and stored at RT				Cytokines processed after 1 h and stored at 4 °C or RT	
Serum	1 h	3 h	6 h	24 h	1 h + 24 h 4 °C	1 h + 24 h RT
IL-4	85.04	94.87	97.00	98.88	99.74	98.27
IL-5	1.08	1.09	1.28	1.36	1.21	1.22
IL-6	8.30	9.43	9.87	9.69	9.79	9.25
IL-8	16.01	20.40	34.20	416.28	15.80	16.18
IL-12	4.27	4.50	4.75	4.88	4.32	4.68
IL-13	9.59	9.82	10.65	9.91	9.60	10.47
IL-17A	9.39	13.50	13.73	13.34	12.01	12.83
IL-23	787.83	838.25	940.41	884.49	816.89	842.91
IFN-γ	12.80	15.60	15.47	15.53	13.86	15.67
TNF-α	14.64	15.63	18.20	17.49	16.00	15.04
BALF	1 h	3 h	6 h	24 h	1 h + 24 h 4 °C	1 h + 24 h RT
IL-6	4.48	3.72	3.27	4.38	3.45	4.06
IL-8	63.78	57.79	82.24	124.02	79.25	86.72
IFN-γ	0.66	0.48	0.38	0.45	0.41	0.39
TNF-α	0.48	0.69	0.71	0.72	0.62	0.75

mean ± standard error of mean (SEM). Differences were considered to be statistically significant for $p < 0.05$.

3. Results

3.1. Cytokine stability in whole blood samples stored at RT

The data generated in pg/ml were analysed in terms of percentage change from the control samples which were whole blood samples separated one hour after blood drawing and immediately frozen at −80 °C. As shown in Table 1 and Fig. 1, there is a general cytokine increase in whole blood after three and six hours of storage at RT. Compared to the control samples, the concentrations of IL-12p70, IL-13, IL-23, IFN-γ, and TNF-α were between 10% and 30% higher, while IL-4, IL-5, IL-6, and IL-17A showed an even higher increase of 40% to 80%. However, due to the large standard deviation of the measured samples, all these variations were not statistically significant.

Interestingly, IL-8 was also increased after three and six hours of storage at RT when compared to the control samples, although not significantly. However, the marked increase of IL-8 after 24 h at RT was statistically significant, with a 350-, 30-, and 15-fold increases compared to controls (one hour at RT), to three hours at RT, and to six hours at RT, respectively (Fig. 1 and Table 1).

3.2. Cytokine stability in BALF samples stored at RT

Similar to whole blood samples, BALF samples were analysed in pg/ml and in terms of percentage change from the control samples. Analysis from BALF was performed out of the 50–100 ml collected aspirate of the four injected portions of 50 ml of isotonic saline solution into the wedged segmental bronchi. We were able to measure levels of IL-6, IL-8, IFN-γ, and TNF-α, while all other cytokines were under the detection limit of our assay. Although IL-6 and IFN-γ were reduced after six and 24 h of storage at RT, due to the high standard deviation, these variations were not significant (Fig. 2 and Table 1). On the contrary, IL-8 showed an almost two fold increase after 24 h storage when compared to all the other storage conditions and to the control samples (Fig. 2 and Table 1).

3.3. Cytokine stability in serum samples stored at RT and at 4 °C after cell separation

For the evaluation of serum after blood cell separation, samples were centrifuged one hour after storage at RT. The first aliquot was immediately stored at −80 °C (control samples), the second and the third aliquots were left at RT or stored 4 °C for 24 h, respectively, before storing both at −80 °C. Concentrations of all ten cytokines did not significantly change when compared to the control group. Cytokine values were similar after 24 h storage at both RT and 4 °C (Fig. 3 and Table 1).

3.4. Cytokine stability in BALF samples stored at RT and at 4 °C after cell separation

For the evaluation of BALF after cell separation, samples were processed as described in the materials and methods section. Although IL-6 and IFN-γ concentrations were decreased after 24 h storage, and TNF-α was increased after 24 h storage at 4 °C, none of the measured differences were statistically significant. In addition, IL-8 concentrations did not show any variation after 24 h storage (Fig. 4 and Table 1).

3.5. Correlation between IL-8 and the initial number of macrophages in BALF

Significant variations have been observed for IL-8 in BALF upon 24 h storage at RT. Therefore, we aimed to investigate if the prevalence of a particular leukocyte subpopulation would affect the *ex vivo* production of IL-8. Macrophages were measured immediately after BALF collection and then compared to the absolute concentrations of IL-8 at the basic level, after storage at RT for one hour, and after storage at RT for 24 h before cell separation. No significant correlation was measured at the basic level, while BALF stored for 24 h showed significant correlations between IL-8 and macrophages (Fig. 5).

