

Effect of cryopreservation on expression of Th1 and Th2 cytokines in blood mononuclear cells from patients with different cytokine profiles, analysed with three common assays: an overall decrease of interleukin-4[☆]

M. Kvarnström^{a,b,*}, M.C. Jenmalm^{a,c}, C. Ekerfelt^{a,b}

^a *Clinical Research Centre, Department of Molecular and Clinical Medicine, Faculty of Health Sciences, Linköping University, S-581 85 Linköping, Sweden*

^b *Division of Clinical Immunology, Faculty of Health Sciences, Linköping University, S-581 85 Linköping, Sweden*

^c *Division of Paediatrics, Faculty of Health Sciences, Linköping University, S-581 85 Linköping, Sweden*

Received 6 November 2003; accepted 24 June 2004

Available online 6 August 2004

Abstract

Studies on cytokine expression in blood cells are commonly performed on cryopreserved cells. Previous studies show that cryopreservation affects cytokine expression, but the findings are not consistent. This may be due to divergent effects of freezing on different cytokines, different stimuli, and different patient groups or to the use of different assays in the studies. This study was designed to investigate the effect of freezing on spontaneous, auto-antigen, allergen, and mitogen induced cytokine secretion from peripheral blood mononuclear cells from several groups of patients expressing different cytokine profiles; multiple sclerosis, atopic children, non-atopic children, and pregnant women. The expression of IFN- γ , IL-4, IL-5, IL-9, IL-10, and IL-13 was analysed with ELISA, ELISPOT and/or real time RT-PCR. Our data provide evidence that the process of cryopreservation and thawing does affect the expression of cytokines, both at the protein and the mRNA level. Moreover, the effect varied among different cytokines, different stimuli, and different patient groups, which partly may be explained by differences in optimal freezing conditions for non-activated and activated cells. An increase of allergen and PHA stimulated IFN- γ secretion in atopic children was found following cryopreservation, but no such increase in auto-antigen induced IFN- γ was seen in MS-patients. The most consistent finding was that expression of IL-4 was generally decreased in spontaneous and auto-antigen/allergen induced

[☆] This project was supported by grants from the “Network for Inflammation research” funded by the Swedish Foundation for Strategic Research, The Swedish Foundation for Health and Care Sciences and Allergy Research and The National Swedish Association against Allergic Diseases.

* Corresponding author. Fax +46-13-13-22-57.

E-mail address: maria.kvarnstrom@imk.liu.se (M. Kvarnström).

expression in cryopreserved cells. In conclusion, this study points out the importance of investigation of the effects of freezing for each cytokine, stimuli and patient group before using frozen cells in studies of *in vitro* cytokine secretion.

©

Keywords: Cryopreservation; Cytokine; IL-4; ELISA; ELISPOT; Real time RT-PCR

Cytokines are signal molecules of great importance in many biological and clinical fields. Studies on cytokine expression in blood cells are commonly performed on cryopreserved cells. Analyses performed on cryopreserved cells have some advantages. First, it is convenient, since samples can be collected over time and analysed later. Second, cryopreservation provides an option to overcome the problems of inter-assay variation, when comparing samples from different occasions, or samples from different groups, since all samples can be analyzed on one occasion. Cryopreservation may, however, influence the expression of cytokines. The effect of cryopreservation is complex, and should be considered for each cytokine analysed, including different stimuli, and further for different patient groups whose cells may be differently affected by freezing due to e.g., activation state. One important aspect is that freezing may affect the spontaneous secretion differently as compared with the secretion induced by antigen stimulation, which could influence the value of the stimulated specific secretion (net-secretion).

The cryopreservation process affects cells in several ways. One important issue is whether the cryopreservation affects the cell populations differently. Allsopp et al. [1] showed that the proportions of CD4+ T-cells, CD8+ T cells, CD14+ monocytes, CD16+ NK cells, and CD20+ B cells did not change after cryopreservation. Similar results were demonstrated by Fujiwara et al. [9], but the activity of the NK-cells was decreased following cryopreservation, although the proportion of NK-cells was unaltered. Preincubation of the cells after cryopreservation did, however, regain the activity.

Previous studies have investigated the influence of storage time, showing that the freezing and thawing processes, and not the time of storage, exert the main effects on the cells [11,19,22].

Reports on the influence of cryopreservation on spontaneous and antigen stimulated cytokine secretion are few, and the findings are conflicting. Furthermore, only certain cytokines have been investigated, and the secretion from cryopreserved cells has not previously been compared with the secretion from fresh cells for several important cytokines, e.g., the ultimate Th-2 cytokine IL-4 has only recently been described by Kreher et al. [14]. Bailey et al. [2] reported release of IL-4 from cryopreserved cells, however no comparison with release from fresh cells was done. We have previously noted a decrease of both spontaneous and allo-antigen induced secretion of IL-4 in PBMC from pregnant women following cryopreservation [5], and wanted to further investigate this observation.

The conflicting findings on the influence of cryopreservation on cytokine expression suggest that the process of freezing and thawing has divergent effects on different cytokines, different stimuli and different patient groups. The use of different assays for detection of cytokine expression in the different studies may also have affected the results. In the present study, we aimed to investigate the effect of freezing on spontaneous, auto-antigen, allergen, and mitogen induced cytokine secretion/expression from peripheral blood mononuclear cells (PBMC) from several groups of patients expressing different cytokine profiles: multiple sclerosis (MS, considered as Th1-predominated), atopic children (Th2-predominated), non-atopic children, and pregnant women (Th2-skewed) [10,16,18]. The cytokine expression was analysed with commonly used cytokine-assays: ELISA, ELISPOT, and/or real time RT-PCR. This design using various assays and stimuli in patient groups with varying cytokine profiles, would give a broad and unbiased mapping of the effects of cryopreservation on cytokine expression. Using samples from pregnant

women allows the study of IL-4, which is hard to detect, since pregnant women are known to show high spontaneous secretion of IL-4.

Material and methods

Patients

MS

Ten patients (8 women and 2 men) age 31–66 years (mean 44 years) with MS according to McDonald et al. [17] were included in the study. Spontaneous and auto-antigen induced secretion of IFN- γ , IL-4, and IL-10 was analysed with ELISPOT.

