RESEARCH ARTICLE

Stability of cytokines in supernatants of stimulated mouse immune cells

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ABSTRACT. Measurements of cytokines in cell culture supernatants are widely used to evaluate the immune response. Cytokine levels in secretomes are usually quantified using enzyme-linked immunosorbent assays (ELISA), which have easy, sensitive, specific, rapid, cost-effective, and reproducible protocols. To our knowledge, the stability of cytokines in secretomes has not been hitherto investigated. We present data that involve; time-dependent changes during storage at +4°C, and the effects of freeze-thaw cycles in samples frozen at -80°C, instant freezing of samples with liquid nitrogen, and addition of protease inhibitors on the stability of certain cytokines (TNF-α, MIP-2, IFN-γ, IL-6, IL-10, IL-17A), in secrotomes of spleen and lymph nodes from tumor-bearing animals. Our results show that IL-6 remains stable, MIP-2, IFN-γ and IL-10 are somewhat stable, while TNF-α and IL-17A are degradable cytokines: instant freezing by liquid nitrogen or adding protease inhibitor does not preserve the stability of these cytokines. From these results it can be concluded that, if possible, TNF-α measurements should be perform in fresh samples, and IL-17A and IL-10 samples can be stored at -80°C, but should be used at the first thaw.

Key words: stability, cytokines, secrotomes, ELISA

Cytokines are a diverse group of soluble, low molecular weight proteins effective at picomolar concentrations. Cytokines have short half-lives, hence they are primarily secreted on demand to act immediately on target cells [1]. Because of their pleotropic effects on many different cell types, accurate measurement of cytokine levels is crucial to the understanding of the pathophysiology of a wide range of diseases.

TNF-α is known as a potent pro-inflammatory cytokine involved in inflammation, autoimmune diseases and cancer [2]. IFN-γ seems to acts as a central regulator of the many inflammatory responses, with both inhibitory and stimulatory effects on the immune system [3]. IL-10 has potent anti-inflammatory effects, and dysregulation of IL-10 secretion contributes to many autoimmune and inflammatory diseases [4]. IL-6 has pleiotropic effects on inflammation, and overexpression of IL-6 has been reported in a variety of autoimmune and inflammatory diseases and hematologic malignancies [5]. IL-17A is thought to be necessary for host defense against infections, and to play a role in autoimmune, inflammatory diseases [6]. MIP-2 (IL-8 in humans), is a chemokine that plays an important role in neutrophilic inflammatory responses implicated in lung disease and metastasis [7]. These cytokines interact with one another in a complex manner [8].

Stimulation of the cytokine responses of immune cells in cell culture medium is used to evaluate immunity in systemic diseases including cancer [9]. Cytokine levels are usually measured using ELISA. ELISA protocols are easy to perform and are highly sensitive and specific, allowing rapid, cost-effective, and reproducible assays for quantification of cytokines in secretomes [10, 11]. Because several cytokines are often measured in cell cultures, storage of the samples is often required, generally under frozen conditions at –80°C. Previously published data, examining the stability of cytokines in human samples, suggested that freeze-thawing alters the stability of certain cytokines [12-14]. In the current study, we assessed the stability of secreted cytokines in the supernatants of stimulated mouse immune cells under different storage conditions, measuring time-dependent changes during storage at +4°C, as well as the effects of freeze-thaw cycles on samples frozen at -80°C. We also examined the effects of instant (snap) freezing of samples in liquid nitrogen, and addition of protease inhibitors, on the stability of a panel of cytokines, specifically TNF-α, MIP-2, IFN-γ, IL-6, IL-10 and IL-17A. Immune cell cultures from tumor-bearing animals, which secrete large amounts of the cytokines of interest, are routinely performed in our laboratory, and were the samples used in the studies described herein.

