

Intracellular T-cell cytokine levels are age-dependent in healthy children and adults

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ABSTRACT. Intracellular detection of cytokines *via* fluorescent antibody staining and flow cytometry has quickly become a standard method in experimental immunology. However, in pediatrics most studies have been hampered by the exclusion of healthy control individuals or have been skewed by neglecting to observe age-dependent differences in cytokine production. We therefore intended to establish normal values for different age groups and to describe the age-dependent development of cytokine profiles. Whole blood from 46 healthy children and 33 adults was analyzed by flow cytometry after stimulation with PMA, ionomycin and Mmonensin, and staining with anti-cytokine and surface antibodies. In the pediatric population, we found a significant positive correlation between age and intracellular cytokine levels of IFN- γ , IL-2, IL-4 and TNF- α in CD4⁺ cells, as well as for IFN- γ and TNF- α in CD8⁺ cells. In adulthood, no such striking trend could be detected, but significant correlation was found for IL-10 in CD4⁺ cells and IFN- γ in CD8⁺ cells as well as for TNF- α in both cell subgroups. We present here the first systematic analysis of intracellular cytokine production in normal, healthy children between the ages of 0 to 18 years compared to results in adults. These data may provide a reference basis for the study of cytokine secretion patterns, and they also demonstrate a significant maturation of the T-cell cytokine production capacity from birth to adulthood.

Keywords: intracellular cytokines, T-cells, children

The analysis of cytokine production levels has been recognized as an important tool for describing the course of an immune response and for discovering pathophysiological mechanisms in immunological diseases [1].

Complementing the use of enzyme-linked immunosorbent assay (ELISA) for the detection of secreted cytokines in supernatants and reverse transcriptase PCR (RT-PCR) [2] and *in situ* RT-PCR [3] at the mRNA level, intracellular detection of cytokines *via* fluorescent antibody staining and flow cytometry has quickly become a standard method in experimental immunology to assess an individual's repertoire of cytokine-producing T-cells [2, 4, 5]. This method has been proven to be useful, reproducible and correlates with traditional methods [6].

Intracellular cytokine detection has been used to analyze samples from patients with almost any disease involving immunological processes, such as asthma [7, 8], atopic diseases [8, 9], rheumatoid arthritis [10], primary and acquired immunodeficiencies [11, 12], inflammatory bowel disease [13] and acute lymphoblastic leukemia [14].

However, in studies in children it can be hard to establish an appropriate control group because of issues such as consent, ethics committees and the difficulty of obtaining large enough blood samples. This might be the reason why some studies included very small control groups and/or control groups that were not age-matched to the patients [13, 15]. Even if sufficiently large and age-matched control samples were included, distinction between age groups was rarely made [9, 16]. Only very few studies have taken into account the fact that there can be variations in intracellular cytokine production between children of different age groups [11]. Even fewer studies have looked systematically at the age-dependence of intracellular cytokine production and have included only one or two pediatric age groups [17, 18].

It is, however, of great importance to acknowledge the variation of cytokine production with age. This has been described not only in studies comparing children and adults [17, 19, 20] but also between younger and aging adults [21, 22].

Although it is clearly impossible to dissect the complex T-cell response into two opposing categories, the dogma of T-helper (Th) and T-cytotoxic (Tc) type 1 and 2 (Th(c)1 and Th(c)2) cell types, characterized by their respective cytokine patterns, has proven very useful for the better understanding of the pathogenesis of a variety of diseases [23]. Interferon- γ (IFN- γ), interleukin-2 (IL-2) and tumor necrosis factor- α (TNF- α) are generally considered to be Th(c)1 type cytokines, while interleukin-4 (IL-4), interleukin-5 (IL-5) and interleukin-10 (IL-10) are representatives of Th(c)2 cytokines [24].

We therefore designed this study to establish normal values for the intracellular production of interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-10 (IL-10), interferon- γ (IFN- γ) and tumor necrosis factor- α (TNF- α) in CD4⁺ and CD8⁺ T-cell subsets for clearly defined age groups of healthy children aged 0 to 18 years, as well as in healthy adults. This was done using one of several, widely applied and easily reproducible methods. Furthermore, we were interested in whether there was a correlation between age and T-cell cytokine production in children and also in age groups beyond adolescence. This revealed significant age-dependent correlations for some, but not all tested cytokines, in children as well as in adults.