Atopic and non-atopic children

Seventeen twelve-year-old children (8 girls and 9 boys) who had been identified from the Linköping part of the phase 2 International Study of Asthma and Allergies in Childhood (ISAAC) were included in the present study. Details of this material has been described elsewhere, including clinical definitions, and description of Skin prick test (SPT) [10]. Thirteen of the children were positive in SPT to the allergens used in this study (5 birch, 4 cat, and 4 both birch and cat) and 4 children had no SPT reactivity. Allergen and phytohaemagglutinin (PHA) induced IFN- γ , IL-4, IL-5, IL-9, IL-10, and IL-13 secretion was analysed by ELISA, and allergen and PHA induced IL-4, and IL-9 mRNA expression by real time PCR.

Pregnant women

Whole blood was obtained from eleven healthy primigravidae women age 24–34 (mean age 28 years) on four occasions: in the first (pregnancy weeks 8–12), second (pregnancy weeks 24–26) and third (pregnancy weeks 36–36) trimesters and 8 weeks after delivery. Due to dropouts, in total 38 blood samples were analysed. Spontaneous secretion of IFN- γ and IL-4 was analysed by ELISPOT.

Ethics

The study of all patients was approved by the Regional Ethics Committee for Human Research

at the University Hospital of Linköping. Informed consent was obtained from all individuals.

Cell preparation

Mononuclear cells were separated from heparinised venous blood by gradient centrifugation on Lymphoprep (Nycomed Pharma AS, Oslo, Norway) or Ficoll Paque density gradient (Pharmacia Biotech, Sollentuna, Sweden), according to Bøyum [3]. PBMC assayed by ELISPOT were diluted in Iscove's modified Dulbecco's medium (IMDM) (Gibco-BRL, Paisley, Scotland, supplemented as described previously [5,8]) containing 5% foetal calf serum (FCS) (Sigma-Aldrich, Stockholm, Sweden). For cell cultures analysed by ELISA and real time RT-PCR, the PBMC were diluted in AIM-V serum free medium (Life Technologies) with 20 μ M β -mercaptoethanol (Sigma-Aldrich, Stockholm, Sweden). The PBMC were counted under a phase contrast microscopy or diluted in Türks solution and counted by light microscopy, and the cell density was adjusted to 1×10^6 lymphocytes/ml.

Cryopreservation

The cells to be analysed frozen, were immediately suspended in cold IMDM or RPMI, containing 10% dimethylsulfoxide (Sigma, St. Louis, MO, USA) and 50% FCS, at a cell density of 5×10^6 lymphocytes/ml in cryo-vials (Nunc, Roskilde, Denmark). The vials were placed in a precooled (+4°C) Cryo 1°C Freezing Container, "Mr. Frosty" (Nalge Nunc International, Rochester, USA) containing isopropanol in –70°C, to achieve a freezing rate of –1°C/min. The following day, the cells were transferred to liquid nitrogen and stored frozen until analysis. The time of storage varied between 1 and 19 months.

Thawing

At the time of cytokine analysis, cells were thawed by gentle rocking of the vials in 37°C water-bath. The cell suspension (1 ml) was immediately transferred to a 15 ml polypropylene tube.

Thereafter, 13 ml IMDM (37°C) or RPMI, containing 10% FCS, was added drop-wise along the tube wall. The cell suspension was then immediately centrifuged at 400g for 10 min in room temperature and the supernatant was discarded, followed by washing twice. The pellets were resuspended in IMDM containing 5% FCS or AIM-V serum free medium with 20 µM β-mercaptoethanol. The cells were counted and the cell density was adjusted to 1×10^6 lymphocytes/ml. The viability of lymphocytes in the frozen and thawed samples was determined by trypan blue exclusion.

Cell culture for ELISA and real time PCR

Aliquots of 1 ml, containing 1×10^6 cells, were cultured in AIM-V serum free medium with 20 µM mercaptoethanol. Optimal allergen doses and time points for cytokine secretion were determined in a pilot study including cells from six adults. Doses tested were 1000, 10,000, and 50,000 standardised units (SU)/ml and time points tested were 24, 48, 72, 96, 144, and 192 h. The cells were stimulated with birch (10,000 SU/ml) and cat allergen (10,000 SU/ml) extracts from ALK. Separate cultures were performed with 2 µg/ml monoclonal antibodies to the IL-4 receptor (R&D Systems, Abingdon, UK), enabling measurement of IL-4 [10]. Two microgram per milliliters of PHA was added to separate cultures as a positive control. The cells were cultured at 37°C with 5% CO₂ for 24 or 144 h. The supernatants were collected after centrifugation and stored at –70°C. For later RNA extraction, the cell pellets were lysed with 300 µl RLT Lysis buffer (from RNeasy 96 RNA extraction kit, Qiagen, Hilden, Germany) and then stored at –70°C.

Background responses (cultures with medium only) were subtracted from the allergen and PHA induced responses. IL-4, IL-5, IL-9, IL-13, and IFN-γ were analysed in the 144 h samples, while IL-10 was analysed in the 24 h samples. The differing kinetics of secretion of IL-10, as compared to the other cytokines, is probably due to different cellular sources. It is likely that monocytes are activated to secrete IL-10 early in the cultures [15].

ELISPOT

Antigens and mitogens used in the ELISPOT

Two peptides from Myelin Oligodendrocyte Glycoprotein (MOG) (kindly provided by Dr. Erik Wallström, Karolinska Institute, Stockholm, Sweden) amino acid sequence 14–39 and 63–87, respectively, were used in a final concentration of 10 µg/ml. The MOG 63–87 peptide evoked the strongest response in MS patients in a study by Wallström et al. [21]. The optimal concentration of the MOG peptides have previously been described [21].

ELISPOT assay

The ELISPOT was performed as previously described for IFN-γ and IL-4 by Ekerfelt et al. [4]. All antibodies were purchased from Mabtech AB, Stockholm, Sweden. Triplicates were used for the spontaneous secretion and all antigens except PHA, which was analysed in duplicate. The spots were counted under a dissection microscope. The mean value of the triplicates was calculated. To obtain the number of antigen specific cells, the value of the non-stimulated cells, the spontaneous secretion, was subtracted from the values of the stimulated cells. Some wells on each plate were incubated with TCM but without cells, but otherwise treated likewise. No spots were found in any of these wells. To avoid interpersonal variations all spots in each patient group were counted by the same person.