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METHODS

Animals and reagents

Female Balb/c mice, eight weeks old, were obtained from Harlan (Israel) and kept under a 12 h light–dark cycle and received a controlled diet. LPS, from *Escherichia coli* 026:B6 (L8274) and ConA, from *Canavalia ensiformis* (jack bean) (L7647) were purchased from Sigma-Aldrich GmbH. Protease inhibitor Cocktail Tablets (05892791001) were purchased from Roche Diagnostics GmbH. Reagents for ELISAs of cytokines were purchased from BioLegend for TNF-α (Cat. No. 430903), IFN-γ (Cat. No. 430803) and, IL-17A (Cat. No. 432506), from BD for IL-6 (Cat. No. 555240) and IL-10 (Cat. No. 555252), from Antigenix America for MIP-2 (Cat. No. RMF431CK).

Mice injected with the 4TBM cancer cell line were used [15]. 4TBM cells were cultured *in vitro* with DMEM-F12 medium, supplemented with non-essential amino acids, 5% fetal bovine serum (FBS), gentamycin (50 μg/mL), 2 mM l-glutamine (final concentration 4.5 mM), and 1 mM sodium pyruvate. 4TBM cells (10^5 cells) were injected orthotopically into the second, right chest mammary pad. Animals were sacrificed 25–28 days following the 4TBM injections. All protocols were approved and performed under the supervision of Akdeniz University Institutional Animal Care and Use Committee.

Mouse immune cell cultures

Mouse immune cell cultures were prepared from tumor-bearing animals. Spleen and lymph nodes from tumor-bearing animals were harvested from animals bearing 4TBM breast carcinoma cells [15]. The cell suspensions were prepared by gently pushing spleen or lymph nodes through a disposable cell strainer (BD Biosciences) in ice-cold phosphate buffered saline (PBS). The cells were washed twice in ice-cold PBS and centrifuged at $1600 \times g$ for 3 min. Cells were resuspended in RPMI-1640 medium containing 10% FBS, gentamycin (50 μg/mL) and 2×10^{-5} M 2-mercaptoethanol, and cultured at 6×10^6 cells per well in 24-well plates [16, 17], with stimulation using 3 μg/mL LPS or 5 μg/mL ConA. Conditioned media were collected 16-20 hours after stimulation for TNF-α, and 36-40 hours after for MIP-2, IFN-γ, IL-6, IL-10, IL-17A, from six separate wells for each cytokine. Cytokines in an aliquot of the conditioned media collected were measured immediately in order to determine levels before processing the samples. The remainder of each sample was aliquotted into eppendorfs to test the different storage conditions as follows:

1. Kept at -80°C and defrosted multiple times
2. Kept at +4°C for various time intervals e.g. 48 h, 1 week, 2 weeks, 4 weeks.
3. Immediately frozen in liquid nitrogen.
4. Protease inhibitor added and kept at +4 °C.

Statistics

The results were statistically assessed by analysis of variance (ANOVA). Multiple *post-hoc* differences were checked by means of Dunnett's or Tukey's tests where appropriate.

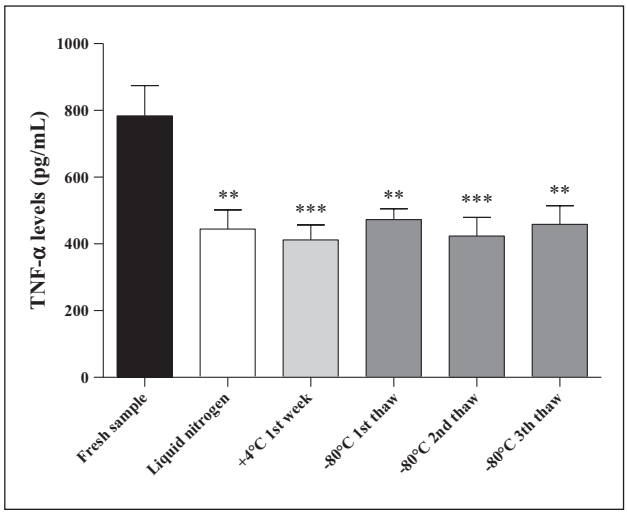

Figure 1
Effect of storage duration at +4°C and freeze-thaw cycles on TNF-α levels (p = 0.0007, one-way ANOVA). Data represented as the mean concentration ± SEM (*p<0.05, ** p<0.001, *** p<0.0001, Dunnett's test).