4. Discussion

In this study we compared the concentration of ten different pro-inflammatory cytokines in whole blood and BALF over time and upon different storage conditions in patients with pulmonary diseases. In whole blood serum, nine out of ten cytokines (IL-4, IL-5, IL-6, IL-12p70, IL-13, IL-17A, IL-23, IFN-γ, and TNF-α) showed increased concentrations upon storage at RT for 24 h before cell separation, although these trends were not statistically significant. Interleukin-8 showed a significant increase before cell separation when samples were stored at RT.

Variability of cytokines in blood samples collected and stored at different conditions has already been demonstrated for individual cytokines with controversial results. In one study conducted using whole blood from healthy donors collected in ethylenediamine tetraacetic acid (EDTA) tubes, cytokines increased over two hours at RT with or without added protease inhibitors [29]. In a similar trial, with whole blood in EDTA tubes, TNF-α increased and IL-6 decreased within six hours [28]. A further study demonstrated that cytokines remained stable in whole blood collected in EDTA tubes from healthy donors, upon storage for three days at RT, however, in patients with an activated immune system, most cytokines increased over time [30]. This diversity is most probably explained by different cellular components of blood samples, which both release and clear cytokines [42]. Cytokine measurement is more elevated if the immune system is activated. All of our patients suffered from a lung disorder with immune-activation. This could explain that in all of our patients, cytokines in serum were slightly increased over time (Fig. 1 and Suppl. Fig. 2). In several studies, no difference in cytokine variability was found if whole blood was collected in EDTA-, sodium heparin- or sodium citrate- tubes [33,43]. However, some data indicated that cytokine concentrations in serum generally increased more than in plasma with anticoagulants, most probably due