ELISA

The production of IFN-γ, IL-4, IL-5, IL-9, IL-10, and IL-13 was detected with ELISA as described previously [10]. Values were expressed as pg/ml (average of duplicate) deduced from the OD of the standard curve after subtracting the blanks. The sensitivity limits for quantitative determinations were 2 pg/ml for IL-10, 3 pg/ml for IL-4, and IL-5, 10 pg/ml for IL-9, 12 pg/ml for IFN-γ, and 16 pg/ml for IL-13.

RNA extraction and reverse transcription PCR of mRNA

Total ribonucleic acid (RNA) was isolated according to the RNeasy 96 Vacuum/Spin Protocol

(Qiagen). In brief, the lysed cells were mixed with ethanol and applied to an RNeasy 96-well plate. Contaminants were washed away by wash buffers, the membrane was dried, and then the RNA was eluted in RNase-free water.

RNA was converted to complimentary DNA using an oligo dT-primer, random decamer primer and M-MLV reverse transcriptase for 1 h at 42°C followed by 10 min at 75°C according to the Reverse-it protocol of the supplier (Abgene Advanced Biotechnologies, Surrey, UK). The samples were run in a GeneAmp PCR System 2400 (Perkin–Elmer Applied Biosystems, Foster City, CA, USA).

Real time PCR

To a 96-well reaction plate (MicroAmp Optical, Perkin–Elmer Applied Biosystems), 24 µl of Reaction Mix (TaqMan Universal PCR Master Mix, forward and reverse primers, and probes, for concentrations see Table 1) was added to each well, followed by 1 µl samples, standards (cDNA from PHA stimulated mononuclear cells) or water as negative control. Oligonucleotide primers (MedProbe, Oslo, Norway) were designed for human IL-4, IL-5, and IL-9 sequence using Primer Express (Perkin–Elmer) (for sequences see Table 3). The TaqMan probe with the flourophore FAM (6-carboxy-flourescein) at the 5'-end and the

quencher TAMRA (6-carboxy-tetramethylrhodamine) at the 3'-end were bought from MedProbe. Both primers and probe were purified with HPLC.

The wells were covered with optical caps (Perkin–Elmer Applied Biosystems), and the plate was centrifuged for some seconds at high speed and thereafter placed into the thermocycler (ABI Prism 7700 Sequence Detector, Perkin–Elmer Applied Biosystems). The thermal cycle conditions were 50°C for 2 min followed by 95°C for 10 min and then 40 cycles were run for 15 s at 95°C and for 1 min at 60°C, as recommended by Perkin–Elmer for the SDS 7700 system.

Samples were run with primers and probes for IL-4, IL-5, or IL-9 and for rRNA in two different wells. The results from the rRNA reactions were used as internal controls, i.e., the amount of IL-4, IL-5, and IL-9 mRNA was calculated relative to the amount of rRNA present in each sample. Standards, from which a standard curve was calculated, were included in each run.

Statistics

Wilcoxon signed rank test was used for comparisons between samples before and after cryopreservation. Correlation between storage time and cytokine secretion was analysed using Spearman rank correlation.

Table 1
Sequences and concentrations of primers and probes

Target primer/probe sequence (5'–3')			Conc. (nM)	Position	Amplicon length
rRNA	FP	CGGCTACCACATCCAAGGAA	200	^a	^a
	Probe	TGCTGGCACCAGACTTGCCCTC	50	^a	^a
	RP	GCTGGAATTACCGCGGCT	100	^a	^a
IL-4	FP	ACAGCCTCACAG AGCAGAAGACT ^b	300	178–201	72
	Probe	TGTGCACCGAGTTGACCGTAACAGACA	200	203–229	
	RP	TGTTCTTGAGGCAGCAAAGA	300	250–229	
IL-5	FP	ATTGAGACTCTGAGGATTCTGTTC	300	183–207	83
	Probe	CTGTACATAAAAAATCACCAACTGTGCACTGAAGAAA	200	208–243	
	RP	CAGTGTGCCTATTCCCTGAAAGA	300	266–243	
IL-9	FP	GATCCAGCTTCCAAGTGCCA	300	128–148	76
	Probe	AAACAGAGACAACCTGGTCACATTAGCACTGCA	175	149–181	
	RP	TGCAGTTGTCAGAGGGAATGC	300	204–181	

^a Recommended by PE Biosystems.

^b Letters in bold indicate an exon–exon junction.

Results

The results of all cytokine analyses are shown in Table 2 (ELISPOT analyses on samples from pregnant women and MS patients) and Table 3 (ELISA and real time RT-PCR on the samples from children). Data not mentioned in the following description of results did not show any differences between fresh and cryopreserved cells.

Effect of cryopreservation on IL-4 secretion and mRNA expression

The samples from the pregnant women showed a significant decrease in the number of spontaneous IL-4 secreting cells following cryopreservation ($p < 0.001$), as shown in Fig. 1A. The auto-antigen and allergen specific secretion, respectively, also decreased as shown in samples from MS-patients, ($p < 0.05$, Fig. 2) and children ($p < 0.05$ at mRNA level and trends at protein levels, Fig. 3). Both fresh and frozen cells from atopic, as compared to non-atopic, children tended to more often produce high levels of allergen induced IL-4 (Fig. 3). In contrast to the antigen induced secretion, the PHA stimulated secretion of IL-4 was increased after cryopreservation in samples from children.

Effect of cryopreservation on IFN- γ secretion

There were no significant differences before and after cryopreservation neither in the numbers of cells spontaneously secreting IFN- γ secreting in the samples from pregnant women (Fig. 1B) or from MS-patients, or in the auto-antigen stimulated secretion in the samples from MS patients (data not shown). The allergen and PHA stimulated IFN- γ secretion in samples from children was increased after cryopreservation (birch: $p < 0.05$, cat, and PHA: $p < 0.01$).