METHODS

Subjects

Inclusion criteria for this study required healthy subjects of Caucasian origin who had no signs of infection, no chronic disease, no family history of immune-mediated disease and who had not received any medication within the 7 days prior, that could have possibly influenced immune competence. In total, samples from 46 children were obtained after written informed consent of their caregivers, and 33 samples were taken from adult volunteers after obtaining their consent. Age groups were constructed to represent groups of interest and to contain approximately the same number of subjects. The age distribution in the age group 0-2 years was as follows: 0-6 months: $n = 2$, 7-12 months: $n = 1$, 13-18 months: $n = 2$, 19-24 months: $n = 4$. The study was approved by the local ethics committee of our institution.

Sample handling

Heparinized venous blood was collected under sterile conditions into an endotoxin-free tube, briefly stored at room temperature and analyzed within 6 hours of collection. Preliminary experiments showed significant changes in cytokine production, if analysis was delayed by more than 6 hours (data not shown).

Cell stimulation and determination of cytokine producing cell populations by flow-cytometry

The flow cytometry intracellular staining protocol used was adapted from the one described by Jung *et al.* [5] and refined by Prussin *et al.* [4, 25].

For *in vitro* cell stimulation, 4 x 100 μ L blood from each subject were suspended with 900 μ L RPMI 1640 culture medium (Biochrom, Berlin, Germany) supplemented with

10% heat-inactivated fetal calf serum (FCS, Biochrom, Berlin, Germany) and L-glutamine (Biochrom, Berlin, Germany), in a 24-well, tissue culture plate and either stimulated with 10 ng/mL phorbol 12-myristate 13-acetate (PMA, Sigma, Munich, Germany), and 0.75 μ g/mL ionomycin (Sigma, Munich, Germany) in the presence of 1.78 μ g/mL monensin (Sigma, Munich, Germany) or not stimulated. Monensin was added to block the export of newly synthesized cytokine from the Golgi apparatus in order to increase the quantity of cytokine available for detection within each cell. Cells were incubated for 6h at 37°C in an atmosphere of 95% air and 5% CO₂.

After transfer to 12 x 75 mm, polystyrene, round-bottom tubes, stimulated cells were washed twice in 3 mL washing buffer (phosphate-buffered saline (PBS) containing 1% FCS) and red cells were lysed with 500 μ L fixing solution (4% paraformaldehyde in H₂O (Serva, Heidelberg, Germany) in PBS) for 10min at 4°C. After centrifugation (1250 U/min, $g = 300$, 5min, 4°C), the supernatant was decanted, followed by washing twice with washing buffer. At this point, samples can be stored for up to 48 hours at 4°C.

Cells were washed once and resuspended in 1ml permeabilization buffer (washing buffer containing 5 μ g/mL saponin (Serva, Heidelberg, Germany) and 2.38 μ g/mL Hepes (Merck, Darmstadt, Germany), incubated for 10 min at 4°C and washed once in permeabilization buffer. For the next step, simultaneous incubation of 50 μ L-aliquots (± 50.000 cells/tube) of unstimulated and stimulated cultures with 7.5 μ L of anti-CD3 PerCP (Becton Dickinson, Heidelberg, Germany), 5.0 μ L CD8 FITC (Coulter Immunotech, Krefeld, Germany) and 10 μ L of the diluted PE-labelled anti-cytokine monoclonal antibodies TNF- α , IFN- γ , IL-2, IL-4, isotype control (Coulter Immunotech, Krefeld, Germany) and IL-10 (BD Biosciences Pharmingen, Heidelberg, Germany) was performed. Anti-cytokine antibodies were diluted with permeabilization buffer 1:20 (TNF- α , IFN- γ , IL-10), 1:40 (IL-2) and 1:4 (IL-4). All samples were stained, in the dark, at 4°C for 25 min.

Finally, the stained cells were washed three times with permeabilization buffer, resuspended in 250 μ L fixing solution and stored for no longer than 24 hours at 4°C until flow cytometric measurement.

Flow cytometric acquisition and analysis

The intensity of fluorescence was analysed by FACScan flow cytometer (Becton Dickinson, Heidelberg, Germany) having preset instrument settings for uniform analysis of each sample. Ten thousand events of CD3⁺-cells were acquired and analysed using CELLQuest software (Becton Dickinson, Heidelberg, Germany).