RESULTS

Effects of storage length at +4°C and freeze-thaw cycles on TNF-α levels

Measurement TNF-α levels revealed that TNF-α was degraded within the first week of storage at +4°C. Levels measured after one week's storage at +4°C were decreased by 47% compared to levels obtained immediately after harvesting the conditioned media (base-line measurements) (from 784 ± 90 to 412 ± 45 pg/mL, p < 0,001). Degradation of TNF-α after freezing at -80°C or in liquid nitrogen was similar to storage at +4°C. Levels of TNF-α were decreased by 40% in first thaw of samples kept at -80°C (445 ± 57 compared to 784 ± 90 pg/mL, p < 0.01). Further thaws did not markedly change the levels measured (*figure 1*).

Effect of storage length at +4°C and freeze-thaw cycles on IL-17A levels

Similar to TNF-α, IL-17A was also degraded upon storage at +4°C (*figure 2*). Levels of IL-17A demonstrated a 42% reduction following one week of storage at +4°C (644 ± 59 to 376 ± 33 pg/mL, p < 0.05). Storage at -80°C did not alter the levels of IL-17A at first thaw. However, additional thaw cycles decreased IL-17A levels (second thaw: 216 ± 41; third thaw 97 ± 6: compared to unthawed levels 644 ± 59 pg/mL, p < 0.001). IL-17A levels after freezing in liquid nitrogen also decreased by 52% (310 ± 90 pg/mL, p<0.01).

Effect of storage length at +4°C and freeze-thaw cycles on MIP-2 levels

MIP-2 levels remained stable within the first two weeks of storage at +4°C, as well as following the first thaw of samples kept at -80°C (*figure 3*). Freezing samples in liquid nitrogen also did not alter MIP-2 levels. MIP-2 degradation however, was observed four weeks after storage at +4°C (35% reduction; 492 ± 18 compared to 760 ± 50 pg/mL, p < 0.001). Similar levels of degradation were observed

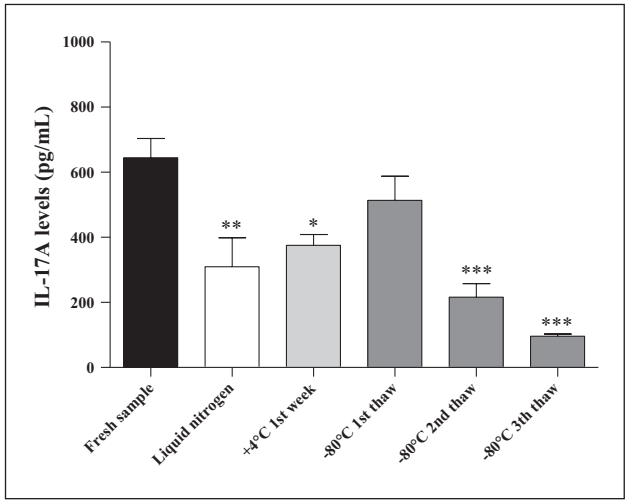

Figure 2
Effect of storage duration at +4°C and freeze-thaw cycles on IL-17A levels (p = 0.0001, one-way ANOVA). Data represented as the mean concentration ± SEM (*p<0.05, ** p<0.001, *** p<0.0001, Dunnett's test).