Effect of cryopreservation on IL-10 secretion

No differences in the IL-10 secretion before and after cryopreservation were seen in samples from MS patients. In children, there was no difference except from an increase in cat stimulated cells ($p < 0.01$).

Effect of cryopreservation on IL-5 secretion and mRNA expression

We found no differences in allergen stimulated IL-5 secretion at the protein level following cryopreservation (Fig. 4). The PHA induced secretion

Table 2
Results of ELISPOT analyses on samples from pregnant women and MS patients

Cytokine		Pregnant women		MS patients	
		Spontaneous	Spontaneous	MOG 14–39	MOG 63–87
IL-4	n	38	10	10	10
	Fresh	63 (10–300)	9.0 (1.7–27.7)	6.3 (–4.7 to 23.0)	5.2 (–2.8 to 15.3)
	Cryopreserved	10.5 (1–169)	6.0 (0.7–18.7)	–0.2 (–4.7 to 8.0)	0.4 (–0.7 to 9.3)
	Wilcoxon	$\downarrow p < 0.001$	ns	$\downarrow p < 0.05$	$\downarrow p < 0.05$
IL-10	n	Not done	8	8	8
	Fresh		47.2 (12.0–101.0)	26.0 (–1 to 46.0)	8.4 (–12.7 to 60.7)
	Cryopreserved		49.9 (6.3–96.5)	15.2 (–4 to 173.4)	0.2 (–17.3 to 89.5)
	Wilcoxon		ns	ns	ns
IFN- γ	n	38	10	10	10
	Fresh	74.5 (12–400)	24.7 (8.7–57.3)	8.2 (–7.6 to 26.7)	8.3 (–2.3 to 101.7)
	Cryopreserved	73.5 (3–225)	22.4 (11.3–33.3)	2.2 (–5.6 to 37.7)	–0.9 (–7.6 to 22.7)
	Wilcoxon	ns	ns	ns	ns

The values are shown as median (range). For stimulated cells, the spontaneous secretion has been subtracted. Cytokine secretion from cells from MS patients and pregnant women were analysed with ELISPOT after 2 days incubation and the secretion expressed as number of cytokine secretion cells per 100,000 lymphocytes. ns, not significant.

Table 3
Results from ELISA and real time RT-PCR on samples from children

Cytokine		Birch	Cat	PHA 24h	PHA 6 days
IL-4mRNA	n	16	17	10	Not done
	Fresh	1.1 (0.0–74.4)	2.5 (0.0–69.4)	55.2 (5.1–116.8)	
	Cryopreserved	0.00 (0.00–0.00)	0.00 (0.00–0.00)	22.4 (17.4–43.7)	
	Wilcoxon	↓ p = 0.01	↓ p < 0.01	(↓) p = 0.06	
IL-4	n	17	16	11	12
	Fresh	0.9 (0.9–67.4)	0.9 (0.9–18.0)	0.9 (0.9–15.3)	24.2 (0.9–101.6)
	Cryopreserved	0.9 (0.9–21.5)	0.9 (0.9–7.3)	18.4 (5.2–52.0)	121.2 (95.3–251.3)
	Wilcoxon	(↓) p = 0.07	(↓) p = 0.06	↑ p < 0.01	↑ p < 0.01
IL-5mRNA	n	16	17	9	Not done
	Fresh	0.0 (0.0–16.4)	2.6 (0.0–38.2)	63.1 (6.8–881.8)	
	Cryopreserved	0.5 (0.3–12.4)	0.5 (0.0–4.4)	45.8 (10.7–124.3)	
	Wilcoxon	ns	↓ p < 0.05	ns	
IL-5	n	17	17	10	14
	Fresh	63.5 (3.1–375.5)	17.4 (3.1–1827.9)	40.4 (6.3–250.5)	96.6 (30.2–230.4)
	Cryopreserved	75.4 (3.1–356.3)	30.6 (3.1–559.8)	106.3 (22.2–2929.0)	860.7 (210.7–3026.8)
	Wilcoxon	ns	ns	(↑) p = 0.06	↑ p < 0.01
IL-9 mRNA	n	16	17	10	Not done
	Fresh	0.0 (0.0–1.5)	0.0 (0.0–0.7)	4.4 (0.0–33.5)	
	Cryopreserved	0.1 (0.0–3.1)	0.0 (0.0–0.9)	28.4 (5.6–88.2)	
	Wilcoxon	↑ p < 0.01	ns	↑ p < 0.01	
IL-9	n	17	17	10	13
	Fresh	4.5 (4.5–309.4)	18.0 (4.5–96.0)	4.5 (4.5–23.8)	4.5 (4.5–118.3)
	Cryopreserved	10.5 (4.5–164.3)	6.7 (4.5–158.2)	60.5 (12.7–287.8)	696.6 (129.2–2865.4)
	Wilcoxon	ns	ns	↑ p < 0.01	↑ p < 0.01
IL-10	n	17	17	11	Not done
	Fresh	67.4 (1.5–117.2)	118.9 (1.5–647.2)	71.3 (1.5–170.9)	
	Cryopreserved	26.1 (8.3–123.7)	66.4 (22.3–340.2)	80.8 (34.3–511.8)	
	Wilcoxon	ns	↓ p < 0.01	ns	
IL-13	n	17	16	9	13
	Fresh	15.6 (15.6–2792.0)	49.8 (15.6–600.0)	84.3 (15.6–366.5)	160.7 (15.6–643.0)
	Cryopreserved	15.6 (1.5–311.8)	28.3 (2.6–263.7)	95.7 (15.6–212.2)	459.9 (15.6–1786.0)
	Wilcoxon	ns	ns	ns	↑ p = 0.01
IFN- γ	n	16	17	10	14
	Fresh	155.8 (12.5–2561.5)	104.8 (12.5–581.5)	791.5 (59.4–3489.0)	625.0 (26.7–6742.0)
	Cryopreserved	345.7 (70.3–2929.5)	362.1 (36.1–2452.6)	6689.0 (2916.0–27740.0)	17145.0 (5256.0–53611.0)
	Wilcoxon	↑ p < 0.05	↑ p < 0.01	↑ p < 0.01	↑ p < 0.01

The values are shown as median (range). For stimulated cells, the spontaneous secretion has been subtracted. mRNA expression and IL-10 secretion was detected after 24h incubation and IL-4, IL-5, IL-9, IL-13, and IFN- γ secretion after 6 days (and for PHA stimulation, also after 24h), the cytokine secretion is expressed as pg/ml. ns, not significant.

did, however, increase (p < 0.05) after 6 days stimulation, and there was a trend (p = 0.06) for an increase after 24h. In contrast, there was a decrease of cat allergen stimulated secretion at the mRNA

level (p < 0.05). Both fresh and frozen cells from atopic, as compared to non-atopic, children tended to more often produce high levels of allergen induced IL-5 (Fig. 4).