Events were gated on lymphocytes *via* forward and side scatter and for CD3⁺ (PerCP, FL3). These gated events were plotted for CD8-FITC (FL1) and the respective cytokine (PE, FL2). The percentage of cytokine-positive cells in stimulated samples was then calculated for either CD3⁺CD8⁺ or CD3⁺CD8⁻ events. CD3⁺CD8⁻ events were defined as CD4⁺ cells. This was done because PMA/ionomycin stimulation can cause the down-regulation of CD4 expression, thereby skewing results for CD4⁺ cells

[26]. CD3⁺CD8⁻ events will therefore include double negative TCR $\gamma\delta$ T-cells, which can make up a proportion of up to 10% in children. For each analysis, dot-plot graphs of forward-scatter versus side-scatter were drawn, and a lymphocyte region R1 was defined. Dot plot quadrant statistics were set on the basis of CD8⁺ (FL-1, FITC) or CD8⁻/CD4⁺ T-cells versus cytokine fluorescence (FL-2). Cytokine-positive CD4⁺ or CD8⁺ T-cells were defined by setting regions, with the lower-limits for cytokine positivity determined from the unstimulated cultures. Data were expressed as the percentage of CD4⁺ or CD8⁺ cells within the lymphocyte gate positive for the respective cytokine. Isotype controls were included in each analysis to exclude non-specific staining.

Statistical analysis

Box plots were created using Sigma plot Version 8 (SYSTAT Software Inc., Richmond, Ca, USA). Statistical analysis was carried out using SPSS software Version 10.0 (SPSS Inc., Munich, Germany). Pearson's correlation coefficient was calculated to measure the association between age and cytokine levels.

RESULTS

Age dependence of intracellular cytokines in children

To define the normal range for intracellular cytokine expression in T-cells in a pediatric population, blood samples from 41 healthy children were analyzed as described above. Results of the analysis are displayed graphically in *figure 1*. The median and the normal range as defined by the 3rd and 97th percentile respectively, are presented in *table 1* for each age group. Generally, there was an obvious trend towards increasing levels of intracellular cytokines

with increasing age. Specifically, in the pediatric group (age <18 years), there was a significant positive correlation between age and intracellular detection of cytokines for IFN- γ , IL-2, IL-4 and TNF- α in CD4⁺ cells ($p \leq 0.002$ for all) as well as for IFN- γ and TNF- α in CD8⁺ cells ($p = 0.002$ and 0.005 respectively).

These data demonstrate that there is a significant increase in cytokine production capacity during childhood for all Th1 and Th2 cytokines except IL-10 in T helper cells. A similar observation can be made for the proinflammatory (or Tc1) cytokines IFN- γ and TNF- α in cytotoxic T-cells.

Age-dependence of intracellular cytokines in adults

In order to find out whether this age-dependent increase in cytokine expression was limited to childhood and adolescence or whether it continued into adulthood, we examined the same cytokines in a population of 33 healthy adult (age ≥ 18 years) volunteers. Results are displayed in *figure 1* and normal ranges can be seen in *table 1*. In the adult group, there was not a striking increase in cytokine expression with age in general, but a significant increment could be detected for some subgroups. Specifically, there was a significant correlation for IL-10 in CD4⁺ cells ($p = 0.045$) and IFN- γ in CD8⁺ cells ($p = 0.001$), as well as for TNF- α in both cell subgroups ($p < 0.001$ for both).

Taken together these findings illustrate that in adulthood, there still is a significant increase in the cytokine production capacity with age, especially for TNF- α .

DISCUSSION

Many researchers have observed that cytokine production in human beings is a process that is in some way influenced by the age of the individual. Many have looked at elderly age groups [22], some have investigated the very young

Table 1
Normal range of intracellular cytokine production in T-lymphocyte subsets in different age groups. Displayed as median values of % cytokine-positive cells in bold type with 3rd and 97th percentile in regular type font