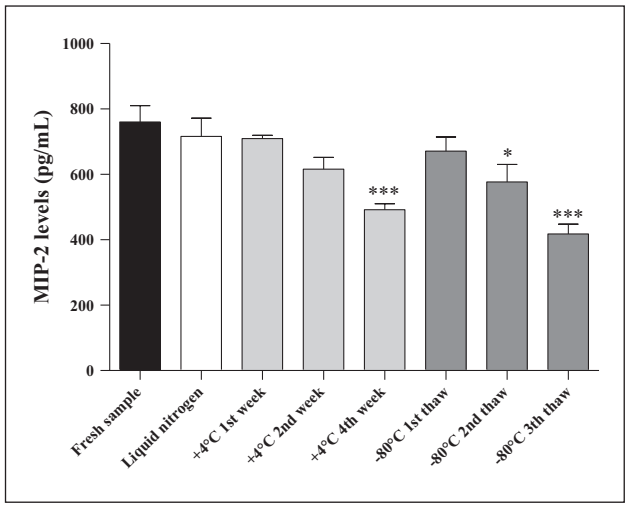

Figure 3
Effect of storage duration at +4°C and freeze-thaw cycles on MIP-2 levels (p = 0.0001, one-way ANOVA). Data represented as the mean concentration ± SEM (*p<0.05, ** p<0.001, *** p<0.0001, Dunnett's test).

after the second and third thawing of samples stored at -80°C (second thaw: 577 ± 53; third thaw 418 ± 30: compared to unthawed samples 760 ± 50 pg/mL, $p < 0.05$ and $p < 0.001$ respectively).

Effect of storage length at +4°C and freeze-thaw cycles on IL-10 levels

IL-10 levels remained stable within the first week of storage at +4°C as well as after the first thaw of samples kept at -80°C. Freezing samples in liquid nitrogen did not alter IL-10 levels. IL-10 degradation was observed after the first week of storage at +4°C and after the first thaw of samples kept at -80°C (*figure 4*). Specifically, a 28% reduction was observed two weeks after storage at +4°C (420 ± 16 compared to 565 ± 36, $p < 0.001$). IL-10 levels were reduced by 14% in samples thawed a second time (401 ± 21 compared to 565 ± 36 pg/mL, $p < 0.001$).

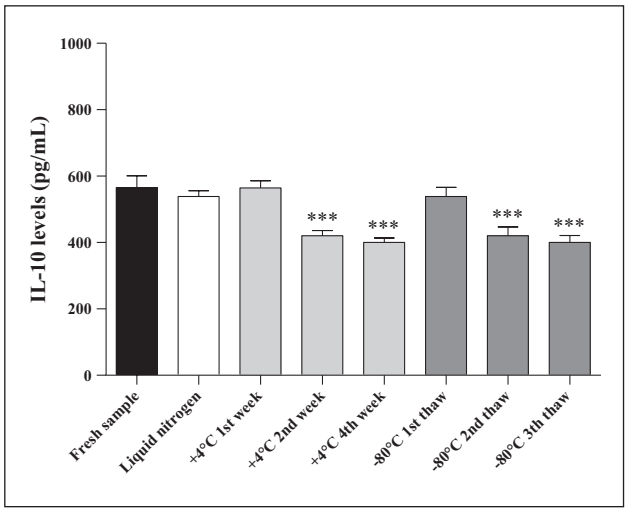

Figure 4
Effect of storage duration at +4°C and freeze-thaw cycles on IL-10 levels (p = 0.0001, one-way ANOVA). Data represented as the mean concentration ± SEM (*p<0.05, ** p<0.001, *** p<0.0001, Dunnett's test).

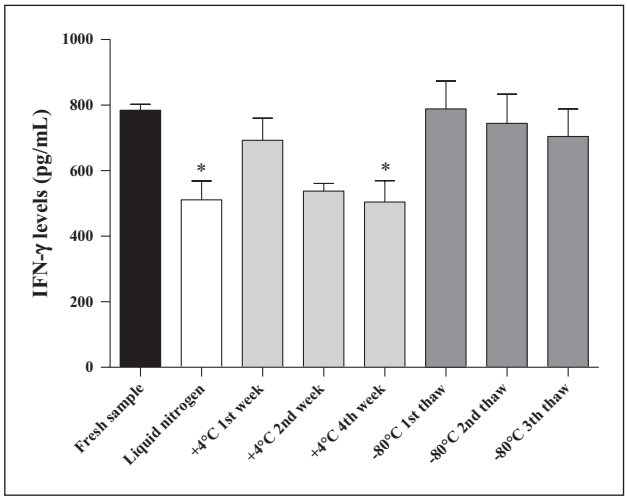

Figure 5
Effect of storage duration at +4°C and freeze-thaw cycles on IFN-γ levels (p = 0.0067, one-way ANOVA). Data represented as the mean concentration ± SEM (*p<0.05, ** p<0.001, *** p<0.0001, Dunnett's test).