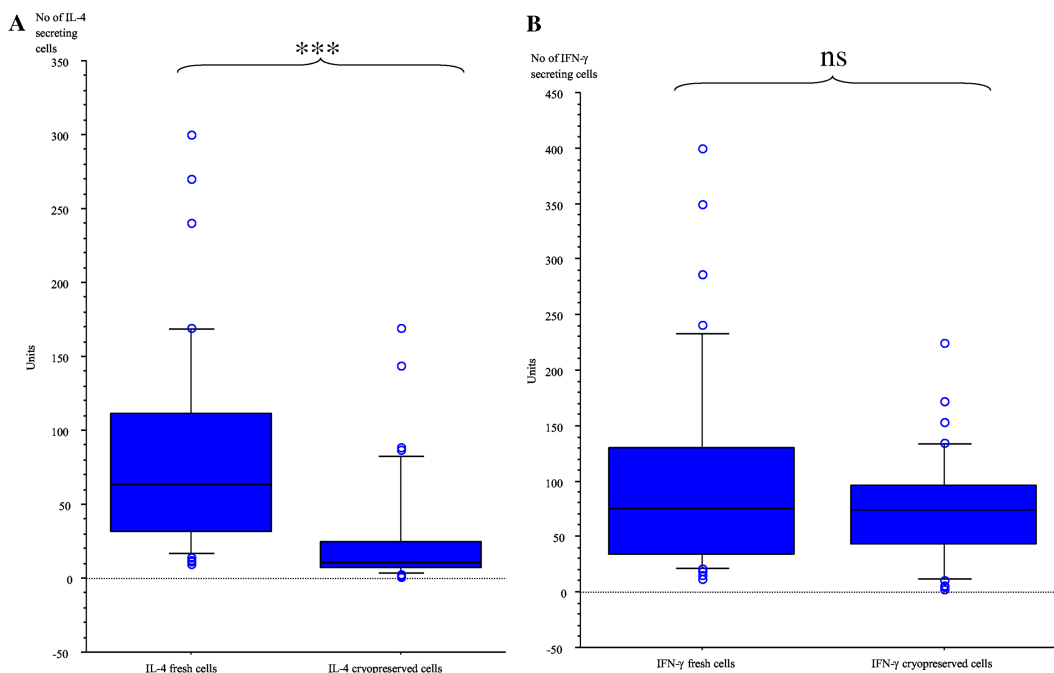


Fig. 1. Spontaneous IL-4 (A) and IFN- γ (B) secretion before and after cryopreservation in samples from pregnant women. *** $p < 0.001$, ns, not significant (Wilcoxon signed rank test). The 10th, 25th, 50th, 75th, and 90th percentiles are indicated, as well as outliers.

Effect of cryopreservation on IL-9 secretion and mRNA expression

Regarding IL-9 expression, there was an increase in both allergen- (birch, at mRNA level)

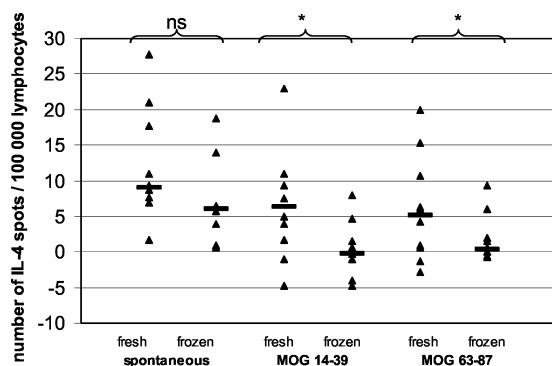


Fig. 2. Number of spontaneous and myelin oligodendrocyte glycoprotein peptide (MOG 14-39, MOG 63-87) induced IL-4 secreting cells before and after cryopreservation in MS patients. MOG-induced secretion is shown as net values (the numbers of spontaneously IL-4 secreting cells are subtracted). * $p < 0.05$, ns, not significant (Wilcoxon signed rank test). Horizontal bars show median values.

and PHA- (at both mRNA and protein level) stimulated secretion of IL-9 after cryopreservation ($p < 0.01$).

Effect of cryopreservation on IL-13 secretion

The only differences between fresh and cryopreserved cells that could be seen regarding IL-13 secretion in samples from children was an increase when cells were stimulated with PHA for 6 days ($p < 0.05$).

Effect of storage time on cytokine secretion

The effect of storage time was investigated in the samples from pregnant women only. The time of storage after cryopreservation did not significantly influence the spontaneous secretion of neither IFN- γ nor IL-4, since there were no significant correlations between the storage times and the changes of cytokine secreting cells in the individual samples (data not shown). There was, however, a trend for increased secretion of IFN- γ over time ($p = 0.074$).