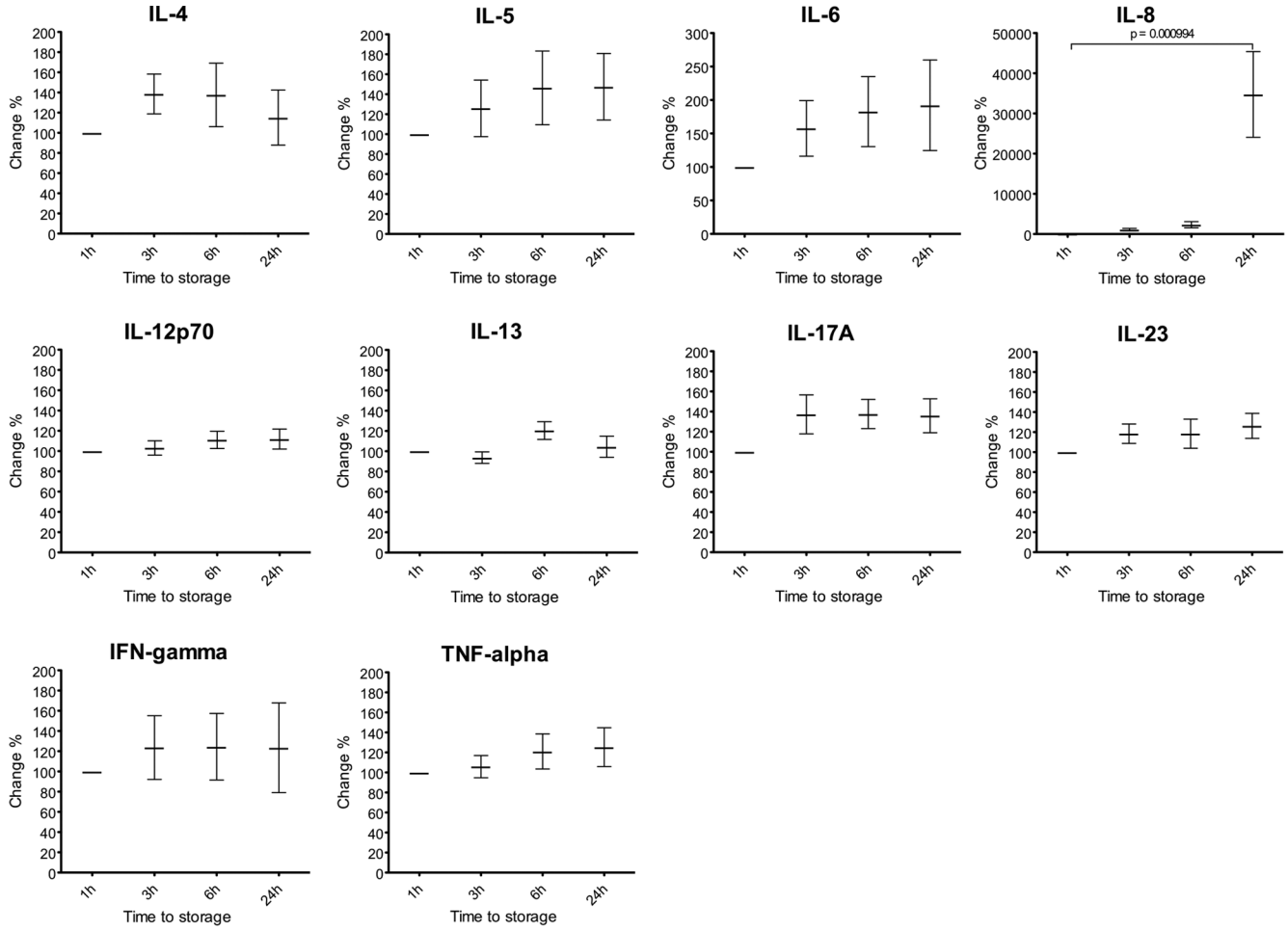

Fig. 1. Whole blood collected from patients (n = 12) with lung diseases. Blood was collected in serum clot activator tubes (CAT) and left unprocessed for 1, 3, 6, and 24 h at RT. After centrifugation, samples were collected in several aliquots and then immediately stored at −80 °C. Data are reported as mean ± standard error of mean (SEM).

to cytokine release from cells during clotting [27,34]. Compared to the other cytokines, IL-8 had the largest increase after 24 h storage at RT before cell separation. This has also been shown in previous studies conducted with whole blood collected from patients with systemic inflammation or from healthy donors upon storage at RT for 24 and 48 h [27,30,31,33]. Activation of monocytes and neutrophils, or dissociation from the surface of erythrocytes and leukocytes, may account for the increases in IL-8 in unprocessed blood samples [43]. In line with the current literature, we can document that after cell separation within one hour, cytokine concentrations remained stable, however, IL-8 constantly increased over time, although not reaching statistical significance. Compared to storage conditions after cell separation, our data demonstrate that cytokines are more stable at 4 °C compared to storage at RT (Fig. 3).

In BALF samples stored at RT before and after cell separation, only four out of ten cytokines in our array were detectable. By injecting four portions of 50 ml of isotonic saline solution into a lung segment, the cytokines in collected lavage fluid were diluted, and this is probably why many of them fell under the lowest detection limit of our assays. For future measurements, cytokines should be analyzed out of each injection of 50 ml BALF. Measurable pro-inflammatory cytokines IL-6, IFN-γ and TNF-α only showed minimal changes over time. However, IL-8 in BALF significantly increased over 24 h (Fig. 2). Interestingly, this *ex vivo* increase was correlated with a higher number of macrophages in

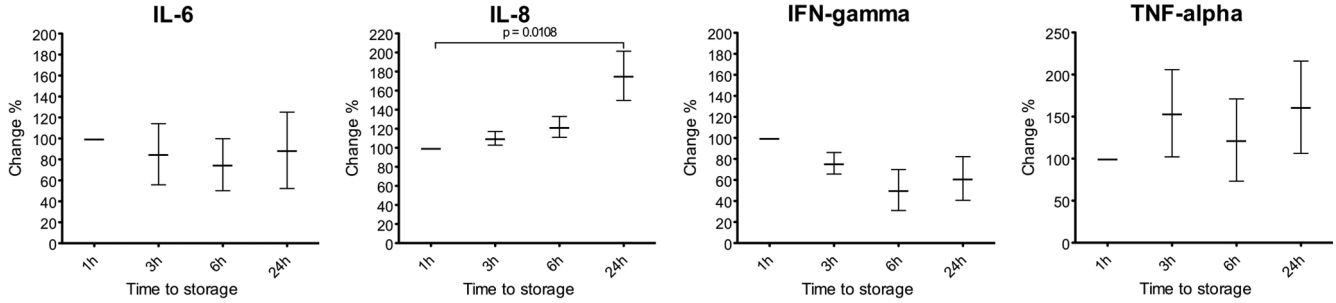

Fig. 2. BALF collected from patients (n = 12) with lung diseases. BALF was left unprocessed for 1, 3, 6, and 24 h at RT. After centrifugation, samples were collected in several aliquots and then immediately stored at −80 °C. IL-8 was measurable in all BALF, IL-6 was measurable in four BALF, IFN-γ and TNF-α were measurable in three BALF. Data are reported as mean ± standard error of mean (SEM).