Effect of storage length at +4°C and freeze-thaw cycles on IFN-γ levels

Levels of IFN-γ were not affected by multiple freeze-thaw cycles or during the first and second weeks of storage at +4°C. IFN-γ degradation however, was observed four weeks after storage at +4°C (36% reduction; 784 ± 19 compared to 504 ± 65 pg/mL, $p < 0.05$), and freezing in liquid nitrogen (35% reduction; 784 ± 19 compared to 511 ± 58 pg/mL, $p < 0.05$) (*figure 5*).

Effect of storage length at +4°C and freeze-thaw cycles on IL-6 levels

IL-6 was the most resistant to storage-induced degradation. Levels of IL-6 were not affected by long-term storage at +4°C, multiple freeze-thaw cycles, or by freezing in liquid nitrogen (*figure 6*).

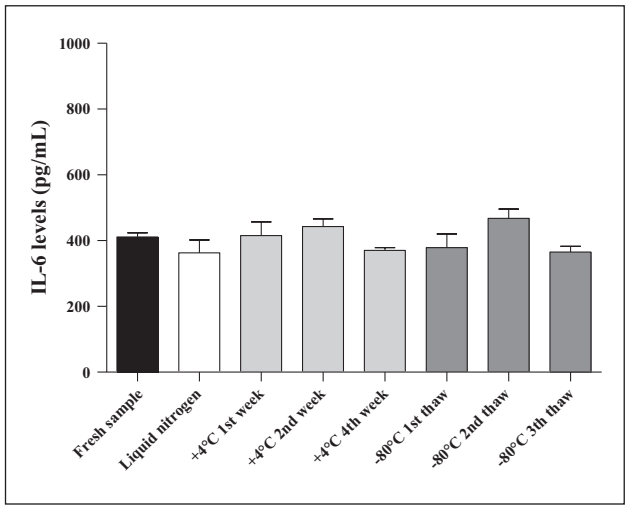

Figure 6
Effect of storage duration at +4°C and freeze-thaw cycles on IL-6 levels (p = 0.1199, one-way ANOVA). Data represented as the mean concentration ± SEM (*p<0.05, ** p<0.001, *** p<0.0001, Dunnett's test).

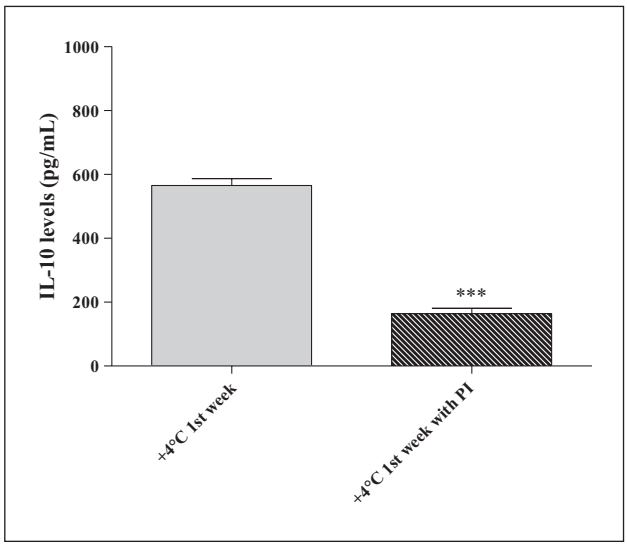

Figure 7
Effect of protease inhibitor at +4°C on IL-10 levels (*** p<0.0001, t-test). Data represented as the mean concentration ± SEM.