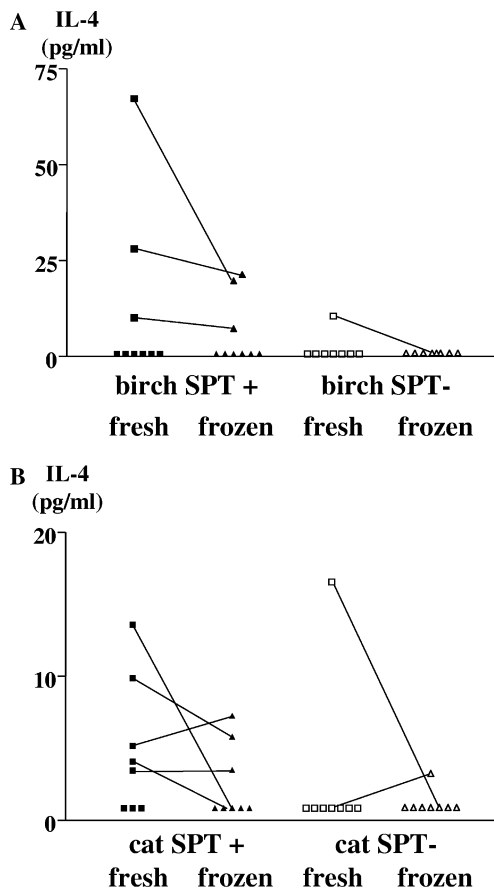


Fig. 3. IL-4 secretion before and after cryopreservation in PBMC from atopic and non-atopic children. Birch (A) and cat (B) allergen induced IL-4 secretion from fresh (squares) and frozen (triangles) PBMC from children with positive (filled symbols) and negative (open symbols) skin prick tests to birch (A) and cat (B). Note that samples below the detection limit have been given the value of the detection limit and are seen close to the *X* axis. Paired samples where differences were seen between fresh and frozen cells are connected with a line.

Discussion

The main finding of this study is a general decrease in IL-4 secretion in cryopreserved cells compared with fresh cells. This decrease was seen in all three patient groups, including spontaneous as well as auto-antigen or allergen induced secretion. The data on allergen-induced IL-4 must, however, be interpreted with caution, since IL-4 was not detectable either in fresh or in frozen cells in many samples. The significant decreases indicated by

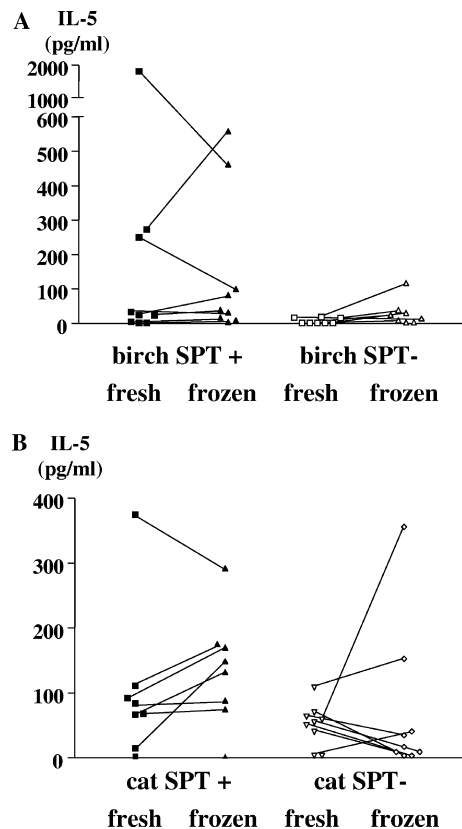


Fig. 4. IL-5 secretion before and after cryopreservation in PBMC from atopic and non atopic children Birch (A) and cat (B) allergen induced IL-5 secretion from fresh (squares) and frozen (triangles) PBMC from children with positive (filled symbols) and negative (open symbols) skin prick tests to birch (A) and cat (B). Note that samples below the detection limit have been given the value of the detection limit and are seen close to the *X* axis. Paired samples where differences were seen between fresh and frozen cells are connected with a line.

Wilcoxon matched pairs signed rank test are thus based on a limited number of samples, where a difference between the paired fresh and frozen samples was evident. A recent report [14] did not show any changes in stimulated IL-4 secretion after freezing, although this was a study on four healthy controls only and the spontaneous secretion was not investigated. The authors point out that it remains to be established if cells from patients with various diseases also are resistant to cryopreservation. The difference compared to the present study could speculatively be due to that our subjects all were in states that are associated

with immune activation. Activated cells may well be more sensitive for the process of cryopreservation. Indeed, activated cells have previously been reported to have a different optimum cooling rate than non-activated cells, where the standard 1 °C/min cooling rate used in the present study is optimal for non-activated cells [7,12], strongly supporting that suboptimal conditions for activated cells may explain the decrease of IL-4 in our study. Nevertheless, this is an important finding since studies on cytokine secretion from cells frozen at the standard cooling rate commonly are performed on patients with diseases associated with immune activation, as this is a reason for the study of cytokine responses.

This study was designed out of practical reasons, to elucidate if analyses of cytokine expression in cryopreserved cells generate similar results as analyses on fresh cells, i.e., if cryopreservation is a proper technique in studies of *in vitro* cytokine secretion in various diseases. It does, however, not allow any conclusions regarding the pure biological effects of cryopreservation on PBMC, since the observed changes in cytokine secretion, due to the laboratory techniques used, can not be certainly attributed to either changes in numbers of responding cells or changes in levels of activity of individual cells. First, ELISPOT identifies responding cells, but the level of activity of the responding cells is not determined [6]. Second, the measurements of expression at the protein and mRNA level cannot be related to expression per cell, since there is a problem with the estimation of cells capable of activation in the cell populations. We used trypan blue exclusion for determination of viability, but a trypan blue negative cell is, although probably alive, not necessarily capable of activation. Indeed, Knight and Farrant [13] have reported that the number of trypan blue negative cells overestimates the number of cells capable of activation. Thus, it is not possible to draw any conclusions regarding if the general decrease in IL-4 is due to alterations of cellular properties in PBMC or to the death of a particular cell population caused by cryopreservation, which previously has been suggested as a cause for alterations in cytokine secretion in studies on the effect of cryopreservation [23].

Regarding the expression of IL-4 mRNA, we found no significant decrease. IL-4 mRNA was, however, not detectable in any of the allergen stimulated cell cultures of cryopreserved cells, in contrast to the cultures of fresh cells. This may indicate a similar decrease of IL-4 mRNA as of IL-4 protein, following cryopreservation. Thus, negative findings on IL-4 expression in cryopreserved cells, must be interpreted with caution. Notably, however, both fresh and frozen cells from atopic, as compared to non-atopic children, tended to more frequently produce high levels of allergen induced IL-4. Thus, a difference between the patient group and controls may be seen even after cryopreservation. Taken together, studies on IL-4-expression would preferably be performed on fresh cells, since IL-4 secretion usually is low. In conditions with high levels of IL-4, like atopy, and pregnancy, secretion of IL-4 in cryopreserved cells may, however, if detected, most likely mirror the secretion patterns of fresh cells.