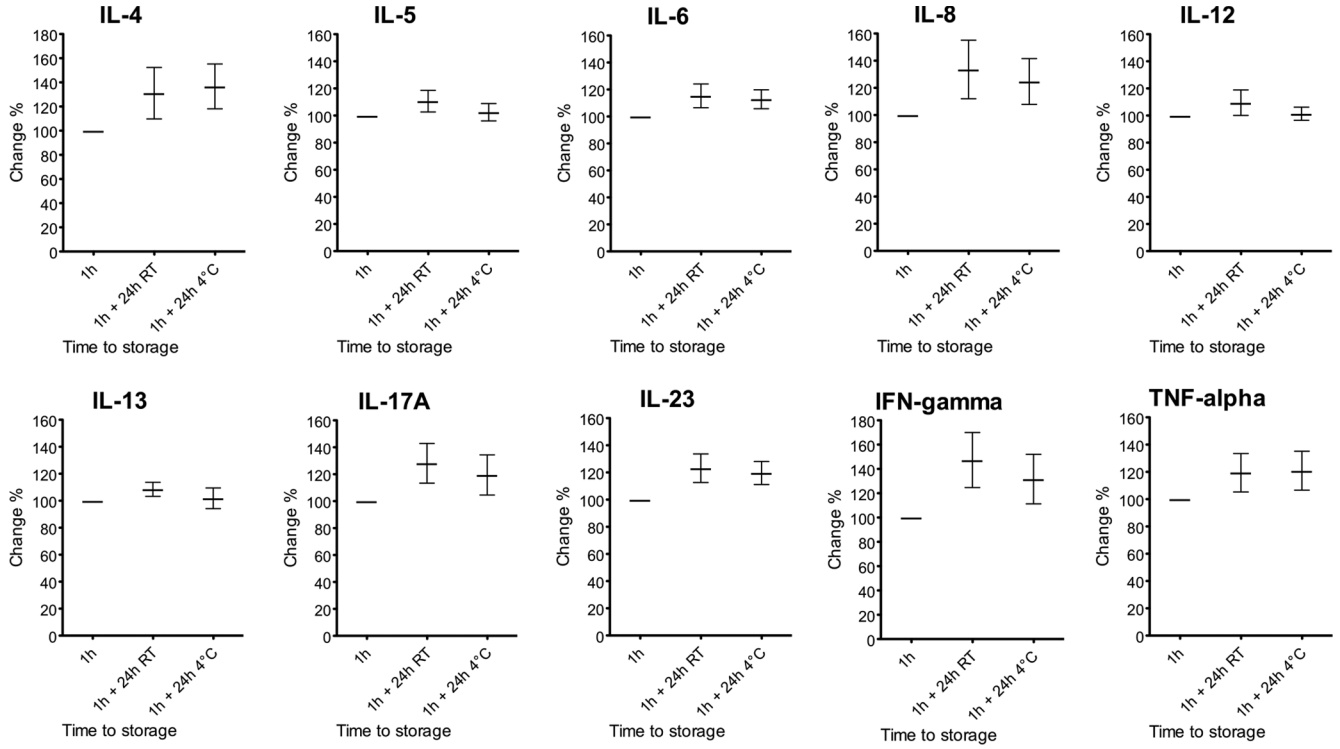

Fig. 3. Whole blood collected from patients (n = 12) with lung diseases. Blood was collected in serum clot activator tubes (CAT) and left unprocessed for 1 h at RT. After centrifugation, control samples were immediately stored at −80 °C, while one group was left for 24 h at RT and another group was left for 24 h at 4 °C. Both groups were then stored at −80 °C. Data are reported as mean ± standard error of mean (SEM).