Effect of protease inhibitor at +4°C on IL-10 levels

To evaluate whether the stability of the cytokines could be increased by protease inhibitors, a protease inhibitor cocktail was added to various conditioned media after harvesting. The presence of protease inhibitors reduced IL-10 levels measured one week after storage at +4°C (164 ± 17 pg/mL compared to 565 ± 36 pg/mL, $p < 0.0001$) (*figure 7*). As a result of these findings, we tested the possibility that protease inhibitors affect results by altering enzyme activity in two different ELISA kits. When the protease inhibitors were added to standards in RPMI-1640 medium, levels of IL-10 and IFN-γ decreased by 44 ± 2% (mean ± SEM as %) and 31 ± 6% (mean ± SEM as %), respectively.

DISCUSSION

The measurement of cytokines secreted from immune cells in culture is widely used to evaluate immune response. When measurement of multiple cytokines is required in large scale experiments, storage of the samples is often required. While several reports have recorded an impact of storage on cytokine levels in human serum samples [12-14], the stability of cytokines in cell culture supernatants had not been previously investigated.

We have shown that there are marked differences in the storage stability of certain cytokines. IL-6 was the most resistant cytokine to storage-induced degradation. Levels of IL-6 were not affected by long-term storage at +4°C, multiple freeze-thaw cycles, or by freezing in liquid nitrogen. Similar findings have been reported in human serum samples [13]. Also, in another study, IL-6 levels in human plasma samples were not affected by addition of anticoagulants at +4°C for 24 h [12]. MIP-2 was quite stable, and remained intact up to four weeks at +4°C, and up to the second freeze-thaw cycle. IFN-γ was also quite stable, being unaffected by freeze-thaw cycles. However, IFN-γ degradation was observed four weeks after storage at +4°C and freezing in liquid nitrogen.

The stability of TNF-α, IL-17A, and IL-10 was affected by storage. Similar differences in the stability of cytokines have been also reported elsewhere [18]. TNF-α was the most labile, with degradation occurring under all storage conditions used. Our findings on TNF-α stability differed from previous observations. TNF-α levels have been reported to increase in human plasma samples after multiple freeze-thaw cycles [13]. In another study [19], TNF-α levels remained stable if stored at +4°C or frozen at -80°C for 24 hours in human blood samples collected over EDTA. IL-17A was the second most labile cytokine and remained stable only up to the first thaw. Our findings on IL-17A stability were similar to that which has been reported previously. Jager *et al*. demonstrated that IL-17A levels drop after multiple freeze-thaw cycles in human blood samples [18]. IL-10 degradation was observed after the first week of storage at +4°C and the first thaw of samples kept at -80°C. Studies on IL-10 in human serum samples demonstrated stability under storage at +4°C for 21 days and even following repeated freeze-thaw cycles up to four cycles [14]. The differences seen between findings reported previously might reflect the effect of differences in the cytokine milieu, plasma-versus-cell culture medium, which may, in itself, affect the stability of cytokines. In addition, we examined for the first time the effect of snap-freezing and found that instant freezing in liquid nitrogen also alters the stability of certain cytokines. Thus, while instant freezing is used for RNA studies, this approach may not be appropriate for the preservation of protein structure and should not be used for this purpose.

Lastly, we observed that protease inhibitors altered cytokine levels, probably by impacting enzyme activity (horse radish peroxidase) in the ELISAs. Our results demonstrated the interference of protease inhibitors with the enzymatic reactions required for ELISA measurements. It is likely that protease inhibitors in the samples adhere to the ELISA plate and dissolve back during the enzymatic reaction. Similar finding have been previously reported for Substance P measurements [20].

In summary, our results demonstrated that IL-6 is stable; MIP-2 and IFN-γ are somewhat stable; and TNF-α,

IL-17A and IL-10 are markedly degradable cytokines in secretomes. We conclude that TNF-α levels should be determined in fresh samples, while IL-17A and IL-10 samples can be stored at -80°C, but should be measured after the first thaw.