Our findings of no difference in spontaneous IFN- γ secretion following cryopreservation is in line with previous studies [8,22]. We found increased allergen and PHA stimulated IFN- γ secretion in atopic children following cryopreservation, the latter corroborating the findings of Venkataraman [23], but contrary to other studies [19,20,22]. On the contrary, no differences in auto-antigen-induced IFN- γ after cryopreservation compared with fresh cells were found in MS-patients. MS is a Th1-associated disease, where auto-reactive IFN- γ secreting cells are supposed to be activated. Thus, PBMC from MS-patients may be differently affected by cryopreservation than cells from the atopic group, which is associated with Th2-responses, due to e.g., the above discussed suboptimal cooling rate for activated cells [7,12]. Hence, when analysing the spontaneous secretion of IFN- γ , frozen cells may be used, whereas stimulated secretion may preferably be analysed in fresh cells.

There are no previous reports on the effect of cryopreservation on IL-5, IL-9, or IL-13. Our results show no effect of freezing on allergen-induced IL-5 or IL-13 secretion. A difference in IL-9 expression was found in birch induced mRNA expression, which was increased in frozen cells.

Since the cytokines IL-5, IL-9, and IL-13 were analysed in one patient group only, further studies need to be done.

We found a decrease in cat-induced secretion of IL-10 in atopic children in frozen cells compared with fresh cells, but no differences in pre versus post freezing levels of birch-induced IL-10 in atopic children or myelin antigen-induced secretion of IL-10 in MS-patients. Spontaneous secretion in these groups was also unaffected by freezing. A previous report showed decreased PHA induced IL-10 secretion after cryopreservation [24]. We could, however, not confirm this finding, but found no differences in PHA-induced secretion of IL-10. This is, however, based on a limited sample of 11 children, which may give insufficient power for detection of a difference. Interestingly, our data on PHA-induced secretion show an overall increase in atopic children after cryopreservation of IFN- γ , IL-4, IL-5, IL-9, and IL-13, but not of IL-10, indicating that IL-10 secretion is differently affected by freezing than the other cytokines. It is intriguing that the effects of cryopreservation differs between antigen- and mitogen-induced responses regarding a certain cytokine within a patient group, which in this study was evident for IL-4, IL-5, IL-10, and IL-13 in atopic children. This may, at least partly, again be due to the discussed above discrepancy in optimal freezing conditions between activated and non-activated cells and maybe also different optimum allergen doses for fresh and frozen cells [7,12]. The antigenic stimulation used in this study was specifically directed against presumably activated disease generating cells, i.e., cells from atopic children were stimulated with allergens and cells from MS-patients with myelin-antigens. PHA, stimulates all T-cells, independently of T-cell receptor specificity, of which most are in a non-activated state in PBMC, however. Speculatively, differences in the fashion and magnitude of stimulation between PHA and allergen/auto-antigen may also contribute to the observed different effects of cryopreservation. PHA is a strong polyclonal stimulus, which in a non-physiological way induces vigorous secretion of most cytokines in PBMC. In contrast, in vitro stimulation with antigens induces low-grade memory responses, in a highly selective, and physiological fashion. Since PHA is a tremendously strong stimulus,

somewhat mimicking bacterial super-antigens, including no need for signals involved in classical T-cell activation e.g., antigen presentation and co-stimulation, it seems likely that some supposed consequences of cryopreservation, which would negatively affect common T-cell activation, may not have negative effects on PHA-stimulation, but rather may enhance PHA-responsiveness if e.g., certain suppressive cells or factors are excluded by cryopreservation [23]. This latter theory is indeed supported by the substantial magnitude of the difference between fresh and frozen cells in response to PHA, where 10–100-fold increases were seen for IL-5, IL-9, and IFN- γ , whereas the allergen stimulated responses at the most showed 5-fold changes.

In conclusion, this study shows a general decrease of IL-4 following cryopreservation. The process of cryopreservation and thawing does affect the expression of cytokines, both at the protein and the mRNA level, and this effect varies among different cytokines, different stimuli and different patient groups. This points out the importance of investigation of the effects of freezing for each cytokine, stimuli, and patient group before using frozen cells in studies of in vitro cytokine secretion.

Acknowledgments

The authors thank Leif Matthiesen, Department of Molecular and Clinical Medicine, Division of Obstetrics and Gynaecology, Linköping University Hospital, Sweden, for recruiting the samples from the pregnant women. We thank Ulrika Bengtsson, Jenny Fredriksson, Anne-Marie Fornander, Kerstin Hagersten, Lena Lindell, Beata Salman, and Ing-Marie Sandberg, Department of Molecular and Clinical Medicine, Division of Paediatrics, Linköping University Hospital, Sweden, for excellent technical assistance. Dr. Jacques van Snick, Ludwig Institute for Cancer Research, Brussels Branch, Belgium, is acknowledged for kindly providing the antibodies to IL-9, and Erik Wallström, Karolinska Institute, Stockholm, Sweden for kindly providing the MOG peptides. We thank Professor Jan Ernerudh, Department of Molecular and Clinical Medicine, Division of Clinical

Immunology, Linköping University Hospital, Sweden, for kind support and critically reading and commenting on the manuscript.