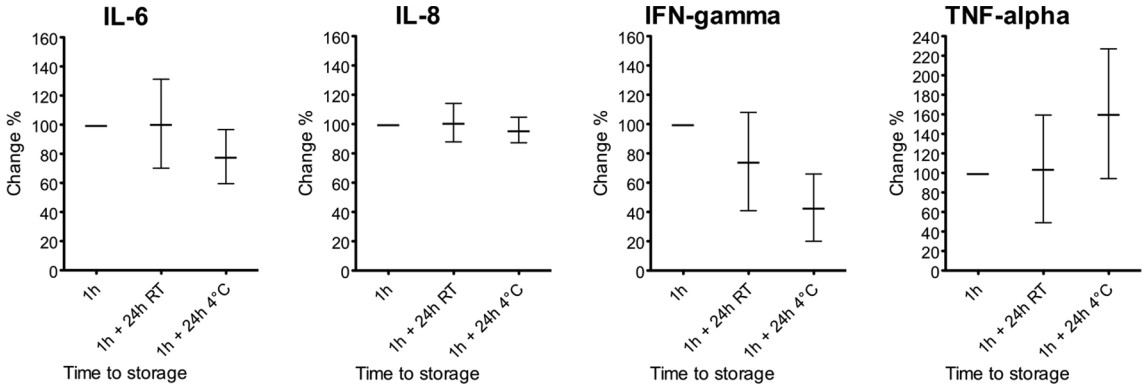

Fig. 4. BALF collected from patients (n = 12) with lung diseases. BALF was left unprocessed for 1 h at RT. After centrifugation, control samples were immediately stored at −80 °C, while one group was left for 24 h at RT and another group was left for 24 h at 4 °C. Both groups were then stored at −80 °C. IL-6, IFN-γ and TNF-α were measurable in four BALF samples. IL-8 was measurable in all BALF. Data are reported as mean ± standard error of mean (SEM).

BALF (Fig. 5). Compared to BALF, the increase of IL-8 did not correlate with the number of any leukocyte subpopulation in whole blood serum. Functional differences between alveolar macrophages and blood monocytes have been described and may be related to their different maturation stage [44]. In addition, it is known that alveolar macrophages are one of the major sources of IL-8 in the lung [45,46]. The correlation between macrophages and IL-8 was observed only after 24 h at RT, but not right after BALF collection. Therefore, it is necessary to quickly process BALF samples right after collection to remove macrophages.

To our knowledge, this is the first study that investigated the concentration and variability of cytokines in whole blood serum and BALF of patients with a pulmonary disorder over time. A downside of this study is the small number of patients and that in BALF only four out of 10 cytokines were measurable. According the small numbers and the low detectability of cytokines in this study, it was not possible to compare cytokine values in serum and BALF in the same patients. Nevertheless, as the aim of this study was to investigate pre-analytical procedures of cytokine measurements in serum and BALF, these issues should be addressed in further studies.

In conclusion, according to the current literature and the results of our study, the most reliable data in cytokine measurements are achieved by processing samples within one hour. When samples are processed within one hour the choice of tubes in collecting whole blood does not matter. After cell separation samples should be stored immediately at −80°, if this is not possible, should be stored at 4 °C. IL-8 in BALF is clearly associated with the amount of macrophages, whereas in serum this correlation was not detected.

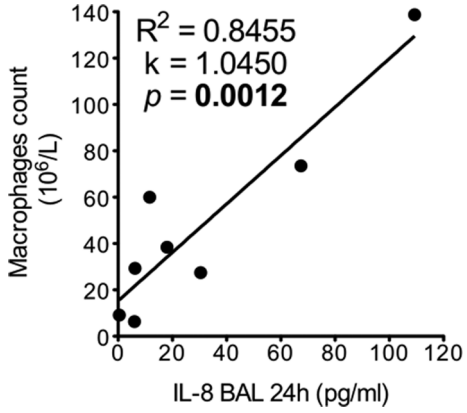

Fig. 5. Correlation between IL-8 and macrophages measured in BALF. BALF was left unprocessed for 24 h at RT. After centrifugation, control samples were immediately stored at −80 °C. The y-axis represents the macrophages count in millions (10^6) per liter (10^6/L), the x-axis represents the measured IL-8 concentration in BALF reported in pg/ml. Linear regression analysis was performed to calculate the goodness of fit (R^2), the slope of the correlation (k), and the p value ($p = 0.0012$), according to [47].

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

We acknowledge the support of the project financing fund, Department of Clinical Immunology, University Hospital Zurich. We are grateful to Catherine Crowley-Kühn for reading and editing the manuscript.

Disclosures

The authors declare that they have no competing interest regarding this study.

Authors' contributions

Study design (US, AV, PB, MV, DF), samples collection (PB, DF), samples measurement (AV, PB), data analysis (AV, PB), and writing of the manuscript (AV, PB, US). All authors approved the final version of the manuscript.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cyto.2019.154768.