	0-2 yrs (n = 9)	2-5 yrs (n = 16)	6-12 yrs (n = 11)	13-17 yrs (n = 10)	18-34 yrs (n = 16)	35-49 yrs (n = 8)	> 50 yrs (n = 9)
IFN CD4	3.73 1.84-12.50	13.89 3.52-27.09	11.60 7.15-41.92	18.65 11.06-30.87	21.62 9.73-40.24	22.87 13.72-38.06	23.21 6.30-43.79
IFN CD8	10.57 1.67-20.38	30.28 7.17-45.74	28.07 12.06-42.73	34.55 16.63-61.95	38.93 23.12-58.46	50.14 27.93-77.92	55.77 13.40-94.28
IL-2 CD4	4.90 1.48-18.40	9.67 1.09-20.13	8.95 1.87-38.58	13.12 5.18-37.60	12.09 0.94-35.82	12.44 3.48-32.13	62.77 0.41-74.19
IL-2 CD8	1.22 0.16-2.64	0.28 2.55-6.67	1.28 0.02-5.81	0.39 0.03-3.66	1.82 0.03-12.24	2.40 0.35-11.19	4.88 0.65-27.36
IL-4 CD4	0.49 0.12-2.05	1.35 0.13-2.44	1.45 0.23-3.18	1.70 0.48-4.07	1.61 0.64-3.99	2.11 1.05-3.81	4.23 0.72-13.14
IL-4 CD8	0.05 0.03-1.33	0.05 0.03-3.02	0.07 0.03-2.45	0.12 0.03-0.66	0.17 0.03-2.12	0.70 0.03-4.86	1.39 0.04-8.45
IL-10 CD4	0.20 0.01-6.35	0.45 0.01-1.81	1.10 0.30-2.04	1.07 0.67-2.44	1.27 0.11-3.24	1.26 0.64-2.61	1.65 0.24-5.13
IL-10 CD8	0.05 0.03-2.03	0.04 0.03-0.63	0.04 0.02-1.16	0.04 0.03-1.07	0.10 0.03-1.37	0.15 0.03-0.79	0.09 0.04-6.63
TNF- α CD4	6.48 4.54-18.77	19.14 6.89-34.03	19.81 9.96-46.98	26.58 13.25-43.50	27.28 16.59-39.28	37.44 15.75-51.84	47.81 22.42-66.62
TNF- α CD8	4.73 1.14-13.40	12.93 6.12-39.09	10.30 0.03-50.89	17.02 8.88-42.83	18.18 10.16-46.16	29.99 18.83-54.76	56.49 11.92-69.25