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REFERENCES

1. Meager A. Measurement of cytokines by bioassays: theory and application. *Methods* 2006; 38: 237.
2. Chu WM. Tumor necrosis factor. *Cancer Lett* 2013; 328: 222.
3. Saha B, Jyothi Prasanna S, Chandrasekar B, Nandi D. Gene modulation and immunoregulatory roles of interferon gamma. *Cytokine* 2010; 50: 1.
4. Ouyang W, Rutz S, Crellin NK, Valdez PA, Hymowitz SG. Regulation and functions of the IL-10 family of cytokines in inflammation and disease. *Annu Rev Immunol* 2011; 29: 71.
5. Nishimoto N. Interleukin-6 as a therapeutic target in candidate inflammatory diseases. *Clin Pharmacol Ther* 2010; 87: 483.
6. Zhu S, Qian Y. IL-17/IL-17 receptor system in autoimmune disease: mechanisms and therapeutic potential. *Clin Sci (Lond)* 2012; 122: 487.
7. Acharyya S, Oskarsson T, Vanharanta S, Malladi S, Kim J, Morris PG, Manova-Todorova K, Leversha M, Hogg N, Seshan VE, Norton L, Brogi E, Massague J. A CXCL1 paracrine network links cancer chemoresistance and metastasis. *Cell* 2012; 150: 165.
8. Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. *Cell* 2010; 140: 883.
9. Yamaguchi Y, Ohshita A, Kawabuchi Y, Ohta K, Shimizu K, Minami K, Hihara J, Miyahara E, Toge T. Adoptive immunotherapy of cancer using activated autologous lymphocytes–current status and new strategies. *Hum Cell* 2003; 16: 183.
10. House RV. Cytokine measurement techniques for assessing hypersensitivity. *Toxicology* 2001; 158: 51.
11. Keustermans GC, Hoeks SB, Meerding JM, Prakken BJ, de Jager W. Cytokine assays: an assessment of the preparation and treatment of blood and tissue samples. *Methods* 2013; 61: 10.
12. De Jongh R, Vranken J, Vundelinckx G, Bosmans E, Maes M, Heylen R. The effects of anticoagulation and processing on assays of IL-6, sIL-6R, sIL-2R and soluble transferrin receptor. *Cytokine* 1997; 9: 696.
13. Flower L, Ahuja RH, Humphries SE, Mohamed-Ali V. Effects of sample handling on the stability of interleukin 6, tumour necrosis factor-alpha and leptin. *Cytokine* 2000; 12: 1712.
14. Kenis G, Teunissen C, De Jongh R, Bosmans E, Steinbusch H, Maes M. Stability of interleukin 6, soluble interleukin 6 receptor, interleukin 10 and CC16 in human serum. *Cytokine* 2002; 19: 228.
15. Erin E, Kale S, Tanriover G, Koksoy S, Duymus O, Korcum AF. Differential characteristics of heart, liver, and brain metastatic subsets of murine breast carcinoma. *Breast Cancer Res Treat* 2013; 139: 677.
16. Moehrlen U, Lechner A, Baumel M, Dostert K, Rohrl J, Meuli M, Mannel DN, Hamacher J. Immune cell populations and cytokine production in spleen and mesenteric lymph nodes after laparoscopic surgery versus conventional laparotomy in mice. *Pediatr Surg Int* 2012; 28: 507.
17. Al-Mariri A, Tibor A, Mertens P, De Bolle X, Michel P, Godfroid J, Walravens K, Letesson JJ. Induction of immune response in BALB/c mice with a DNA vaccine encoding bacterioferritin or P39 of Brucella spp. *Infect Immun* 2001; 69: 6264.
18. de Jager W, Bourcier K, Rijkers GT, Prakken BJ, Seyfert-Margolis V. Prerequisites for cytokine measurements in clinical trials with multiplex immunoassays. *BMC Immunol* 2009; 28: 10.
19. Friebe A, Volk HD. Stability of tumor necrosis factor alpha, interleukin 6, and interleukin 8 in blood samples of patients with systemic immune activation. *Arch Pathol Lab Med* 2008; 132: 1802.
20. Erin N, Ulusoy O. Differentiation of neuronal from non-neuronal Substance P. *Regul Pept* 2009; 152: 108.