References

[1] M. Akdis, A. Aab, C. Altunbulakli, K. Azkur, R.A. Costa, R. Crameri, et al., Interleukins (from IL-1 to IL-38), interferons, transforming growth factor β, and TNF-α: Receptors, functions, and roles in diseases, J. Allergy Clin. Immunol. 138 (2016) 984–1010, https://doi.org/10.1016/j.jaci.2016.06.033.
[2] C.A. Dinarello, Historical insights into cytokines, Eur. J. Immunol. 37 (2007) S34–S45, https://doi.org/10.1002/eji.200737772.
[3] M.D. Turner, B. Nedjai, T. Hurst, D.J. Pennington, Cytokines and chemokines: at the crossroads of cell signalling and inflammatory disease, Biochim. Biophys. Acta 2014 (1843) 2563–2582, https://doi.org/10.1016/j.bbamcr.2014.05.014.
[4] J.A. Stenken, A.J. Poschenrieder, Bioanalytical chemistry of cytokines – A review, Aca 853 (2015) 95–115, https://doi.org/10.1016/j.aca.2014.10.009.
[5] O. Takeuchi, S. Akira, Pattern recognition receptors and inflammation, Cell 140 (2010) 805–820, https://doi.org/10.1016/j.cell.2010.01.022.
[6] A. Valaperti, Drugs targeting the canonical NF-κB pathway to treat viral and autoimmune myocarditis, Curr. Pharm. Des. 22 (2016) 440–449, https://doi.org/10.2174/1381612822666151222160409.
[7] Q. Guan, J. Zhang, Recent advances: the imbalance of cytokines in the pathogenesis of inflammatory bowel disease, Med. Inflamm. 2017 (2017) 4810258, https://doi.org/10.1155/2017/4810258.
[8] K.D. Moudgil, D. Choubey, Cytokines in autoimmunity: role in induction, regulation, and treatment, J. Interferon Cytokine Res. 31 (2011) 695–703, https://doi.org/10.1089/jir.2011.0065.
[9] Z. Culig, Cytokine disbalance in common human cancers, Biochim. Biophys. Acta 2011 (1813) 308–314, https://doi.org/10.1016/j.bbamcr.2010.12.010.
[10] B. Burkholder, R.-Y. Huang, R. Burgess, S. Luo, V.S. Jones, W. Zhang, et al., Tumor-induced perturbations of cytokines and immune cell networks, Biochim. Biophys. Acta 2014 (1845) 182–201, https://doi.org/10.1016/j.bbcan.2014.01.004.
[11] C. Thi Hong Nguyen, N. Kambe, I. Kishimoto, I. Ueda-Hayakawa, H. Okamoto, Serum soluble interleukin-2 receptor level is more sensitive than angiotensin-converting enzyme or lysozyme for diagnosis of sarcoidosis and may be a marker of multiple organ involvement, J. Dermatol. 44 (2017) 789–797, https://doi.org/10.1111/1346-8138.13792.
[12] S.E. Verleden, A. Martens, S. Ordies, A.P. Neyrinck, D.E. Van Raemdonck, G.M. Verleden, et al., Immediate post-operative broncho-alveolar lavage IL-6 and IL-8 are associated with early outcomes after lung transplantation, Clin. Transplant. 32 (2018) e13219–e13229, https://doi.org/10.1111/ctr.13219.
[13] F. Capone, E. Guerriero, A. Sorice, G. Colonna, G. Ciliberto, S. Costantini, Serum cytokinome profile evaluation: a tool to define new diagnostic and prognostic markers of cancer using multiplexed bead-based immunoassays, Med. Inflamm. 2016 (2016), https://doi.org/10.1155/2016/3064643.
[14] T. Xia, X. Xu, N. Zhao, Z. Luo, Y. Tang, Comparison of the diagnostic power of cytokine patterns and procalcitonin for predicting infection among paediatric haematology/oncology patients, Clin. Microbiol. Infect. 22 (2016) 996–1001, https://doi.org/10.1016/j.cmi.2016.09.013.
[15] M. Ostadkarampour, A. Eklund, D. Moller, P. Glader, C. Olgart Höglund, A. Lindén, et al., Higher levels of interleukin IL-17 and antigen-specific IL-17 responses in pulmonary sarcoidosis patients with Löfgren's syndrome, Clin. Exp. Immunol. 178 (2014) 342–352, https://doi.org/10.1111/cei.12403.