References

- [1] C.E. Allsopp, S.J. Nicholls, J. Langhorne, A flow cytometric method to assess antigen-specific proliferative responses of different subpopulations of fresh and cryopreserved human peripheral blood mononuclear cells, *J. Immunol. Methods* 214 (1998) 175–186.
- [2] T. Bailey, S. Stark, A. Grant, C. Hartnett, M. Tsang, A. Kalyuzhny, A multidonor ELISPOT study of IL-1 beta, IL-2, IL-4, IL-6, IL-13, IFN-gamma and TNF-alpha release by cryopreserved human peripheral blood mononuclear cells, *J. Immunol. Methods* 270 (2002) 171–182.
- [3] A. Bøyum, Isolation of mononuclear cells and granulocytes from human blood. Isolation of mononuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1g, *Scand. J. Clin. Lab. Invest Suppl.* 97 (1968) 77–89.
- [4] C. Ekerfelt, J. Ernerudh, M. Vrethem, Th1-like responses to peptides from peripheral nerve myelin proteins in patients with polyneuropathy associated with monoclonal gammopathy, *Clin. Exp. Immunol.* 108 (1997) 516–522.
- [5] C. Ekerfelt, L. Matthiesen, G. Berg, J. Ernerudh, Paternal leukocytes selectively increase secretion of IL-4 in peripheral blood during normal pregnancies: demonstrated by a novel one-way MLC measuring cytokine secretion, *Am. J. Reprod. Immunol.* 38 (1997) 320–326.
- [6] C. Ekerfelt, J. Ernerudh, M.C. Jenmalm, Detection of spontaneous and antigen induced human IL-4 responses in vitro; comparison of ELISPOT, a novel ELISA and real time RT-PCR, *J. Immunol. Methods* 260 (2002) 55–67.
- [7] J. Farrant, S.C. Knight, G.J. Morris, Use of different cooling rates during freezing to separate populations of human peripheral blood lymphocytes, *Cryobiology* 9 (1972) 516–525.
- [8] D. Friberg, J. Bryant, W. Shannon, T.L. Whiteside, In vitro cytokine production by normal human peripheral blood mononuclear cells as a measure of immunocompetence or the state of activation, *Clin. Diagn. Lab. Immunol.* 1 (1994) 261–268.
- [9] S. Fujiwara, M. Akiyama, M. Yamakido, T. Seyama, K. Kobuke, M. Hakoda, S. Kyoizumi, S.L. Jones, Cryopreservation of human lymphocytes for assessment of lymphocyte subsets and natural killer cytotoxicity, *J. Immunol. Methods* 90 (1986) 265–273.
- [10] M.C. Jenmalm, J. Van Snick, F. Cormont, B. Salman, Allergen-induced Th1 and Th2 cytokine secretion in relation to specific allergen sensitization and atopic symptoms in children, *Clin. Exp. Allergy* 31 (2001) 1528–1535.
- [11] C.A. Kleeberger, R.H. Lyles, J.B. Margolick, C.R. Rinaldo, J.P. Phair, J.V. Giorgi, Viability and recovery of peripheral blood mononuclear cells cryopreserved for up to 12 years in a multicenter study, *Clin. Diagn. Lab. Immunol.* 6 (1999) 14–19.
- [12] S.C. Knight, J. Farrant, G.J. Morris, Separation of populations of human lymphocytes by freezing and thawing, *Nat. New Biol.* 239 (1972) 88–89.
- [13] S.C. Knight, J. Farrant, Comparing stimulation of lymphocytes in different samples: separate effects of numbers of responding cells and their capacity to respond, *J. Immunol. Methods* 22 (1978) 63–71.
- [14] C.R. Kreher, M.T. Dittrich, R. Guerkov, B.O. Boehm, M. Tary-Lehmann, CD4+ and CD8+ cells in cryopreserved human PBMC maintain full functionality in cytokine ELISPOT assays, *J. Immunol. Methods* 278 (2003) 79–93.
- [15] C. Macaubas, P.D. Sly, P. Burton, K. Tiller, A. Yabuhara, B.J. Holt, T.B. Smallacombe, G. Kendall, M.C. Jenmalm, P.G. Holt, Regulation of T-helper cell responses to inhalant allergen during early childhood, *Clin. Exp. Allergy* 29 (1999) 1223–1231.
- [16] L. Matthiesen, C. Ekerfelt, G. Berg, J. Ernerudh, Increased numbers of circulating interferon-gamma- and interleukin-4-secreting cells during normal pregnancy, *Am. J. Reprod. Immunol.* 39 (1998) 362–367.
- [17] W.I. McDonald, A. Compston, G. Edan, D. Goodkin, H.P. Hartung, F.D. Lublin, H.F. McFarland, D.W. Paty, C.H. Polman, S.C. Reingold, M. Sandberg-Wollheim, W. Sibley, A. Thompson, S. van den Noort, B.Y. Weinshenker, J.S. Wolinsky, Recommended diagnostic criteria for multiple sclerosis: guidelines from the international panel on the diagnosis of multiple sclerosis, *Ann. Neurol.* 50 (2001) 121–127.
- [18] V. Navikas, H. Link, Review: cytokines and the pathogenesis of multiple sclerosis, *J. Neurosci. Res.* 45 (1996) 322–333.
- [19] J.G. Smith, X. Liu, R.M. Kaufhold, J. Clair, M.J. Caulfield, Development and validation of a gamma interferon elispot assay for quantisation of cellular immune responses to varicella-zoster virus, *Clin. Diagn. Lab. Immunol.* 8 (2001) 871–879.
- [20] V. Sobota, J. Bubenik, M. Indrova, V. Vlk, J. Jakoubkova, Use of cryopreserved lymphocytes for assessment of the immunological effects of interferon therapy in renal cell carcinoma patients, *J. Immunol. Methods* 203 (1997) 1–10.
- [21] E. Wallström, M. Khademi, M. Andersson, R. Weissert, C. Linington, T. Olsson, Increased reactivity to myelin oligodendrocyte glycoprotein peptides and epitope mapping in HLA DR2(15)+ multiple sclerosis, *Eur. J. Immunol.* 28 (1998) 3329–3335.
- [22] S.Y. Wang, M.L. Hsu, C.H. Tzeng, H.C. Hsu, C.K. Ho, The influence of cryopreservation on cytokine production by human T lymphocytes, *Cryobiology* 37 (1998) 22–29.
- [23] M. Venkataraman, Effects of cryopreservation on immune responses. VIII. Enhanced secretion of interferon-gamma by frozen human peripheral blood mononuclear cells, *Cryobiology* 32 (1995) 528–534.
- [24] M. Venkataraman, Effects of cryopreservation on immune responses. IX. Stimulus-mediated dichotomy in IL-10 production by frozen human peripheral blood mononuclear cells, *J. Hematother.* 5 (1996) 301–308.