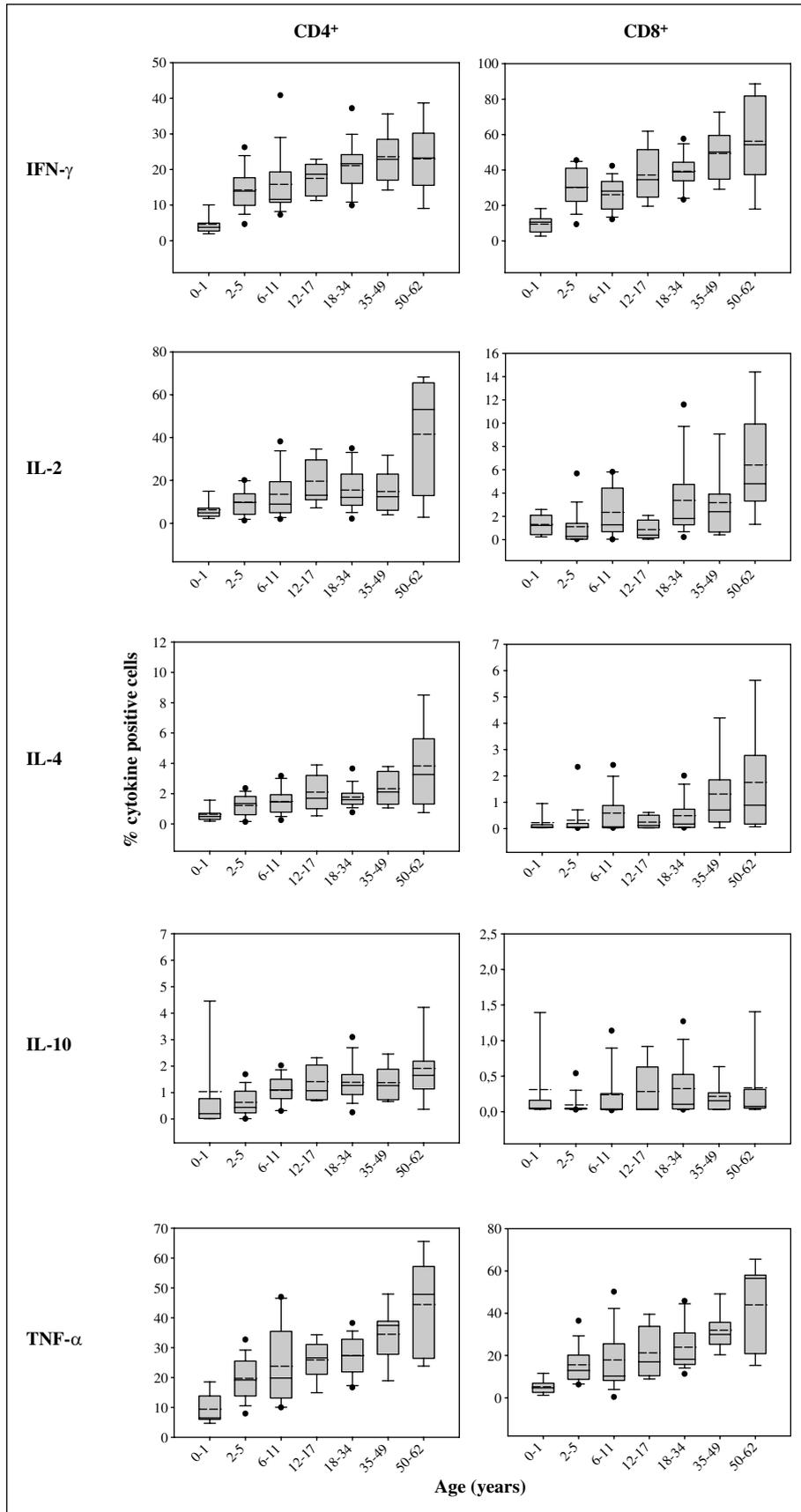


Figure 1

Intracellular cytokine production in T-lymphocyte subsets of healthy individuals. The boxes represent the 25th and 75th percentile. Also displayed: median (solid line), mean (dashed line), lowest and highest value outside of the interquartile distance (whiskers) and outlying values inside of 5th and 95th percentile (solid dots).

[20]. While these studies did not reveal a clear trend in older adults [22], there was some indication that cytokine production in very young children or neonates is lower than in older children or adults [17, 18]. When looking at intracellular cytokine staining after non-specific stimulation *in vitro*, in individuals from 0 to 65 years of age in this study, we found a significant correlation of increasing cytokine levels with age in the pediatric age group for IFN- γ , IL-2, IL-4 and TNF- α in CD4⁺ cells and for IFN- γ and TNF- α in CD8⁺ cells. This corresponds well with the finding by Chipeta *et al.* and Krampera *et al.* that IFN- γ is increased in older children compared to umbilical cord blood [17, 18]. We also observed that IFN- γ secretion is very low in young children, which could be argued to be a sign of an inherent mechanism of down-regulation of Th1 type cytokines that have been shown to be deleterious towards the placenta during pregnancy [27]. Berdat *et al.* reported that serum levels of TNF- α showed a non-significant trend towards increasing with age, in children aged 0 to 5 years [28], which we show to be significant on an intracellular level in both CD4⁺ and CD8⁺ cells in all age groups from 0 to 65 years. Building on data in mice, it has been suggested that there is a shift from a predominantly Th1 phenotype in the young, towards increased production of Th2 type cytokines in the elderly [29]. The data from our study do not support this hypothesis as we observed increasing levels of Th2 as well as Th1 type cytokines with age, and found the most striking increase in pro-inflammatory cytokines such as IFN- γ and TNF- α .

The reason for the fact that we found a higher proportion of cytokine-producing CD4⁺ and CD8⁺ cells with increasing age was not revealed by the techniques used in these experiments. One could however speculate that this is a sign of a more memory-like T-cell phenotype in older humans after having encountered countless infectious and other antigens during their lifetime, an observation that has been made by others [30].

This study was designed to establish a useful baseline of normal values for T-cell, intracellular cytokine production in children and adults, using a widely applied and easily reproducible flow cytometric technique. These results revealed a clear association between cytokine expression and age, especially in young children. These data might therefore be able to provide a reference for future studies aimed at characterizing immune mechanisms in disease and health.

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