[16] C. Tortorella, G. Piazzolla, M. Matteo, V. Pinto, R. Tinelli, C. Sabbà, et al., Interleukin-6, interleukin-1beta, and tumor necrosis factor alpha in menstrual effluents as biomarkers of chronic endometritis, Fertil. Steril. 101 (2014) 242–247, https://doi.org/10.1016/j.fertnstert.2013.09.041.
[17] S.J. Frangiamore, A. Saleh, M. Farias Kovac, M.J. Grosso, X. Zhang, T.W. Bauer, et al., Synovial fluid Interleukin-6 as a predictor of periprosthetic shoulder infection, J. Bone Joint Surgery-Am. Vol. 97 (2014) 63–70, https://doi.org/10.2106/JBJS.N.00104.
[18] P. Rider, Y. Carmi, I. Cohen, Biologics for targeting inflammatory cytokines, clinical uses, and limitations, Int. J. Cell Biol. 2016 (2016) 9259646–9259711, https://doi.org/10.1155/2016/9259646.
[19] B.S. Qazi, K. Tang, A. Qazi, Recent advances in underlying pathologies provide insight into interleukin-8 expression-mediated inflammation and angiogenesis, Int. J. Inflamm. 2011 (2011) 908468, , https://doi.org/10.4061/2011/908468.
[20] K. Hosoki, S. Ying, C. Corrigan, H. Qi, A. Kurosky, K. Jennings, et al., Analysis of a panel of 48 cytokines in BAL fluids specifically identifies IL-8 levels as the only cytokine that distinguishes controlled asthma from uncontrolled asthma, and correlates inversely with FEV1, PLoS One 10 (2015) e0126035, , https://doi.org/10.1371/journal.pone.0126035.
[21] A.T. Corcoran, N. Yoshimura, V. Tyagi, B. Jacobs, W. Leng, P. Tyagi, Mapping the cytokine profile of painful bladder syndrome/interstitial cystitis in human bladder and urine specimens, World J. Urol. 31 (2012) 241–246, https://doi.org/10.1007/s00345-012-0852-y.
[22] L.J.A.G. Ricker, A. Kijlstra, A.G.H. Kessels, W. de Jager, A.T.A. Liem, F. Hendrikse, et al., Interleukin and growth factor levels in subretinal fluid in rhegmatogenous retinal detachment: a case-control study, PLoS One 6 (2011) e19141, , https://doi.org/10.1371/journal.pone.0019141.
[23] F. Kreiner, H. Langberg, H. Galbo, Increased muscle interstitial levels of inflammatory cytokines in polymyalgia rheumatica, Arth. Rheum. 62 (2010) 3768–3775, https://doi.org/10.1002/art.27728.
[24] A. Granath, A. Norrby-Teglund, R. Uddman, L.-O. Cardell, Reduced iNOS expression in adenoids from children with otitis media with effusion, Pediatr. Allergy Immunol. 21 (2010) 1151–1156, https://doi.org/10.1111/j.1399-3038.2010.01020.x.
[25] A. Conway Morris, K. Kefala, T.S. Wilkinson, O.L. Moncayo-Nieto, K. Dhaliwal, L. Farrell, et al., Diagnostic importance of pulmonary interleukin-1beta and interleukin-8 in ventilator-associated pneumonia, Thorax 65 (2010) 201–207, https://doi.org/10.1136/thx.2009.122291.
[26] E. Küpeli, D. Karnak, S. Beder, O. Kayacan, H. Tutkak, Diagnostic accuracy of cytokine levels (TNF-alpha, IL-2 and IFN-gamma) in bronchoalveolar lavage fluid of smear-negative pulmonary tuberculosis patients, Respiration 75 (2008) 73–78, https://doi.org/10.1159/000110744.
[27] J.-E. Lee, J.-W. Kim, B.-G. Han, S.-Y. Shin, Impact of whole-blood processing conditions on plasma and serum concentrations of cytokines, Biopreserv. Biobanking 14 (2016) 51–55, https://doi.org/10.1089/bio.2015.0059.
[28] L. Flower, R.H. Ahuja, S.E. Humphries, V. Mohamed-Ali, Effects of sample handling on the stability of Interleukin 6, tumour necrosis factor-α and leptin, Cytokine 12 (2000) 1712–1716, https://doi.org/10.1006/cyto.2000.0764.
[29] S. Ayache, M. Panelli, F.M. Marincola, D.F. Stroncek, Effects of storage time and exogenous protease inhibitors on plasma protein levels, Am. J. Clin. Pathol. 126 (2006) 174–184, https://doi.org/10.1309/3WM7-XJ7R-D8BC-LNKX.
[30] R.P. Jackman, G.H. Utter, J.W. Heitman, D.F. Hirschkorn, J.P. Law, N. Gefter, et al.,

Effects of blood sample age at time of separation on measured cytokine concentrations in human plasma, Clin. Vaccine Immunol. 18 (2011) 318–326, https://doi.org/10.1128/CVI.00465-10.
[31] K. Skogstrand, C.K. Ekelund, P. Thorsen, I. Vogel, B. Jacobsson, B. Nørgaard-Pedersen, et al., Effects of blood sample handling procedures on measurable inflammatory markers in plasma, serum and dried blood spot samples, J. Immunol. Methods 336 (2008) 78–84, https://doi.org/10.1016/j.jim.2008.04.006.
[32] N. Aziz, R. Detels, J.J. Quint, Q. Li, D. Gjertson, A.W. Butch, Stability of cytokines, chemokines and soluble activation markers in unprocessed blood stored under different conditions, Cytokine 84 (2016) 17–24, https://doi.org/10.1016/j.cyto.2016.05.010.
[33] A. Friebe, H.-D. Volk, Stability of tumor necrosis factor alpha, interleukin 6, and interleukin 8 in blood samples of patients with systemic immune activation, Arch. Pathol. Lab. Med. 132 (2008) 1802–1806, https://doi.org/10.1043/1543-2165-132.11.1802.
[34] G.C.E. Keustermans, S.B.E. Hoeks, J.M. Meerding, B.J. Prakken, W. de Jager, Cytokine assays: an assessment of the preparation and treatment of blood and tissue samples, Methods 61 (2013) 10–17, https://doi.org/10.1016/j.ymeth.2013.04.005.
[35] M. Plebani, L. Sciacovelli, A. Aita, A. Padoan, M.L. Chiozza, Quality indicators to detect pre-analytical errors in laboratory testing, Clinica Chimica Acta 432 (2014) 44–48, https://doi.org/10.1016/j.cca.2013.07.033.
[36] G. Lippi, J.J. Chance, S. Church, P. Dazzi, R. Fontana, D. Giavarina, et al., Preanalytical quality improvement: from dream to reality, Clin. Chem. Laborat. Med. 49 (2011) 5–14, https://doi.org/10.1515/CCLM.2011.600.
[37] N. Aziz, Measurement of circulating cytokines and immune-activation markers by multiplex technology in the clinical setting: what are we really measuring? For. Immunopathol. Dis. Therap. 6 (2015) 19–22, https://doi.org/10.1615/ForumImmunDisTher. 2015014162.
[38] S.I. Rennard, R. Aalbers, E. Bleecker, H. Klech, L. Rosenwasser, D. Olivieri, et al., Bronchoalveolar lavage: performance, sampling procedure, processing and assessment, Eur. Respir. J. Suppl. 26 (1998) 13S–15S.
[39] R. Baughman, Technical aspects of bronchoalveolar lavage: recommendations for a standard procedure, Semin. Respir. Crit. Care Med. 28 (2007) 475–485, https://doi.org/10.1055/s-2007-991520.
[40] L.M. Sedger, M.F. McDermott, TNF and TNF-receptors: From mediators of cell death and inflammation to therapeutic giants – past, present and future, Cytokine Growth Factor Rev. 25 (2014) 453–472, https://doi.org/10.1016/j.cytogfr.2014.07.016.
[41] R.C. Russo, C.C. Garcia, M.M. Teixeira, F.A. Amaral, The CXCL8/IL-8 chemokine family and its receptors in inflammatory diseases, Exp. Rev. Clin. Immunol. 10 (2014) 593–619, https://doi.org/10.1586/1744666X.2014.894886.
[42] G. Kroemer, I. Moreno de Alborán, J.A. Gonzalo, C. Martínez, Immunoregulation by cytokines, Crit. Rev. Immunol. 13 (1993) 163–191.
[43] W. de Jager, K. Bourcier, G.T. Rijkers, B.J. Prakken, V. Seyfert-Margolis, Prerequisites for cytokine measurements in clinical trials with multiplex immunoassays, BMC Immunol. 10 (2009) 52, https://doi.org/10.1186/1471-2172-10-52.
[44] C. Dinakar, A. Malur, B. Raychaudhuri, L.T. Buhrow, A.L. Melton, M.S. Kavuru, et al., Differential regulation of human blood monocyte and alveolar macrophage inflammatory cytokine production by nitric oxide, 82 (1999) 217–222. doi: http://doi.org/10.1016/S1081-1206(10)62600-2.
[45] J.E. Losa Garcia, F.M. Rodriguez, M.R. Martin de Cabo, M.J. Garcia Salgado, J.P. Losada, L.G. Villaron, et al., Evaluation of inflammatory cytokine secretion by human alveolar macrophages, Med. Inflamm. (1999) 1–9.
[46] P. Gosset, I. Tillie-Leblond, S. Oudin, O. Parmentier, B. Wallaert, M. Joseph, et al., Production of chemokines and proinflammatory and antiinflammatory cytokines by human alveolar macrophages activated by IgE receptors, J. Allergy Clin. Immunol. (1999) 1–9.
[47] M. Ding, A. Cavallin, N.-O. Hermansson, P. Berntsson, L. Jinton, S.R. Blomqvist, Comparing flow cytometry QBeads PlexScreen assays with other immunoassays for determining multiple analytes, SLAS Discov. (2018) 676–686, https://doi.org/10.1177/2472555218771610.